Developments in Applied Phycology 6

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The Physiology of Microalgae

Springer

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Preface

Algae play an enormously important role in ecology and, increasingly, in biotechnology. Microalgae in the world oceans, for instance, are responsible for nearly half of the CO_2 fixed (and O_2 released) by photosynthesis annually and form the basis of most marine and other aquatic food chains. With the potential of global warming and associated ocean acidification, the effects of these changes on phytoplankton communities and the flow-on effect on the marine ecosystems are of major interest. The impact of anthropogenic activities on aquatic environments, especially the effects of eutrophication and associated algal blooms and their mitigation, is of great importance. Through their application in wastewater treatment, microalgae are also part of the solution to reduce the detrimental effects of the discharge of wastewaters.

Microalgae are also of significant commercial importance. A number of species are important for the growing aquaculture industry, serving as critical food for larval fish and abalone and for shellfish. Since the early 1980s there has been a growing microalgal-based biotechnology industry, producing natural pigments such as β-carotene and astaxanthin and long-chain polyunsaturated fatty acids. More recently, microalgae have, once again, become the focus for the development of renewable biofuels, and this has also reinvigorated interest in the commercial production of other microalgal products and new applications of microalgae. A deep understanding of algal physiology is one of the most important factors in the development of new species and products for commercialisation.

In 1962 the first book to comprehensively review the research on the physiology and biochemistry of algae edited by Ralph Lewin was published (Lewin 1962), following on from the earlier small, but important, monograph on algal metabolism of Fogg (1953). Both of these books are still worth reading. The next major volume on this topic was *Algal Physiology and Biochemistry* edited by WDP Stewart published in 1974 (Stewart 1974). All of these books covered both the microalgae and the macroalgae.

Stewart in the preface to his volume noted:

> Ten years ago it would have been possible to include in a book of this type, over 90 per cent of the relevant aspects of algal physiology and biochemistry but this is no longer the case.

It has now been 41 years later, and clearly it is impossible to include in a single book all relevant aspects of algal physiology, and it is therefore not surprising that since the publication of Stewart's book, no comprehensive book on algal physiology has been published, only reviews on particular topics and general chapters in a number of broader ranging books on algae. However, we strongly feel that there is a need for a reasonably comprehensive up-to-date reference work on algal physiology and biochemistry for the use of researchers in the field, both old and new. Such a reference work is probably now more important than ever, as few people have the time and capacity to keep up to date with the massive literature that has accumulated on algal metabolism and related topics. The days of generalist phycologists are past, and for a variety of reasons, researchers have needed to become more specialised. However, whatever the specific field of algal research, it is often important and instructive to consider one's work in a broader context.

Given the mass of knowledge on algae and their physiology and biochemistry that has been accumulated in the last 40 years, we had to make two decisions in the planning of this book. First, we decided to limit the scope to the microalgae, i.e. those algae one generally needs a microscope to see. Second, as it is impossible to cover all possible topics, we selected what we consider the major aspects of microalgal physiology. There are many important topics which are not covered, but we hope that these will be part of future volumes.

We invited a range of leading researchers to write authoritative review chapters on critical aspects of algal physiology and biochemistry. These range from the studies on the cell cycle and advances in our understanding of cell wall biosynthesis, through fundamental processes such as light harvesting and assimilation of carbon and other nutrients, to secondary metabolite production and large-scale cultures of microalgae and genomics. We also tried to ensure that all species names used were those currently accepted, and we have included a chapter which lists both the old and new names (as well as a plea to provide adequate information on strains used when publishing) to help researchers in finding all relevant literature on a particular species. The authors were given a relatively free hand to develop their topic, and we feel that the variety of approaches leads to a more interesting and useful book. We are very grateful to all those people we have cajoled into contributing to this enterprise and the many people who aided by reviewing particular chapters.

Our intention is that this book serves as a key reference work to all those working with microalgae, whether in the laboratory, in the field, or growing microalgae for commercial applications. The chapters are intended to be accessible to new entrants into the field (i.e. postgraduate students) as well as being a useful reference source for more experienced practitioners. We hope that the book thoroughly deals with the most critical physiological and biochemical processes governing algal growth and production and that any omissions do not disappoint too many readers. It is our hope that you find the information here as stimulating as we do – microalgae are exciting organisms to work with!

Murdoch, WA, Australia — Michael A. Borowitzka
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Dundee, UK — John A. Raven
June 2015

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Part I

The Algae Cell

The Cell Cycle of Microalgae

Vilém Zachleder, Kateřina Bišová, and Milada Vítová

Abbreviations

CDK	cyclin-dependent kinase
chl-RNA	chloroplast ribosomal RNA
cyt-RNA	cytosolic ribosomal RNA
CKI	inhibitor of cyclin-dependent kinase
CP	commitment point
DP	dimerization partner
E2F	transcription factor
FdUrd	5-fluorodeoxyuridin, inhibitor of thymidylate synthase
NAL	nalidixic acid, an inhibitor of DNA gyrase, (1-ethy1-1,4-dihydro-7-methy1-4-oxo-1,8-naphtyridine-3-carboxylic acid)
nuc-DNA	nuclear DNA
pt-DNA	chloroplast (plastid) DNA
Rb	retinoblastoma protein

1 Introduction

Algae are a unique group of organisms displaying a wide variety of reproductive patterns. Various division patterns can be found from simple division into two cells, similar to yeast (binary fission), to the formation of four and up to several thousand daughter cells in a single cell cycle in green algae dividing by multiple fission. In some algal species, both binary and multiple fission can be observed in the same organism, either under different growth conditions (Badour et al. 1977) or at different phases of the life cycle (van den Hoek et al. 1995). Furthermore, wide-ranging body organizational structures exist in algae, from unicellular organisms (microalgae) to multicellular ones resembling higher plants (macroalgae) with a very complex body shape built by morphologically distinct cells having various physiological roles. This section will deal only with the vegetative cell cycle of unicellular green algae, existing as single cells or gathered into coenobia (where daughter cells arising from a single mother cell stay connected together), colonies or filaments, but independent of each other. Although 60 years have passed since the first studies of the algal cell cycle (Tamiya et al. 1953), possible ways in which algae can still contribute to research into the biology of cell cycles are far from exhausted. The seemingly narrow range of these organisms provides such a broad variety of reproductive patterns that, in spite of extensive literature, they still represent a challenge for future researchers in cell cycle biology. The aim of this section is to summarize the significant progress made, from early historical findings up until the last few years, and to highlight the hidden potential of algae for the future.

About 60 years ago, chlorococcal algae of the genus *Chlorella* were among the first microorganisms to be successfully grown in synchronous cultures (Lorenzen 1957; Tamiya et al. 1953) and used for biochemical and physiological analyses of the cell cycle. The first experiments were therefore carried out at the same time that Howard and Pelc first separated the cell cycle into four phases G1, S, G2 and M (Howard and Pelc 1953). From the early years, other green algae, *Desmodesmus* (*Scenedesmus*) and *Chlamydomonas* also formed prominent cell cycle models (Lien and Knutsen 1979; Lorenzen 1980; Šetlík et al. 1972; Tamiya 1966). Their multiple fission reproductive patterns are, as is described below, rather different from the patterns terminated by binary fission that are characteristic of most eukaryotic cells. The multiple fission cell cycle and mechanisms governing its regulation are the most important contributions that algal cell cycle studies have made to the general field of cell cycle research.

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2 Types of Cell Cycle of Microalgae

2.1 Cycle Type C1

The purpose of the cell cycle is to consistently reproduce all cellular structures in order to produce a new daughter cell. Such a reproductive sequence normally comprises the following steps: growth, DNA replication, nuclear division, and cellular division or protoplast fission (Mitchison 1971). In the growth step, the cell builds up functional structures and accumulates reserves. At the end of this, the cell attains a critical size and content of essential constituents, including energy reserves; such a cell becomes competent to proceed through the reproductive sequence even in the absence of further growth. This is referred to as attainment of **commitment point** to divide. While the rate at which a cell attains commitment is tightly correlated with growth rate (in autotrophic algae, via photosynthesis), once the cell is committed, the two processes become independent. It is therefore convenient to divide the cell cycle of algae into **pre-commitment** and **post-commitment** periods. From now on, the term **DNA replication-division sequence** will be used for the sequence of processes and events that take place after the commitment point. Each step in the DNA replication-division sequence is comprised of a preparatory and an executive phase. The latter include DNA replication, and the morphologically well characterized stages of mitosis and cytokinesis. The events constituting the preparatory phases, in spite of intensive studies, are not yet completely characterized. Accumulation of deoxynucleotides in a pool, and of a sufficient number of molecules of a replicating enzyme, must precede actual DNA replication as a prerequisite for mitosis and cytokinesis. It is not difficult to establish the timing of the executive phases of individual events, however, exactly where and when the events of their corresponding preparatory phases are located and triggered is, in most cases, uncertain. The general impression is that the preparatory processes of DNA replication and nuclear and cellular division start soon after the commitment point and overlap with each other.

The classical cell cycle describes the basic organization of the cycle in cells dividing by binary fission (Howard and Pelc 1953); it is illustrated as a sequence of four phases: G1, S, G2 and M (Fig. 1a). This cell cycle organization, where the mother cell divides into two daughter cells, is common to most algae, particularly the filamentous ones (Fig. 2). For some algae, the mother cell can also divide into more than two daughter cells, in a process called multiple fission. Binary fission is denoted here as the C_1 cell cycle. This terminology is based on the fact that the cells can generally divide into 2^n, where n is an integer. For binary fission, $n=1$, thus this cell cycle can be designated as C_1. The more general cell cycle pattern, C_n, or multiple fission, is described in detail in the next section. The classical cell cycle (C_1) scheme can be modified in some organisms, like the budding yeast, where S and M phases overlap without an intervening G2 phase (Forsburg and Nurse 1991), or under some conditions such as in embryonic development, where rapid cell cycles consist of only alternating S and M phases without any gap phases (Hormanseder et al. 2013; Newport and Kirschner 1982, 1984). However, the basic rule of one mother cell giving rise to two daughters is always kept. Similarly to these organisms, cell cycle organization in green algae also requires additional features to be added to the classical cell cycle scheme (Fig. 1). The first novel characteristic is the commitment point (CP).

The existence of commitment points in algae became clear from experiments involving transfer into the dark. If algal cells are put into the dark at different time-points during their G1 phase, their behavior differs significantly. Cells darkened at early times stay the same, even after prolonged time periods. In contrast, at later time-points, the cells acquire the ability to divide in the dark without an external energy supply (John et al. 1973; Šetlík et al. 1972). The point (or stage) in the cell cycle when cells became competent to duplicate reproductive structures (DNA, nuclei) and to divide was, in early works, called variously the "point-of-no-return" (Moberg et al. 1968), "induction of division" (Šetlík et al. 1972) "transition point" (Spudich and Sager 1980) or "commitment point" (John 1984, 1987); recently only the last term has been generally accepted. Clearly, commitment point (CP) is of outmost importance for cell cycle progression and the algal cell cycle can be very simply split into pre- and post-commitment periods. The rules governing CP are similar to those found for Start in yeasts and the restriction point in mammalian cells (Fujikawa-Yamamoto 1983; Sherr 1996; Sherr and Roberts 1995). CP is thus considered a functional equivalent of both key decision points (John 1984).

The second typical feature of the algal cell cycle is directly related to the existence of CP. The "gap" phase following attainment of CP, prior to DNA replication, starts completely differently from the G1 phase preceding the commitment point. It corresponds to the preparatory phase for DNA replication. This phase also occurs in other organisms, (sometimes termed the late G1 phase), where its character, distinct from the preceding G1 phase, is well recognized. In cell cycle models illustrated in Fig. 1, this phase is termed a pre-replication phase (pS) (Zachleder et al. 1997). The main characteristic of this phase of the algal cell cycle (in contrast to the G1 phase) is that no growth processes or external energy supplies are required. Formation of the pre-replication protein complex in chromosomes, and the activation of S-phase CDKs (cyclin-dependent kinase), seems to be part of this phase in frogs and yeasts (Nasmyth 1996; Sherr 1995, 1996; Sherr and Roberts 1995). Maximum activities of

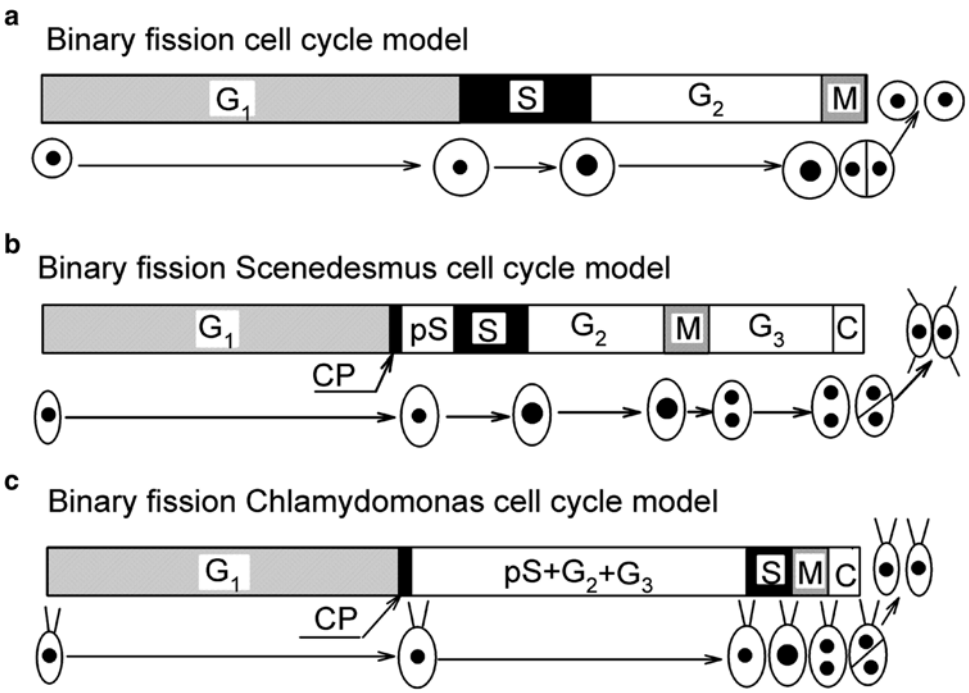

Fig. 1 Diagrams showing different types of cell cycle phases, including the classical cell cycle model and those found in *Desmodesmus* (*formerly Scenedesmus*) *quadricauda* and *Chlamydomonas*, which divide into two daughter cells. (**a**) Classical type cell cycle after Howard and Pelc (1953), (**b**) Scenedesmus-type cell cycle after Šetlík and Zachleder (1984), and (**c**) Chlamydomonas-type cell cycle after Zachleder and van den Ende (1992). Individual *bars* show the sequence of cell cycle phases and events during which growth and reproductive processes take place. Only one sequence of events leading to the duplication of cell structures occurs during the cycle of cells dividing into two daughter cells (*Panels* **a**, **b**, **c**). Thus all of the schemes correspond to a C_1 type of cell cycle (number of daughter cells is 2^1). Schematic pictures of the cells indicate their size during the cell cycle and the *black circles* inside illustrate the size and number of nuclei. Large *black spots* indicate a doubling of DNA. The *lines* at the terminal cells of *Desmodesmus* (*Scenedesmus*) coenobia represent spines typical for the species *D. quadricauda*. The *lines* at the *top* of the *Chlamydomonas* cells represent flagella, which are retracted by the cells before DNA replication begins. **G1**: the phase during which the threshold size of the cell is attained. It can be called a **pre-commitment period** because it is terminated when the commitment point is reached. **CP**: the stage in the cell cycle at which the cell becomes committed to triggering and terminating the sequence of processes leading to the duplication of reproductive structures (**post-commitment period**), which consists of: **pS**: the pre-replication phase between the commitment point and the beginning of DNA replication. The processes required for the initiation of DNA replication are assumed to happen during this phase. **S**: the phase during which DNA replication takes place. **G2**: the phase between the termination of DNA replication and the start of mitosis. Processes leading to the initiation of mitosis are assumed to take place during this phase. **M**: the phase during which nuclear division occurs. **G3**: the phase between nuclear division and cell division. The processes leading to cellular division are assumed to take place during this phase. **C**: the phase during which cell cleavage and daughter cells formation occurs. In *Chlamydomonas*, apparent G2 and G3 phases are missing; it can, however, be assumed that all the required processes happen during the prolonged gap phase, which is thus denoted pS+G2+G3, for more details see text (Modified after Zachleder et al. 1997)

CDKs were also observed at commitment points in *Chlamydomonas reinhardtii* (Zachleder et al. 1997).

In some algae, there is a relatively long phase separating nuclear division and cleavage of the cells. This requires a third modification of the classical cell cycle. The term G3 phase seems to be an appropriate designation for this phase (Fig. 1b) (Zachleder et al. 1997).

Chlamydomonas has a very specific cell cycle, somehow resembling that of some embryos. It lacks apparent G2 and G3 phases since the S- and M-phases and cell cleavage occur nearly immediately after each other. However, all the preparatory processes for DNA replication, nuclear and cellular division must, by definition, precede the processes themselves. This is in line with the continuum concept of Cooper (1979, 1984), which is described in more detail below, stating that the preparatory processes do not necessarily immediately precede their respective phases but are performed continuously throughout the cell cycle, and the gap phase is only a manifestation of processes not yet completed. It can therefore be assumed that the processes from "missing" phases take place during the gap phase, between the time of commitment point attainment and the initiation of DNA replication. This phase has been designated as pS+G2+G3 (Fig. 1c) (Zachleder et al. 1997).

2.2 Cycle Type C_n

In the previous section, the C_1 cell cycle type was introduced, where the mother cell divides into two daughter cells; many

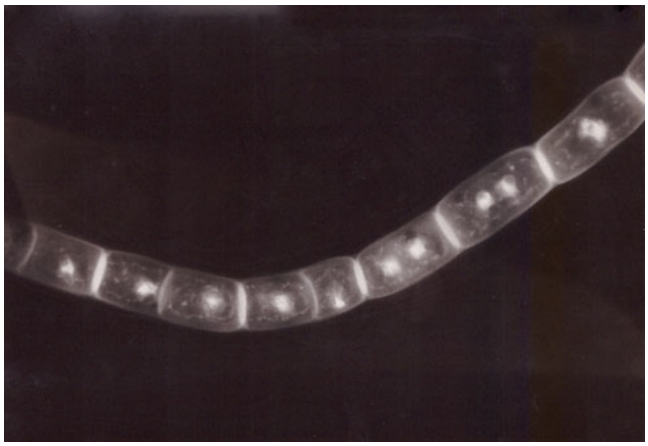

Fig. 2 Fluorescence photomicrographs of the green filamentous alga *Microspora* sp. (Ulotrichales) stained with DAPI. Different phases of the cell cycle and nuclear division can be seen in individual cells of the filament. Nucleoids are localized in chloroplasts along the cell wall (After Zachleder and Cepák 1987c)

algae divide into more than just two daughter cells in a modified cell cycle, denoted as the multiple fission cycle. Generally, any division will occur into 2^n daughter cells (cycle type C_n), where *n* is an integer from 1 to 15. The C_1 and C_n cell cycle types are, in some species, interchangeable and the one that will be used for division depends solely on growth rate. Cells grown under unfavorable growth conditions, with a low growth rate, will divide into only 2 (n = 1, C_1) daughter cells while the same cells, when grown under optimal conditions, can divide into 8 (n = 3, C_n) or 16 (n = 4, C_n) daughter cells. Although C_n cell cycle types also occur in other organisms, their exclusive use for vegetative reproduction of cells in many taxonomic groups of algae is unique. C_n cycles are characteristic for most cells in the algal orders Chlorococcales and Volvocales, such as *Chlorella*, *Desmodesmus*, *Scenedesmus*, and *Chlamydomonas*. These algae became popular in cell cycle studies (Lorenzen 1957; Tamiya 1966) because they can be easily synchronized by alternating light and dark periods, a procedure that is considered natural and where induced synchrony is very high. Due to the presence of multiple DNA replications, nuclear and cellular division, the cycle is much more complex than the classical scheme, and has a number of modifications. Importantly, there is extensive overlapping of genome duplication by DNA replication, genome separation by nuclear division, and cell division. It is even more complex since cell cycle processes are coordinated with equivalent processes in both mitochondria and chloroplasts. It has become increasingly evident that the "classical" scheme, as originally proposed by Howard and Pelc (1953), is inadequate for interpretation of C_n cell cycles types. Interestingly, the C_n cell cycle shares some common features with the prokaryotic cell cycle (Šetlík et al. 1972). This notion was supported by Cooper who, based on extensive studies of bacterial and eukaryotic cell cycles (Cooper 1990; Cooper and Helmstetter 1968; Helmstetter and Cooper 1968; Helmstetter et al. 1968; Liskay et al. 1979, 1980; Singer and Johnston 1981), proposed a unifying concept that assumes some common principles in the control of eukaryotic and prokaryotic cell cycles (Cooper 1979, 1984). Similarly to the reproductive sequence concept introduced above, he argues that the cell cycle, generally perceived as a "cycle" since the same sequence of events happens in mother and daughter cells, is not a "cycle" but rather a sequence of events repeating themselves in each cell (Cooper 1979, 1984, 1987). Moreover, since it is not a "cycle" but rather a continuum, some of the events comprising each sequence may occur within the mother cell; this is particularly true of the growth step and all the preparatory phases of the DNA replication-division sequence. Research findings on the cell cycle of algae dividing by multiple fission fit well into Cooper's unifying hypothesis. An understanding of cell cycle events as a sequence of processes not necessary bound to some specific gap phases of a classical cell cycle, nor to the boundary of a single cell cycle, represents the best way for grasping mechanisms by which complex algal cell cycles are governed.

In line with Cooper's predictions, the main difference between cell cycles of organisms dividing by binary or multiple fission is that in the latter case, multiple commitment points are attained during a single cell cycle. Each of the commitment points is preceded by growth to a threshold size (critical size), followed by a single DNA replication-division sequence. For each consecutive commitment point, a certain critical cell volume exists at which the commitment point is attained. A critical cell volume for a given commitment point is approximately twofold that of the previous one (Šetlík et al. 1972; Šetlík and Zachleder 1984). Growth is clearly a prerequisite for attaining consecutive commitment points. When a DNA replication-division sequence committed by the first commitment point attains a certain phase (preparation for protoplast fission), further commitment points cannot be attained and all committed reproductive sequences are terminated by the formation of daughter cells. However, until this phase, additional commitment points will be attained, provided that growth is sustained by continuous or prolonged illumination.

Obviously, to describe such a complex cell cycle in terms of the classical G1, S, G2, and M phases (Howard and Pelc 1953) will require major modifications (Fig. 3).

The gap phases, according to Cooper, are simply a manifestation of the fact that the preparatory processes for DNA replication (late G1 phase) and nuclear and cellular division (G2 phase) are not yet complete. Additionally, in many algal species or strains, particularly those with C_n type cycles,

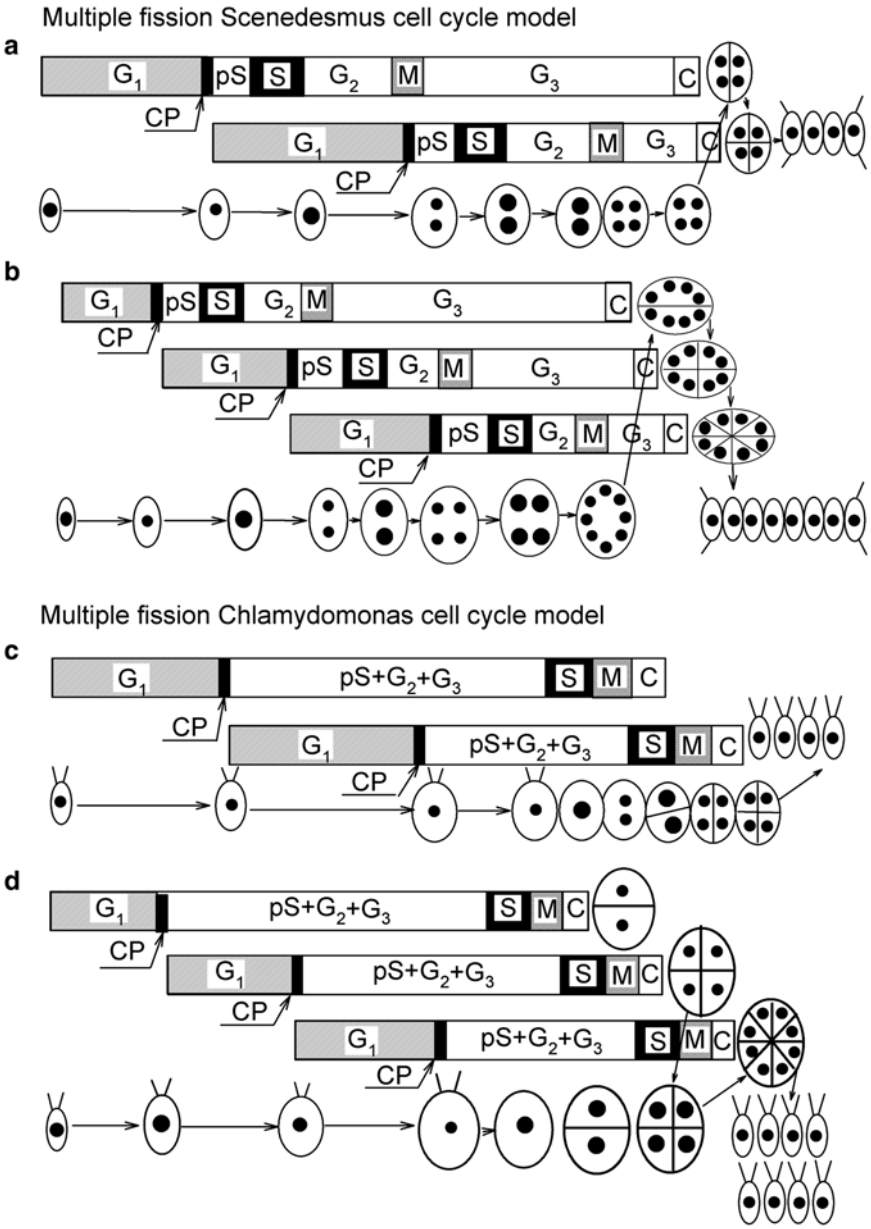

Fig. 3 Diagrams showing different types of cell cycle phases found in *Desmodesmus* (*Scenedesmus*) and *Chlamydomonas* dividing by multiple fission (cell cycle type C_n). (**a**, **b**) Scenedesmus-type cell cycle after Šetlík and Zachleder (1984), and (**c**, **d**) Chlamydomonas-type cell cycle after Zachleder and van den Ende (1992). For description of figure characteristics see Fig. 1. Two (**a**, **c**) or three (**b**, **d**) partially overlapping sequences of growth and reproductive events occur within a single cycle in cells dividing into four daughter cells (**a**, **b**) or eight daughter cells (**b**, **d**) (Modified after Bišová and Zachleder 2014)

nuclear divisions are followed by additional "gap" phases, during which time, processes leading to cytokinesis (protoplast fission and daughter cell formation) occur, and are designated as G3 phase (Zachleder et al. 1997). Various external or internal factors can stop further cell cycle progress during this phase, just after nuclear division is terminated, implying a control mechanism involved in the regulation of cell division. Also arising from Cooper's concept of a continuum is the fact that if some of the gap phases are missing for a particular cell cycle type, it can be assumed that processes usually performed during these phases run concurrently with processes of other phases. For example, in organisms where cell division occurs immediately after mitosis, the processes leading to cell division can be assumed to take place during G2, together with the processes leading to mitosis. In algae dividing into more than two daughter cells, the cell cycle model must also be modified to take into account overlapping or parallel courses of entire phases of consecutive sequences of growth and reproductive events (Fig. 3).

In the C_n types of cell cycle, two distinct patterns of cell cycle phases can be distinguished:

One is typical for *Desmodesmus* and *Scenedesmus* and can be called a **consecutive** pattern (**Scenedesmus-type cell cycle**). As presented schematically in Fig. 3, the cells replicate DNA shortly after attaining a commitment point, then nuclear division follows. If more than one commitment point is attained, several rounds of DNA replication and nuclear divisions occur consecutively during the cell cycle, and cells

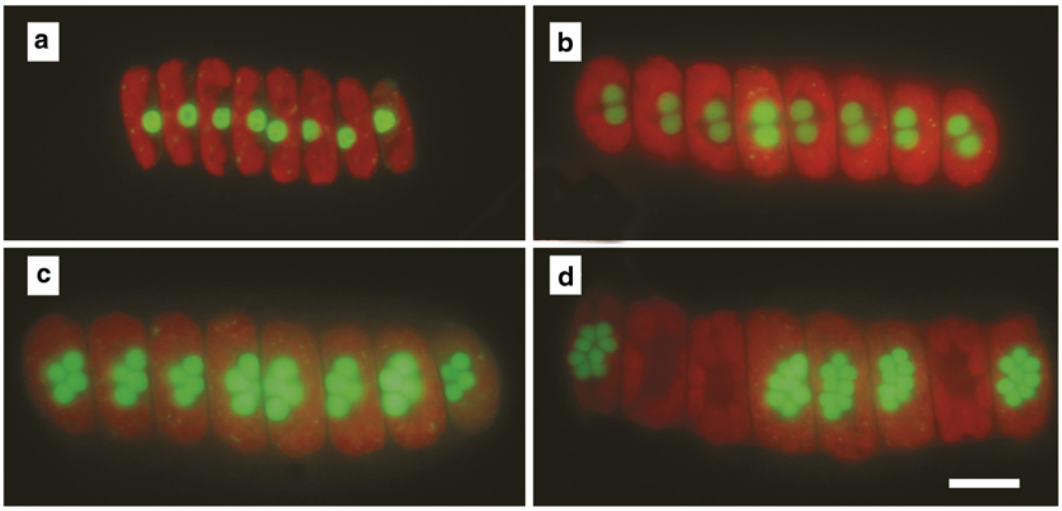

Fig. 4 Fluorescence photomicrographs of eight-celled coenobia of *Desmodesmus* (*Scenedesmus*) *quadricauda* during the cell cycle, stained with 0.3 % SYBR *green* I dye. Nuclei are visible as *yellow-green spots*. Chloroplasts are visible as a *red color*, which is autofluorescence of chlorophyll. (**a**) Uninuclear daughter coenobium, (**b**) binuclear coenobium. (**c**) Tetranuclear coenobium. (**d**) Mother octanuclear coenobium. Cells already dividing protoplasts remained unstained. *Scale bar* = 10 μm (Modified after Vítová et al. 2005)

become polynuclear because mitoses follow, a relatively short time after the attainment of consecutive commitment points (Figs. 3a, b and 4). Then, during the cycle in which *Desmodesmus quadricauda*[1]cells divided into eight daughters, the nuclei are distributed in an octuplet coenobium. The uninuclear daughter cell (Fig.4a) passed the first commitment point, quickly followed by the first committed mitosis, to become binuclear (Fig. 4b). It then consecutively attained another two commitment points and a second mitosis came about. The cell continued in the cycle as tetranuclear (Fig. 4c), with the third mitoses occurring after the preceding third commitment point (Fig. 4d), and octanuclear cells entered protoplast fission, forming an octuplet daughter coenobium.

The second cell cycle pattern is typical for *Chlamydomonas* and can be called a **clustered** pattern (**Chlamydomonas-type cell cycle**). As can be seen schematically in Fig. 3c, d and in the photos in Fig. 5, no nuclear division occurred until very late in the cell cycle (the same is true for DNA replication, see next section). However, similarly as in the Scenedesmus-type cell cycle, several commitment points can be attained during the cell cycle, leading to multiple rounds of DNA replication, mitoses and protoplast fissions clustered at the very end of the cell cycle. In Fig. 3, the time course of three consecutive *Chlamydomonas* reproductive processes is shown. Photomicrographs of multiple clustered nuclear divisions, followed nearly immediately by daughter cell formation, are presented for the cell cycle where 4 commitment points were attained and nuclei divided four times, forming 16 daughter cells by the end of the cell cycle (Fig. 5).

Arising from the preceding text, progress in commitment point studies provides key information on regulation of the cell cycle. The principle of determination of commitment point in algal culture is based on the fact that attaining commitment point is dependent on light as an energy source while the post-commitment processes (DNA replication, nuclear and cellular division) are light-independent. Subcultures are exposed to light periods of increasing length and the average number of cells formed in successively darkened subpopulations is followed. This number depends on the light intensity and the length of illumination. Cells in the successively darkened samples do not start division immediately upon darkening since they must first undergo all the preparatory processes for cell reproduction. In samples withdrawn from the culture early in the cycle, it may take several hours before cell division sets in. But under physiological conditions, they will ultimately divide, i.e. they are committed to divide. The results collated from synchronized algal populations with time are called commitment diagrams or commitment curves (Fig. 6). To construct them, samples are withdrawn from a synchronously growing culture at regular intervals (as a rule, 1 or 2 h), and incubated in darkness under aeration at the temperature of the culture (Fig. 7). After a

[1]Wherever possible the currently accepted names for species are used. The name used in the paper cited is also indicated. For details of names see chapter "Systematics, Taxonomy and Species Names: Do They Matter?" of this book (Borowitzka 2016).

Concerning this chapter, genus *Scenedesmus* was re-assessed giving rise to two genera: *Scenedesmus* and *Desmodesmus* (An et al., 1999). Species formerly known as *Scenedesmus quadricauda* was re-classified as *Desmodesmus quadricauda*. The species has been for many years used as an important model organisms and has been referred mostly as *Scenedesmus quadricauda*. For the sake of clarity, the text referring to such publications states the current genus name *Desmodesmus* with the former name *Scenedesmus* in parentheses.

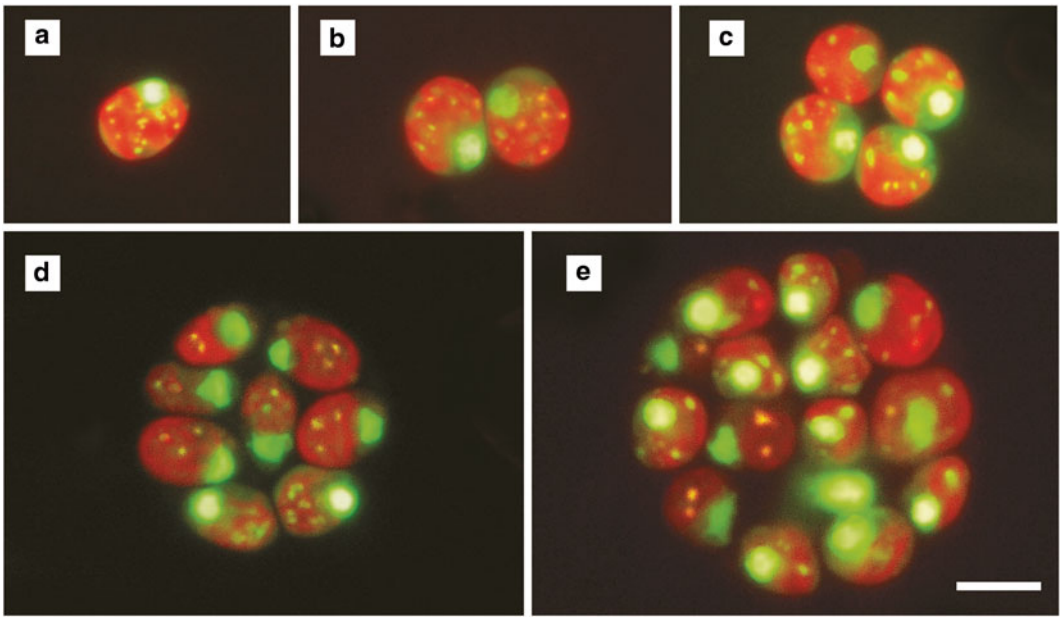

Fig. 5 Fluorescence photomicrographs of *Chlamydomonas reinhardtii* showing multiple division of protoplasts during the cell cycle. Stained with 0.3 % SYBR *green* I dye. (**a**) Uninuclear daughter cell. The nucleus is visible as a *yellow-green spot* and chloroplast nucleoids as tiny *yellow-green dots*. The chloroplast is visible in *red color*, which is due to autofluorescence of chlorophyll. (**b**) The first division of the protoplast; protoplast divided onward into two. (**c**) The second division of protoplasts; two protoplasts divided onward into four. (**d**) The third division of protoplasts; four protoplasts divided onward into eight. (**e**) The fourth division of protoplasts; eight protoplasts divided onward into 16 cells. *Scale bar* = 10 µm (Modified after Vítová et al. 2005)

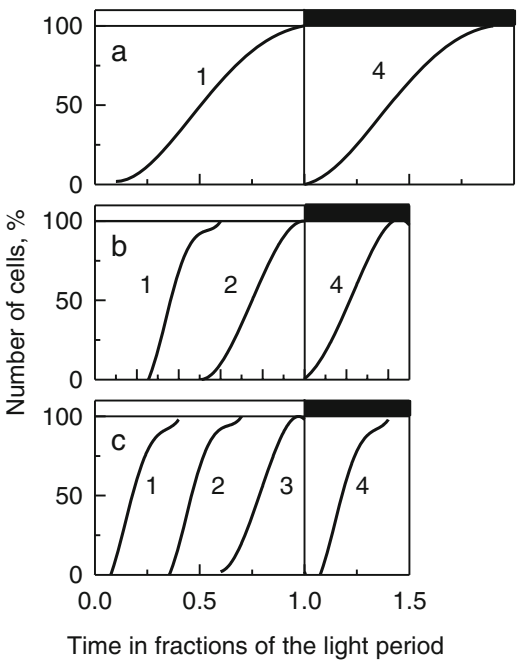

Fig. 6 Schematic drawing of commitment diagrams for three algal populations growing at low (**a**), medium (**b**), and high (**c**) growth rates. Detailed explanation in the text. Curves **1**, **2**, **3**: percentage of cells in the population committed to division into two, four and eight daughter cells, respectively; curves **4**: percentage of cells in the population which have released daughter cells. *White and black strips* above the panels indicate light and dark periods (After Šetlík and Zachleder1983)

time period required to complete all committed processes (e.g. to finish all committed DNA replication-division sequences), they are examined under the microscope. The proportion of divided cells in the population is determined and, in doing so, mother cells that yielded different numbers of daughter cells are recorded separately. The numbers so obtained are plotted against the times at which the respective sample was darkened. In the case of coenobial species such as *Scenedesmus*, counting is very convenient since it can be done even in liquid medium; for other species, the cells are spread on a solid support (e.g. agar plates) and the resulting daughter cell microcolonies attached to the surface are counted. The resulting sigmoidal curves trace the increase in the percentage of committed cells with time. It is important to recognize that the shapes of the curves represent the variability in progress through the cell cycle among cells of the population, and thus characterize the degree of synchrony (Fig. 6).

The number of daughter cells (N_d) in most algae that divide into 2^n daughter cells is usually greater than 2 but the maximum number is rarely more than 32, usually n = 25 (Figs. 8 and 9). The alga *Kentrosphaera* can produce about 2^{10} daughter cells, as illustrated in Fig. 10. There are, however, species such as coenobial algae of the family Hydrodictyaceae (*Hydrodictyon*) and colonial algae of the family Volvocaceae (*Volvox*) that may divide and produce up to several thousands of offsprings (Figs. 11 and 12). Species

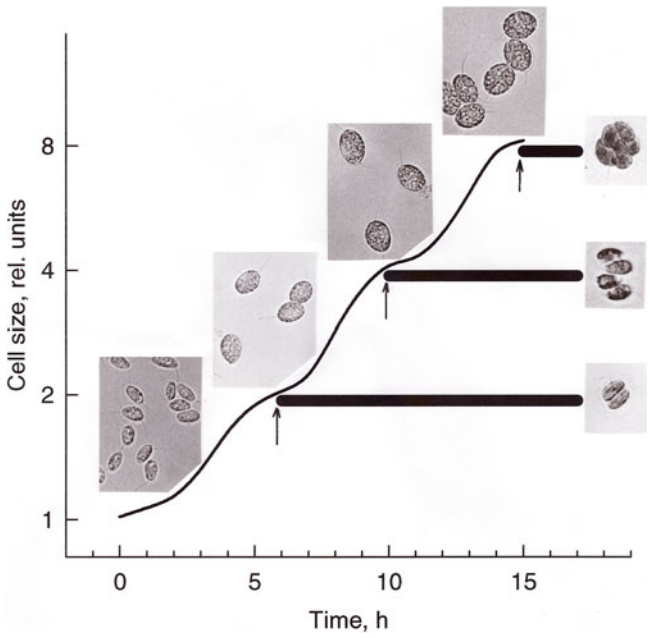

Fig. 7 Schematic illustration of the determination of commitment points to cellular division in synchronous populations of *Scenedesmus armatus*. The idealized *curve* represents the growth of cells in continuous light during the cell cycle; at the times marked by *arrows*, the subcultures were put into dark periods (indicated by *horizontal black stripes*); the microphotographs above the *curve* show typical cells from synchronized cultures at the time of transfer of subcultures into the dark; the *vertical lane* of photomicrographs illustrates (on agar plates) the micro-colonies of daughter cells that were released from one mother cell during the corresponding dark interval. The moments of transfer into the dark correspond to the attainment of the 1st (5 h of light), the 2nd (10 h of light) and the 3rd (15 h of light) commitment points; two, four, and eight daughter cells were released during the dark period, respectively (After Vítová and Zachleder 2005)

with cycle type C_n promise to provide significant results (Kirk 1998), although knowledge of their cell cycles is still limited. The cell cycle type for all algae in the family Hydrodictyaceae and Volvocaceae is of the C_n type. The value of *n* among members of families varies with growth conditions, but does not decrease below a certain lower limit; for the genus *Eudorina* n = 4–6, for the genus *Volvox*, the values of *n* are between 8 and 14, and a similar range characterizes the genus *Hydrodictyon*. Of importance is the fact that algae closely related to these genera have a lower value of *n* under certain conditions, and can also divide into two daughter cells. Thus, over several related species, transition covers the whole range from n = 1 to n = 14. Related to the genus *Volvox*, there are genera for which typical colonies consists of 2 (*Didymochloris*), 4 (*Pascherina*), 8 or 16 (*Ulva*, *Spondylomorum*) cells and their closest relatives are *Gonium*, with 4–16 cells in the colony, *Pandora* with 8 or 16, and *Eudorina* with 1664. The genus *Pediastrum* belongs to the same family as the genus *Hydrodictyon*, whose cells divide into 2–128 daughter cells (n = 1–7), and the genus *Sorastrum* with 8128 daughter cells (n = 3–7). For comprehensive information on suborder Volvocinae algae, see the book "Volvox" (Kirk 1998).

3 Nuclear DNA Synthesis in the Cell Cycle

More than 60 years ago, analyses on the course of DNA synthesis in the synchronized chlorococcal alga *Chlorella ellipsoidea* (Iwamura and Myers 1959), and in volvocalean alga *Chlamydomonas reinhardtii* (Chiang and Sueoka 1967a, b) were first published. This was followed by studies on DNA replication in *Chlorella* (Wanka 1962, 1967; Wanka and Geraedts 1972; Wanka et al. 1972), *Desmodesmus* (*Scenedesmus*) *quadricauda* (Šetlík et al. 1972), and *Chlamydomonas reinhardtii* (Knutsen et al. 1974; Lien and Knutsen 1979).

The number of steps (rounds) of DNA replications is set by the number of commitment points attained and is determined by growth rate. In autotrophically growing cultures, it is light intensity-dependent; the higher the light intensity, the more DNA is synthesized (Donnan and John 1983; Iwamura 1955; Šetlík et al. 1988; Zachleder et al. 1988). While attaining a commitment point is light intensity-dependent, DNA replication itself is light intensity-independent. The ability of cells to replicate DNA can be assessed in dark samples taken from light grown cultures, where the committed DNA is replicated during sufficiently long dark intervals. If plotted against the time of darkening, "committed DNA" can be monitored. It was repeatedly found that rounds of DNA replication are committed in steps. A clear step-wise increase was observed not only in species with a Scenedesmus-type cell cycle but also in species with a Chlamydomonas-type cell cycle, such as *Chlamydomonas reinhardtii* (Donnan and John 1983), supporting the fact that DNA replication is indeed committed separately after each commitment point.

Based on published data, the course of DNA replication in synchronized populations of algae can be divided into two groups, consecutive and clustered.

3.1 Consecutive Rounds of DNA Replication

The increase in DNA content in synchronous populations begins to rise quite early in the cell cycle and has an apparent stepwise character with steps corresponding to consecutive DNA replication rounds (Fig. 13). This course is characteristic for algae with a Scenedesmus-type cell cycle (see preceding chapter) and it has been described in detail in synchronous cultures of *Desmodesmus* (*Scenedesmus*) *quadricauda* (Ballin et al. 1988; Šetlík et al. 1972; Zachleder et al. 1988, 2002; Zachleder and Šetlík 1988); it was also reported in some strains of *Chlorella*, e.g. *Chlorella vulgaris* v. *vulgaris* (Umlauf and Zachleder 1979) and the thermophilic strain of *Chlorella pyrenoidosa* (Vassef et al. 1973).

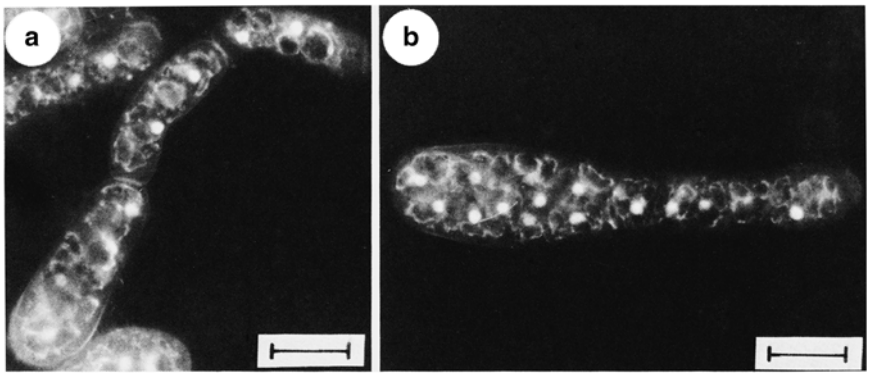

Fig. 8 Fluorescence photomicrographs of the yellow-green alga *Bumilleriopsis filiformis* (Mischococcales) stained with DAPI at different developmental stages of the cell cycle. (**a**) Binuclear and tetranuclear cells. (**b**) Multinuclear cells. Spherical arrangement of nucleoids in the individual chloroplasts can be seen. *Scale bar* = 20 µm (After Zachleder and Cepák 1987c)

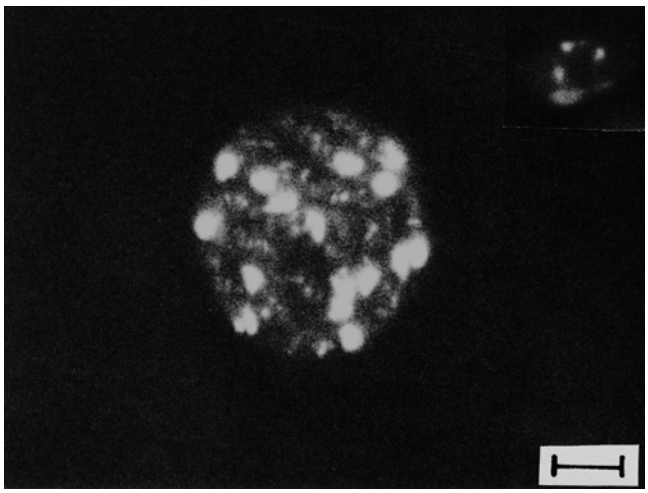

Fig. 9 Fluorescence photomicrographs of the green alga *Nautococcus piriformis* (Tetrasporales) stained with DAPI. A mature mother cell with 16 nuclei and numerous nucleoids. A young cell with one nucleus and four nucleoids is inserted in the top right-hand corner. *Scale bar* = 10 µm (After Zachleder and Cepák 1987c)

If DNA replication occurs in a stepwise mode, the consecutive DNA replications for each committed sequence are distinctly separated by time intervals during which it is assumed that extensive gene transcription occurs. Stepwise DNA replication is clearly connected with periodic fluctuations in the ratios of RNA:protein, and cell volume:DNA, since the ratios repeatedly rise to double values in the intervals between steps of DNA replication and decrease during DNA replication itself (Fig. 14) (Šetlík and Zachleder 1981).

In some synchronized cultures of different species, DNA content increased within a relatively lengthy phase of the cell cycle, and its progression was sigmoidal, with no apparent or only slight steps indicating changes in the rate of DNA replication. This course has been described in some species of *Chlorella* (Iwamura and Myers 1959; Senger and Bishop 1966, 1969) and of *Volvox* (Tucker and Darden 1972; Yates et al. 1975). Even in these cases, however, it cannot be excluded that in a single cell, rounds of DNA replication are separated by relatively long time intervals. Even in synchronized populations, the lack of apparent separation between DNA replication cycles and the DNA content curve could be caused by high variability in cell generation times (Šetlík et al. 1972).

A very important result was that even in synchronized cultures, where the time course of DNA synthesis had a smooth sigmoidal shape without apparent steps, a stepwise increase in DNA content in cells incubated in the dark was found (Zachleder and Šetlík 1988); this was denoted as "committed" DNA synthesis (Fig. 15).

3.2 Clustered Rounds of DNA Replication

For this pattern of DNA synthesis, the time interval in which a single round of DNA synthesis takes place is not much longer than the time required for multiple replications corresponding to the number of duplications; it is characteristic of cells with a Chlamydomonas-type cell cycle (see preceding chapter). The DNA content in synchronized populations increases sharply, in one wave at the end of the cell cycle, to multiples corresponding to the number of daughter cells released by division.

The first publication on this type of DNA replication in the cell cycle of *Chlamydomonas reinhardtii* was in 1967 (Chiang and Sueoka 1967a, b). However, *Chlamydomonas reinhardtii*, belonging to cells with a C_n type of cell cycle, was grown in a synchronous culture under sub-optimal growth conditions that supported only a twofold increase in DNA and consequent division into two daughter cells (cell cycle type C_1). Nevertheless, one wave of DNA synthesis occurring at the end of the cell cycle (Fig. 16) is characteristic of all species with a Chlamydomonas C_n type of cell cycle, even with a much higher value of *n*.

The courses of multiple DNA replications in different strains and mutants of synchronized *Chlamydomonas reinhardtii*, as well as under phosphate limiting conditions, were described in several papers by Knutsen and Lien (1981), Knutsen et al. (1974), and Lien and Knutsen (1973, 1976, 1979); an example of the course of DNA replication multi-

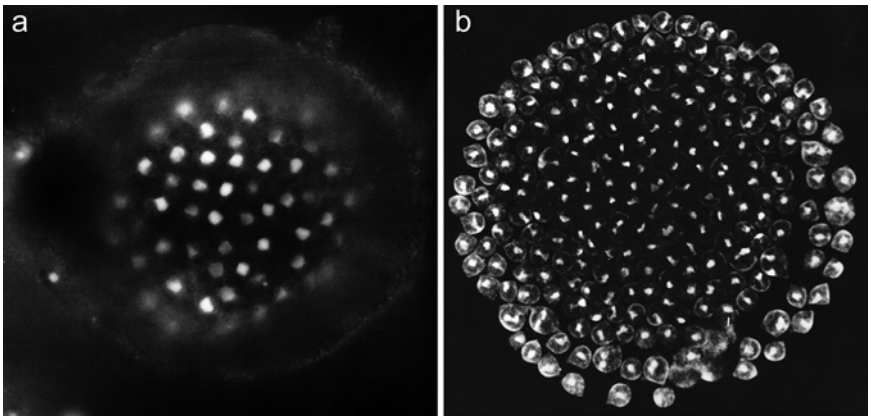

Fig. 10 Fluorescence photomicrographs of the chlorococcal alga *Kentrosphaera* sp. stained with DAPI. (**a**) A giant mother cell with an enormous number of nuclei (showing only those seen in one focal plane). (**b**) Freshly released daughter cells from one mother cell

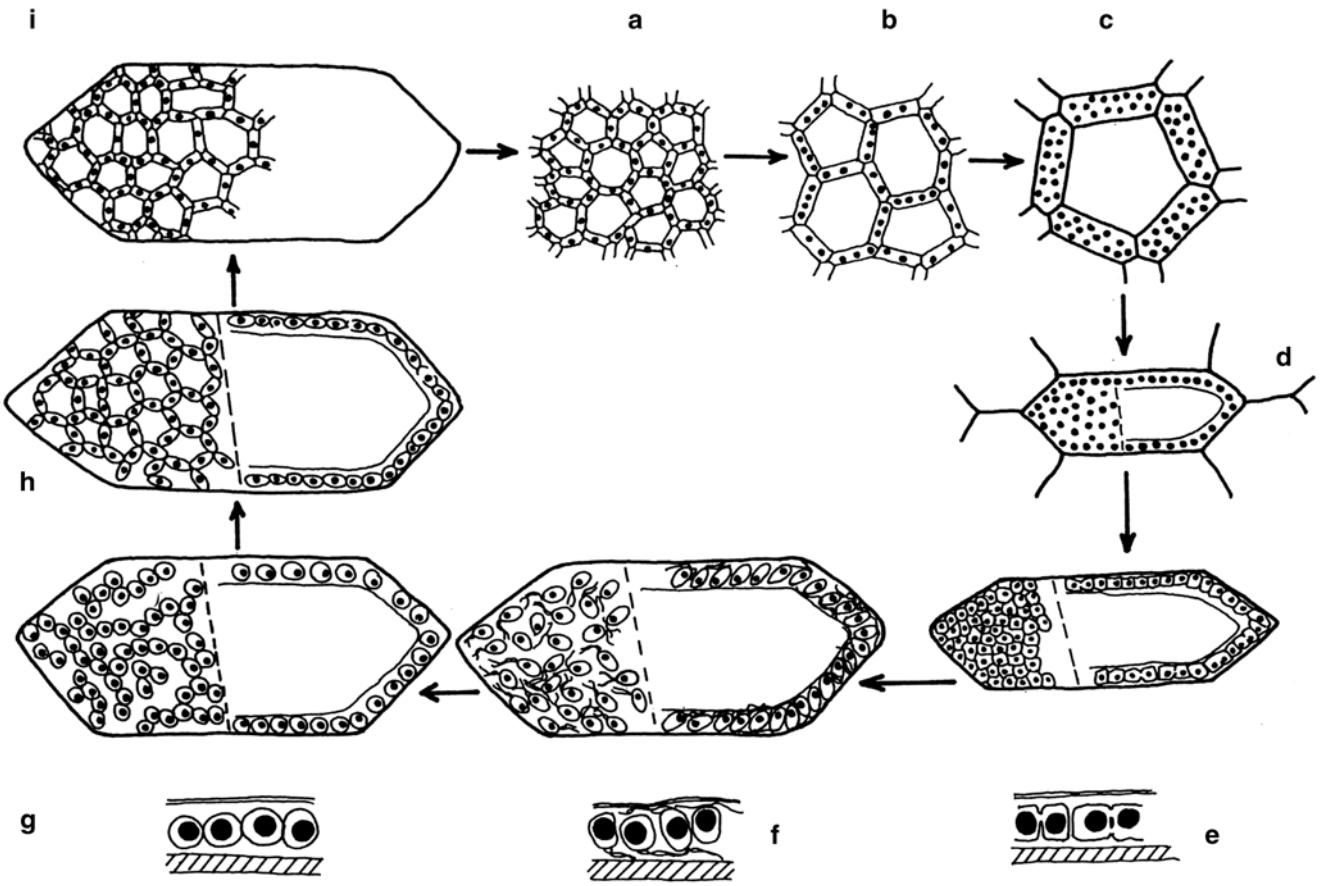

Fig. 11 *Hydrodictyon reticulatus* scheme of the cell cycle. (**a**) Uninuclear daughter cell released from mother cell wall, (**b**–**d**) multiple nuclear division in growing cells, (**e**) division into uninuclear protoplasts, (**f**) formation of biflagellar cell-wall-less zoospores, (**g**) conversion into zoospores without flagella, (**h**) forming of areolate coenobium (After Šetlík and Zachleder 1981)

plying to 16-fold (n=4) in a strain of *Chlamydomonas reinhardtii* is illustrated in Fig. 17. The level of DNA before replication was estimated to be 2×10^{-13} g cell^{-1} and this amount remained constant for the first 89 h of the light phase. Thereafter, during the next 4 h, DNA/cell increased to the same extent as the increase in the average number of offspring, usually 16-fold (Lien and Knutsen 1979).

A similar time course of DNA replication was described not only in *Chlamydomonas reinhardtii* (Lien and Knutsen 1973, 1976) but also in the thermophilic species *Chlorella pyrenoidosa* (Hopkins et al. 1972) and in *Eudorina elegans* (Kemp and Lee 1975).

In all cases, the replication steps followed each other almost immediately and there was no time lag between them

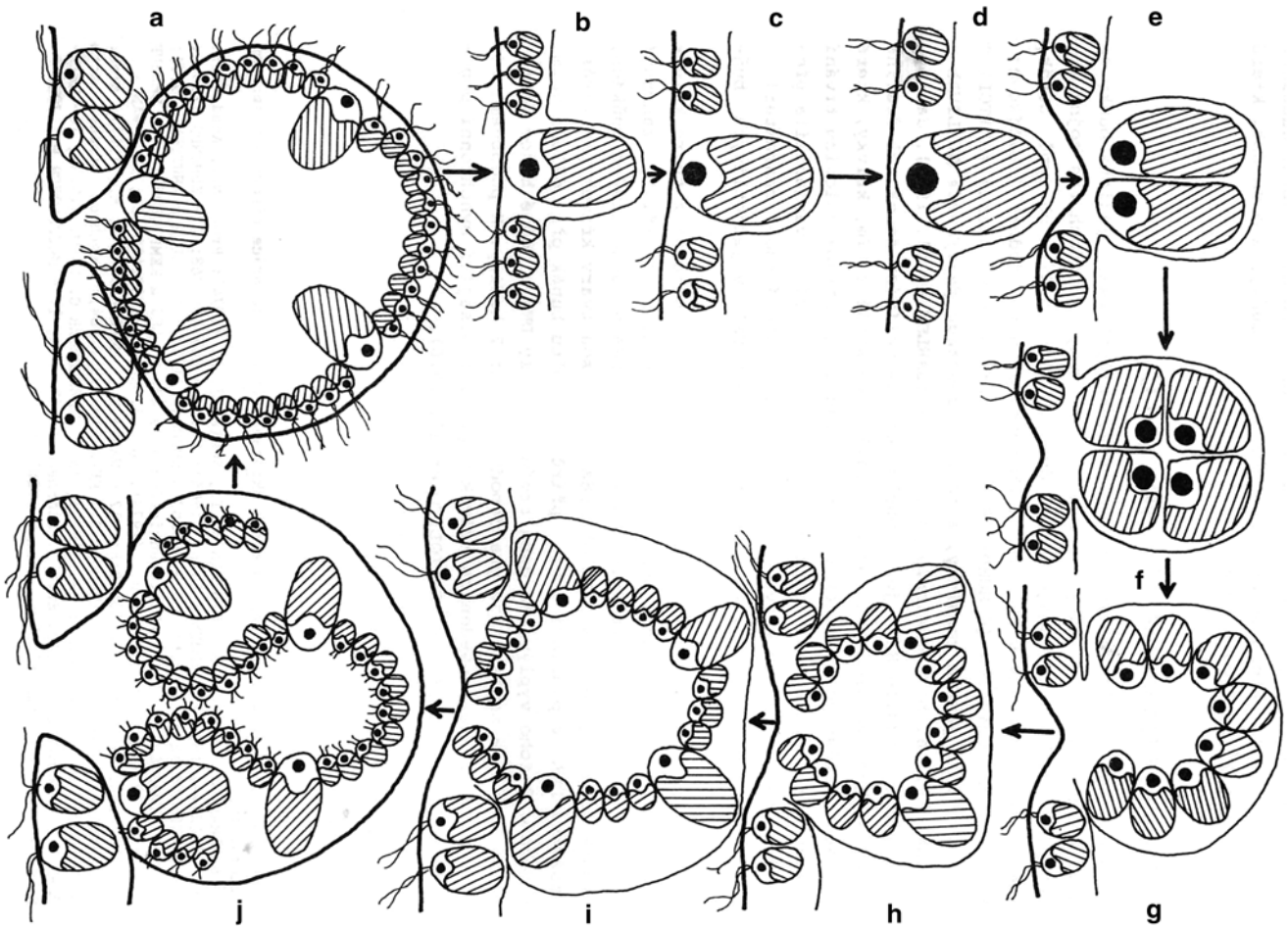

Fig. 12 *Volvox* scheme of the cell cycle. A diagram of the development of a new colony of gonidium for the genus *Volvox*. The young colony, which is released from the wall of the mother cell (**a**) nonflagellated gonidium, considerably larger than the other cells (**a**–**d**) and begins to divide (**e**, **f**). At one of early stages of synchronous division (**g**) unequal cells are formed. One type of cell does not divide more but grows in volume (gonidia), the other continues in division and remains small in volume (vegetative cells). By division of vegetative cells inside the mother cell (**h**, **i**) the final number of cells and the future colony are attained. The colony is inverted (**j**) so that the internal poles of cells occurs on the surface and form flagella. The vegetative cells do not divide any more and will eventually die after colonies are released (After Šetlík and Zachleder 1981)

to allow for any other processes, including massive gene transcription.

Although the two patterns of DNA replication seem well separated, they can merge with each other under specific growth conditions. DNA synthesis in synchronous populations of *Chlamydomonas reinhardtii* growing in the absence of phosphorus occurs in several steps, as opposed to the standard increase in a single wave (Lien and Knutsen 1973). On the other hand, the thermophilic species, *Chlorella vulgaris*, grown under a threshold temperature of 43 °C, has nuclear and cellular divisions blocked, but DNA replication occurs in steps (Šetlík et al. 1975).

4 Regulation of Cell Cycle of Algae

In general, the cell cycle consists of two distinct, but closely interacting, sequences of processes and events. These have been historically termed the "growth cycle" and the "DNA-division cycle" (Mitchison 1971, 1977). In the context of C_n cell cycle types, the "growth cycle" corresponds to a pre-commitment period and the DNA replication-division sequence to a post-commitment period (as already defined in preceding chapters). Most macromolecular syntheses occur during the pre-commitment period, which results in an increase in cell mass and the formation of cell structures.

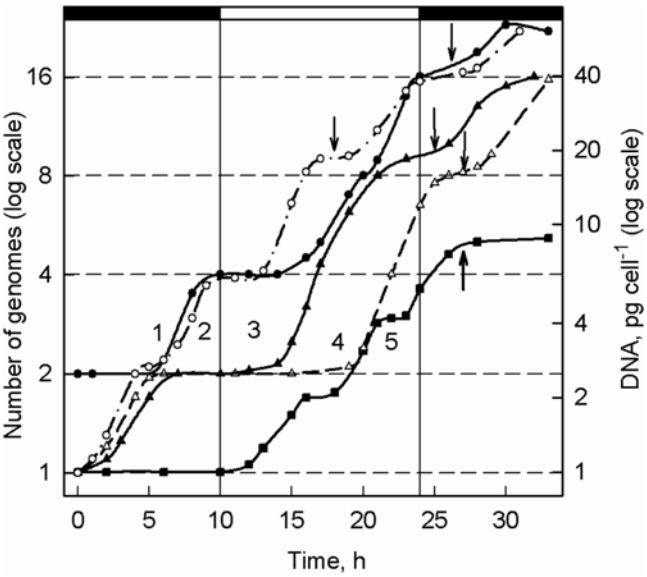

Fig. 13 The stepwise course of DNA replication under conditions of different growth rates and light-dark regimes in the cell cycle of *Desmodesmus* (*Scenedesmus*) *quadricauda*. Positions of the midpoints of cell divisions are indicated by *arrows* and the light-dark periods (for curves 2–5) are indicated by *strips* above the figure and by *vertical lines*. Curve *1*: A synchronized culture grew in continuous light for two cell cycles (dark periods were omitted). The course of DNA synthesis in the second cycle is illustrated. Growth rate = 28 pg of protein cell^{-1} h^{-1}. Curve *2*: The population of the fastest growing (i.e. the biggest) cells was selected by sedimentation from the original strain and allowed to grow under alternating light-dark periods (14:10 h). Growth rate = 32 pg of protein cell^{-1} h^{-1}. Curve *3*: The same culture as illustrated by curve 1 grown under a light-dark regime (14:10 h). Growth rate = 24 pg of protein cell^{-1} h^{-1}. Curve *4*: The daughter cells were obtained from the culture darkened at the 6th h of light (6:8 h). Growth rate = 120 pg of protein cell^{-1} h^{-1}. Curve *5*: The culture grown under alternating light-dark 14:10). Growth rate = 20 pg of protein cell^{-1} h^{-1} (After Šetlík and Zachleder 1983)

The main events in the post-commitment period (DNA replication-division sequence) are: replication of DNA, nuclear division, and cytokinesis, including processes leading to their initiation (for more detail see Sect. 2). While the rate of growth processes depends primarily on the rate of energy supply and raw materials for synthetic processes from outside of the cell, reproductive processes are carried out under standard conditions at a strictly determined rate that is specific to a given organism and depends mostly on temperature (see below).

The main regulatory point separating sequences of pre- and post-commitment is the commitment point. In autotrophically grown algae, it is convenient to define the commitment point as a transition point when the cell becomes capable of division in the dark; more generally, in the absence of an external energy supply. This indicates that algae have a regulatory mechanism ensuring that the reproductive sequence is triggered only if the cell is capable of completing the whole sequence without any external source of energy. However, it must be noted that commitment point is not a point but rather a short part of the cell cycle that consists of several segments: commitment point for DNA replication, commitment point for nuclear division and commitment point for cytokinesis. Usually, all these segments follow so close to each other that the difference is not noticeable. In some situations however, only one or two of them are committed and the cells become temporally arrested with polyploid (only DNA replication committed) nuclei or with multiple nuclei (DNA replication and nuclear but not cellular divisions committed).

The coordination of growth and DNA replication-division sequences appears to be controlled by the achievement of a threshold cell size necessary for the initiation of DNA replication (Nasmyth et al. 1979; Nasmyth 1979). Another cell size control is supposed to be a prerequisite for the onset of nuclear division (Fantes and Nurse 1977; Fantes 1977). It is, however, assumed that it is not the cell size itself, but some other more specific processes that can be coupled or coordinated with the increase in cell size. Synthesis of RNA and protein are the most important features of the growth cycle and both processes are considered to play a major role in the control of cellular reproductive processes via regulation at the commitment point (Alberghina and Sturani 1981; Darzynkiewicz et al. 1979a, b; Johnston and Singer 1978).

The importance of regulation at the commitment point is evident from the behavior of cells blocked in G1 phase due to limiting nutrients or energy supply. Algal cells taken from the stationary phase of asynchronous cultures (which are usually limited by light) are synchronized in G1 phase and thus are often used as inocula for synchronous cultures (Tamiya et al. 1953; Tamiya 1964). Synchronous populations of *Chlamydomonas reinhardtii* and chlorococcal algae grown from the beginning of the cell cycle in mineral medium deficient in nitrogen, sulfur or phosphorus are also blocked in G1 phase (Ballin et al. 1988; Lien and Knutsen 1973; Šetlík et al. 1988; Tamiya 1966; Zachleder et al. 1988; Zachleder and Šetlík 1982, 1988, 1990). Diatoms can be arrested in G1 phase by a deficiency in silicon, which they need to build cell walls; consequently it is crucial for the start of DNA replication (Darley and Volcani 1969; Sullivan and Volcani 1973). Thus, as long as the critical size required for attaining commitment point is reached, no DNA replication-division sequence can take place.

The interdependency between growth processes and cell cycle progression can be assessed by studies of RNA and bulk protein synthesis in synchronized cultures. In control cultures of *Desmodesmus* (*Scenedesmus*) *quadricauda*, the RNA and protein content increased in several steps, each of them corresponding to a doubling of the preceding value (Šetlík et al. 1972; Šetlík and Zachleder 1984; Zachleder et al. 1975; Zachleder and Šetlík 1982, 1988). The number of stepwise increases in both RNA and protein matched the

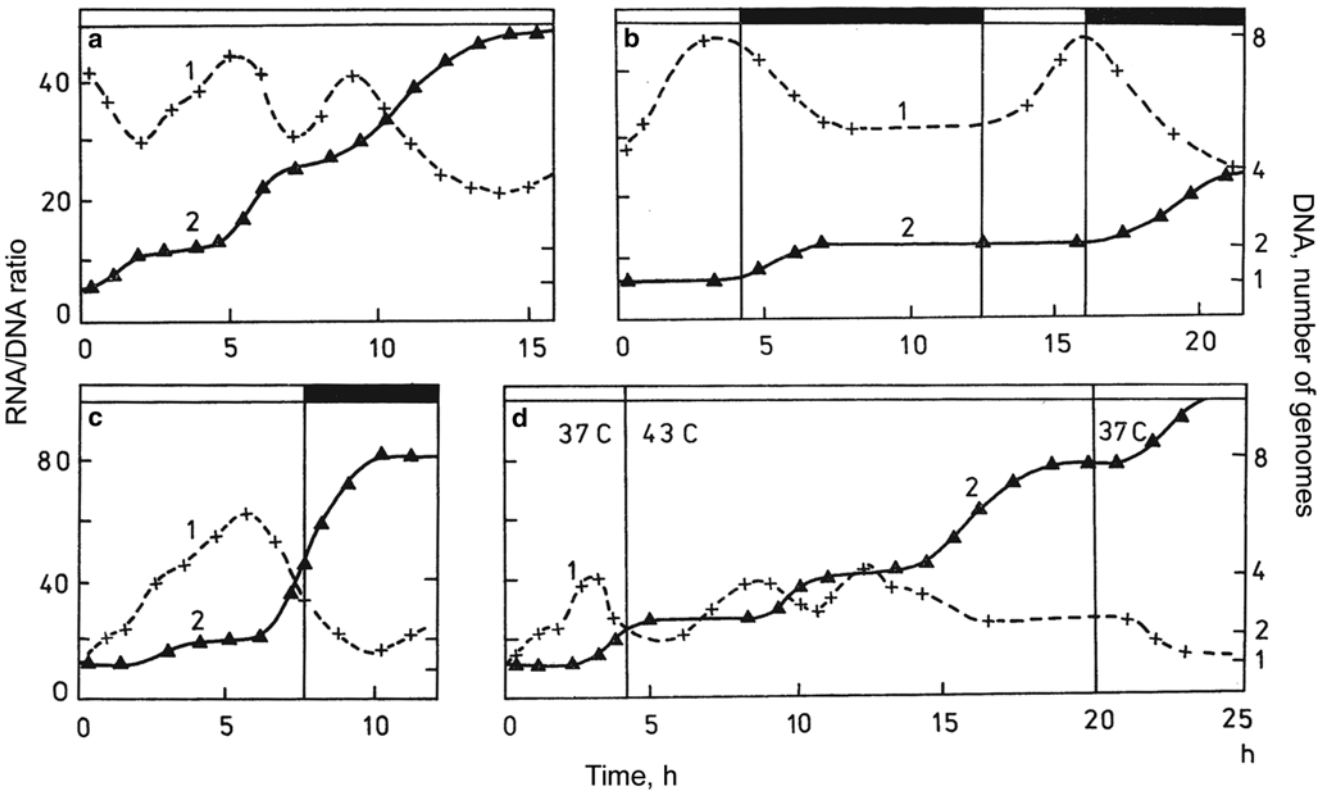

Fig. 14 Changes in RNA to DNA ratio in synchronized populations of *Desmodesmus* (*Scenedesmus*) *quadricauda*. (**a**) Continuous light (**b**) Inserted dark interval separated two growth steps. (**c**) Culture growing under alternating light and dark periods. (**d**) Inserted interval of supra-optimal temperature slowed down the DNA replication rate so that the replication steps are well separated in time. Dark intervals are indicated by *black stripes* and separated by *vertical lines*. *1* the course of the ratio of RNA to DNA, *2* the course of DNA replication (After Šetlík and Zachleder 1981)

number of DNA replication-division sequences that were initiated (Figs. 18 and 19). For both RNA and protein, the maximum of each doubling precedes attaining the commitment point; this implies a threshold amount of both macromolecules has to be reached prior to the cell attaining commitment point.

Similarly, stepwise accumulation of RNA was shown to occur in *Chlamydomonas reinhardtii* (Knutsen and Lien 1981; Lien and Knutsen 1979). The number of steps of RNA accumulation affects the number of DNA replication rounds. Each of these steps, representing an approximate doubling of RNA, is followed shortly thereafter either by a corresponding replication of DNA, as in *Desmodesmus* (*Scenedesmus*) *quadricauda* (Ballin et al. 1988; Šetlík et al. 1988; Zachleder et al. 1988; Zachleder and Šetlík 1982, 1988, 1990) or multiple replication rounds at the end of the cell cycle corresponding to the number of RNA accumulation steps, as in *Chlamydomonas reinhardtii* (Knutsen and Lien 1981; Lien and Knutsen 1979). So the initiation of the DNA replication-division sequence, e.g. DNA replication, nuclear division and cell division, as well as their number, is tightly controlled by growth processes, i.e. by RNA and protein synthesis.

It was mentioned above that the entire DNA replication-division sequence is not always committed and completed so the cells remain undivided with polyploid or have multiple nuclei. How does this occur? Usually in a growth sequence, RNA synthesis precedes protein synthesis for different time intervals. RNA synthesis starts earlier and, in contrast to bulk protein synthesis, can be performed for some time in the dark. By an appropriate choice of cultivation conditions, the two processes can be uncoupled (Fig. 20). It is clear that DNA replication rounds are completed in proportion to the amount of RNA, while nuclei divide in proportion to the amount of protein (Zachleder and Šetlík 1988). Thus, *Desmodesmus quadricauda* requires a longer growth period for the commitment point to nuclear division than for the commitment point to DNA replication.

Is this growth-cell cycle relationship specific for algae? Not at all. A threshold RNA amount is required for DNA replication in mammalian cells (Adam et al. 1983; Baserga 1990; Darzynkiewicz et al. 1979a, b, 1980; Fujikawa-Yamamoto 1982, 1983; Johnston and Singer 1978) and blocking of RNA synthesis prevents DNA replication in both mammals (Baserga et al. 1965; Lieberman et al. 1963) and yeast (Bedard et al. 1980; Lieberman 1995; Singer and Johnston 1979, 1981). This suggests a more general mechanism governing the coordination between growth and cell cycle progression.

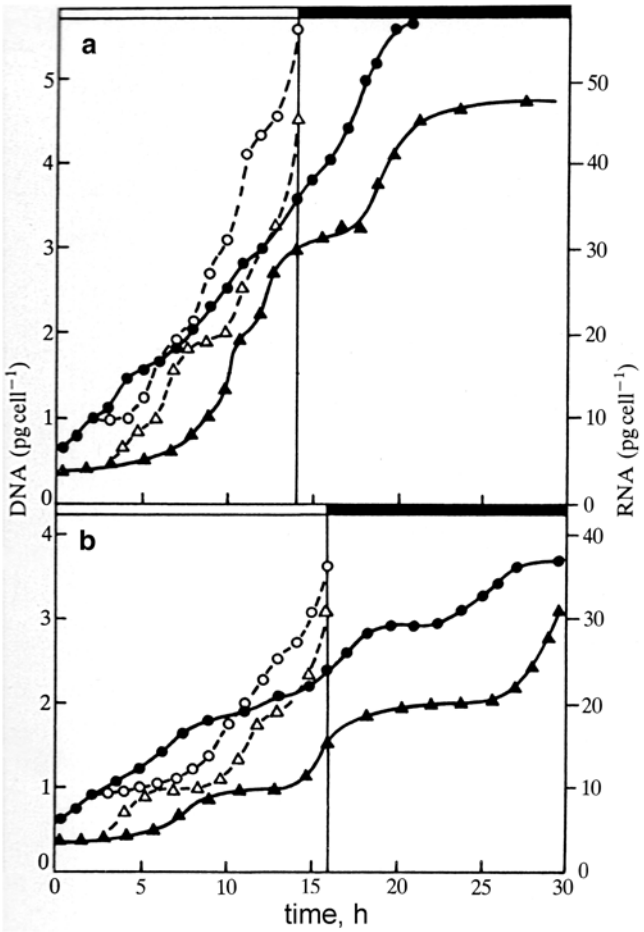

Fig. 15 Time course of RNA and DNA synthesis and their committed values in synchronous cultures of *Desmodesmus* (*Scenedesmus*) *quadricauda* grown under optimal growth conditions (**a**) and under conditions of slowed growth (**b**). Light and dark periods are indicated by *white* and *black strips* at the top of each panel and are separated by a vertical line. (●) RNA; (▲) DNA; (○) committed RNA; (Δ) committed DNA (After Zachleder and Šetlík 1988)

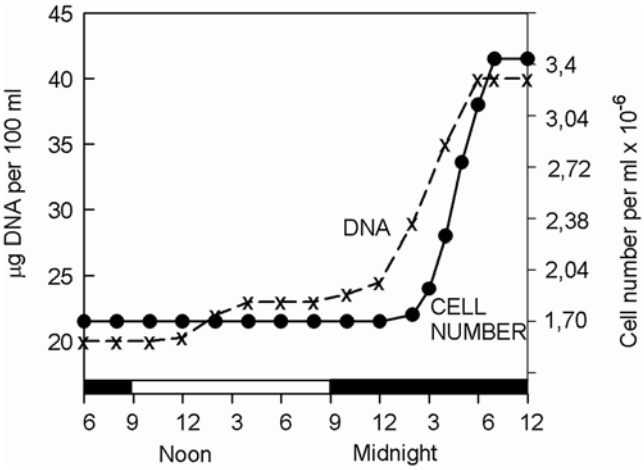

Fig. 16 Time course of DNA synthesis and cell number in a synchronized culture of *Chlamydomonas reinhardtii*. Two daughter cells were released at the end of the cell cycle under given growth conditions (After Chiang and Sueoka 1967b)

However, a critical question remains. "Do running DNA replication-division sequences control growth processes?" To answer this, the DNA replication-division has to be blocked and the effect of this treatment on growth needs to be assessed. In *Desmodesmus (Scenedesmus) quadricauda*, when 5-fluorodeoxyuridine was added to daughter cells, DNA replication and all subsequent reproductive events, such as nuclear and cellular division, were inhibited. On the other hand, both RNA and protein synthesis continued at a slower rate than in untreated cultures, but attained a 16-fold increase in their initial content, while DNA content was kept at its initial value (Fig. 21) (Zachleder 1994). Clearly, growth processes are a prerequisite for attaining a commitment point and initiating a DNA replication-division sequence, but completion of these initiated processes is growth-independent. Moreover, there is no impact on growth even if the committed DNA replication-division sequence cannot be completed.

From the point of cell cycle regulation, it is very interesting that not only synthesis itself but also stepwise oscillations in the rate of synthesis of both macromolecules were preserved, even in the absence of reproductive processes (Fig. 21). This indicates that all processes required for the commitment point were probably consecutively performed, in spite of the fact that committed processes like DNA replication and nuclear and cell division themselves were blocked. This also implies that growth and cell cycle processes are regulated by distinct mechanisms. The effect of growth on cell cycle progression is probably coincidental in providing sufficient reserves for completion of a DNA replication-division sequence, but having no direct interaction. The molecular mechanisms underlying cell cycle progression are discussed in Chap. 5.

5 Molecular Mechanisms Regulating Cell Cycle Progression

The understanding of molecular mechanisms governing cell cycle regulation comes from two genetic screens performed in budding yeast (Culotti and Hartwell 1971; Hartwell 1971; Hartwell et al. 1970, 1973, 1974) and in fission yeast (Beach et al. 1982; Fantes and Nurse 1977; Nasmyth and Nurse 1981; Nurse 1975; Nurse et al. 1983; Nurse and Fantes 1977; Nurse and Thuriaux 1977; Nurse et al. 1976; Thuriaux et al. 1978) that identified master regulators of the cell cycle, denoted as CDC28 and cdc2, respectively. The two genes differed in the parts of cell cycle that they regulated and at

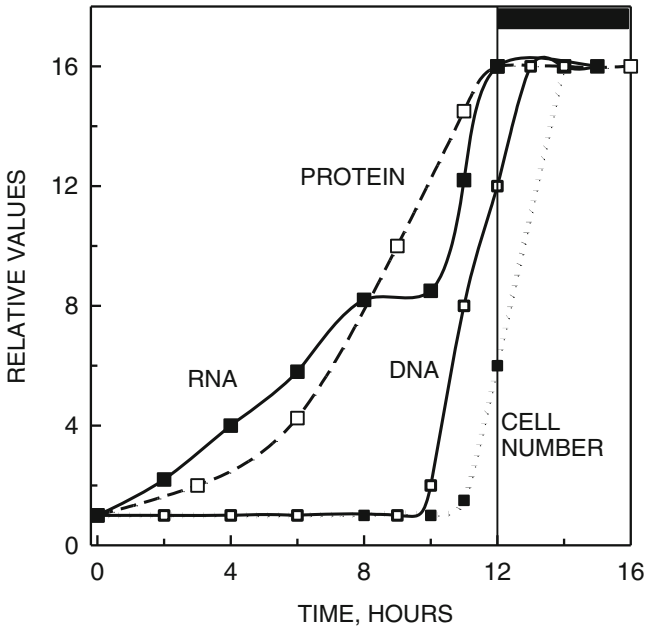

Fig. 17 Time course of increases in various parameters as indicated in the graph in a synchronized culture of *Chlamydomonas reinhardtii* (Modified after Lien and Knutsen 1979)

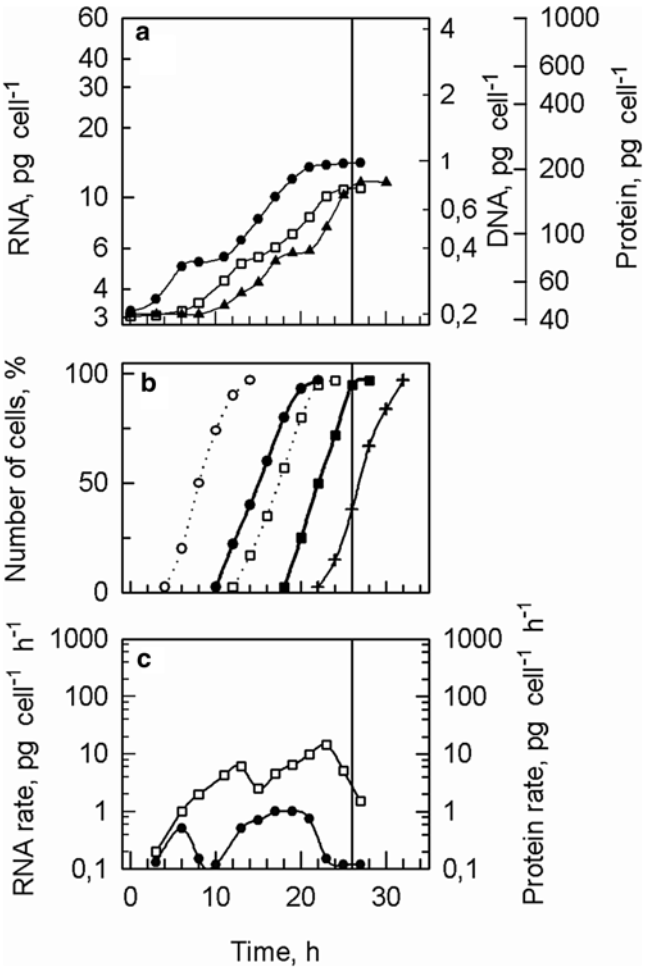

Fig. 18 Time course of growth and reproductive processes in synchronous populations of *Desmodesmus* (*Scenedesmus*) *quadricauda* grown at low irradiance. Mean irradiance 45 W m^{-2}, continuous light, temperature 30 °C. (**a**) Variation in RNA (●), protein (□), and DNA (▲) amounts per cell. The first and second cell cycles are separated by a *vertical dotted line*. (**b**) Course of commitment to nuclear and cellular division and termination of these processes. *Dotted curves*: percentage of cells that attained commitment for the first (○) and second (□) nuclear divisions. *Solid curves*: percentage of cells in which the first (●) and second (■) nuclear divisions were terminated and percentage of cells that released their daughter cells (+). (**c**) Oscillations in the rates of accumulation of RNA (●) and protein (□) (After Zachleder 1995)

first sight seemed unrelated. However, in less than two decades it became clear that not only were the two genes homologous but that homologs of the gene, encoding protein kinases (Hindley and Phear 1984; Moreno et al. 1989; Reed et al. 1985; Simanis and Nurse 1986), are encoded in the human genome and have similar functions (Langan et al. 1989; Lee and Nurse 1987). Further experiments have proven the strikingly high conservation of cell cycle regulators among eukaryotes. The core cell cycle machinery includes homologs of CDC28/cdc2, denoted as cyclin-dependent kinases (CDKs). Both yeasts require only one CDK (CDC28 or cdc2) to drive the cell cycle (Mendenhall and Hodge 1998; Moser and Russell 2000). Other eukaryotes usually require more than a single gene. In humans and other mammals, there are several CDK homologs: three of them (CDK1/cdc2, CDK2 and CDK3) are considered genuine CDC28/cdc2 homologs since they possess the same canonical PSTAIRE motif in their cyclin-binding domains; another homolog/s, CDK4/6, encodes a P(I/L)ST(V/I)RE variant of the conserved motif (Lee and Yang 2003; Meyerson et al. 1992; Pines 1996; Reed 1997). Higher plants encode two classes of cell cycle regulating CDKs, A- and B-type. CDKAs possessing a PSTAIRE motif represent the genuine CDC28/cdc2 orthologs (Ferreira et al. 1991; Hirt et al. 1991) (Mironov et al. 1999) while CDKBs are a plant-specific family of CDKs with a unique expression pattern (Dewitte and Murray 2003; Dewitte et al. 2003; Mironov et al. 1999). The first green algal homologs of CDC28/cdc2 were identified in *Chlamydomonas reinhardtii* by antibody cross-reactivity (John et al. 1989). Protein abundance of putative CDC28/cdc2 increased as the cells entered the commitment point and slower migrating phosphorylated forms of the protein appeared as they entered mitosis, indicating involvement of this protein in cell cycle regulation (Fig. 22). Kinase activity of CDKs is assessed by the extent of phosphorylation of histone H1, which is considered a CDK-specific substrate. In *Chlamydomonas reinhardtii*, the peak of kinase activity correlates with the attainment of commitment points and with nuclear divisions (Fig. 23), confirming the existence of putative CDK and suggesting its involvement in cell cycle regulation (Zachleder et al. 1997). A more detailed analysis of

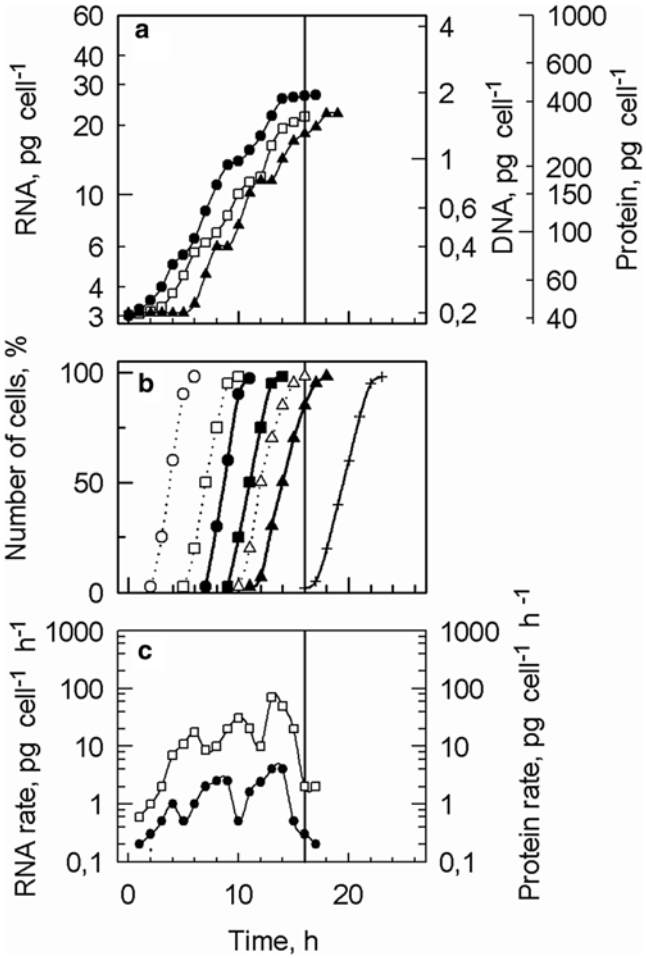

Fig. 19 Time course of growth and reproductive processes in synchronous populations of *Desmodesmus* (*Scenedesmus*) *quadricauda* grown at high irradiance. Mean irradiance 85 W m^{-1}, continuous light, temperature 30 °C. (**a**) Variation in RNA (●), protein (□), and DNA (▲.) amounts per cell (log scale). (**b**) Course of commitment points to nuclear and cellular division and termination of these processes. *Dotted curves*: percentage of cells that attained commitment point for the first (○), second (□), and third (Δ) nuclear divisions. *Solid curves*: percentage of cells in which the first (●), second (■), and third (▲) nuclear divisions were complete and percentage of cells that released eight daughter cells (+). (**c**) Oscillations in the rates of accumulation of RNA (●) and protein (□) (After Zachleder 1995)

putative CDKs in *Scenedesmus quadricauda* revealed that the two types of CDK complexes could be separated, one with activity related to growth and attainment of commitment point, and the second one with activity related exclusively to nuclear division (Bišová et al. 2000; Tulin and Cross 2014).

After complete sequencing of the *C. reinhardtii* genome (Merchant et al. 2007), comprehensive analysis identified homologs of all major CDKs (and cyclins, see below) present in higher plants, including plant-specific B-type CDK as well as some *C. reinhardtii*-specific CDKs with so far unknown functions (Bišová et al. 2000). The existence of multiple CDKs, apparently involved in cell cycle regulation, and the existence of plant-specific CDKB indicate that organization of *C. reinhardtii* cell cycle genes is more plant-like and metazoan-like than are yeast (Bišová et al. 2000).

Similarly completion of genome sequencing of the green alga *O. tauri* (Derelle et al. 2006), the red alga *Cyanidioschyzon merolae* (Matsuzaki et al. 2004), diatoms *Thalassiosira pseudonana* (Armbrust et al. 2004) and *Phaeodactylum tricornutum* (Bowler et al. 2008) showed plant-like, genome-encoded cell cycle genes (http://merolae.biol.s.u-tokyo.ac.jp/) (Huysman et al. 2010; Robbens et al. 2005). Specific information on algal cell cycle regulators is scarce and often unravels algal-specific functions. One of the best characterized CDKs are those of *O. tauri*, where B-type CDK seems to be the main regulator of the cell cycle, in contrast to higher plants where A-type CDK is the main player (Corellou et al. 2005). OtCDKB can also be phosphorylated on tyrosine. This is in contrast to OtCDKA that is not phosphorylated although it also contains the conserved tyrosine residue; another striking difference when compared to higher plants, where the situation is quite the opposite. Isolation of temperature sensitive cell division cycle mutants in *CDKA1* and *CDKB1* genes in *C. reinhardtii* brought an understanding of their functional differences. Of the two, CDKA seems to be the key enzyme regulating cell cycle progression since it is crucial for initiation of DNA replication and cytokinesis and presumably also commitment point. On the contrary, CDKB is only required to complete the processes initiated by CDKA activity, for spindle formation, nuclear division and subsequent rounds of DNA replication (Tulin and Cross 2014). This is in line with the hypothesis of CDKB being the key regulator of mitosis in higher plants (De Veylder et al. 2011).

As the name implies, CDKs depend on and interact with another subunit, cyclin (Sherr et al. 1994). Cyclins were first discovered as proteins that were periodically degraded at each division in sea urchin eggs (Evans et al. 1983) and later, were proved to be key components of the M-promoting factor and partners of CDKs (Hunt 1989; Meijer et al. 1989; Minshull 1989; Minshull et al. 1989a, b). The three main cyclin classes comprise proteins transcribed during G1 (D-type), S (A-type) and M (B-type) phases; while the latter two are orthologous in animals and plants, the D-type cyclins are not conserved between the two kingdoms although they share the same transcriptional pattern (for review, see Abrahams et al. 2001; Mironov et al. 1999; Murray 2004; Renaudin et al. 1996). The CDKs are expressed constitutively, with the sole exception of plant-specific B-type CDK (Boudolf et al. 2004; Corellou et al. 2005; Fobert et al. 1996; Lee et al. 2003; Magyar et al. 1997; Menges et al. 2002; Porceddu et al. 2001; Segers et al. 1996; Sorrell et al. 2001) and the activity of any particular CDK-cyclin complex is, to a large extent, determined by cyclin availability. Cyclin

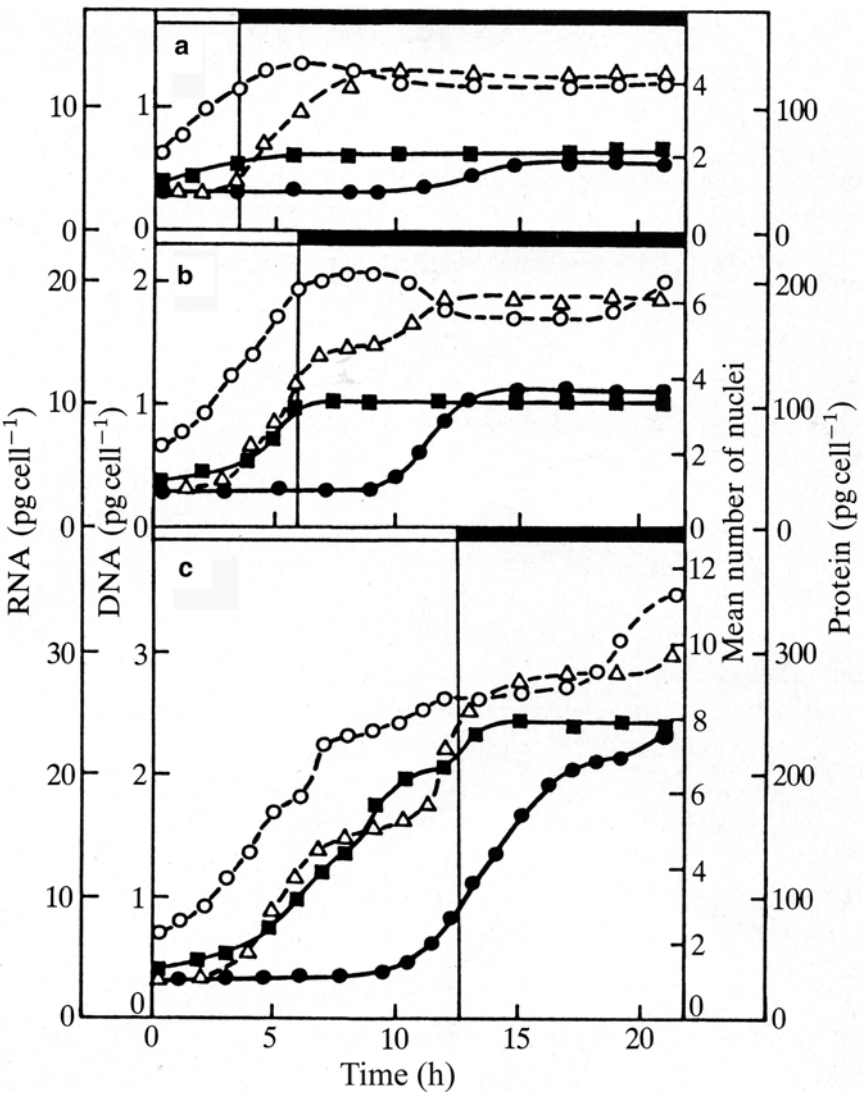

Fig. 20 Effect of darkening after different light intervals on the time course of RNA, DNA and protein synthesis and of nuclear divisions in synchronous cultures of *Desmodesmus* (*Scenedesmus*) *quadricauda* grown under optimal growth conditions. I = 95 W m^{-2}, D = 0.10 h^{-1}. The cultures were put into dark after attaining the first (**a**), second (**b**) and third (**c**) commitment points to divide into two, four and eight nuclei. Light and dark periods are indicated by *white and black strips* at the top of each panel and are separated by a *vertical line*. (○) RNA; (Δ) DNA; (■) protein; (●) nuclei (After Zachleder and Šetlík 1988)

abundance is regulated both by phase specific transcription and degradation. Transcriptional patterns of different algal cell cycle genes mimic the transcriptional patterns of their higher plant counterparts (Bisova et al. 2005; Corellou et al. 2005; Farinas et al. 2006; Huysman et al. 2010; Shrestha et al. 2012), on top of which they are also differentially expressed during the light dark cycle (Bisova et al. 2005; Huysman et al. 2010, 2013; Moulager et al. 2007, 2010). A diatom specific cyclin, dsCYC2, a partner of CDKA, is induced in a rate-dependent manner, by blue light. Interestingly, it seems to be responsible for regulation of the rate of cell division, specifically in light/dark cycles and not in continuous light (Huysman et al. 2013). This suggests that, at least in diatoms, light has not only a trophic but also a signaling role in cell cycle regulation. In *O. tauri*, *OtCYCA* was transcribed ubiquitously during the cell cycle (Corellou et al. 2005; Farinas et al. 2006) but its translation was light and cAMP-dependent. OtCycA interacts with retinoblastoma protein (Rb) during S phase and thus regulates S phase entry (Moulager et al. 2007, 2010). Both G1 cyclin (cyclin A) and Rb are known sizers in other organisms. G1 cyclin Cln3 has been accepted as a sizer in budding yeast (Rupeš 2002) and Rb protein was genetically identified as a sizer in *C. reinhardtii* (for details see below) (Umen and Goodenough 2001). The proven interaction of these two proteins, and most importantly their involvement in growth-dependent S phase entry, combines the two suspected sizers into a pathway (as was hypothesized) and underlines the usefulness of *O. tauri* as a model system.

The degradation machinery consists of two families of E3 ubiquitin ligases, the Skp/cullin/F-box-containing complex and the anaphase-promoting complex/cyclosome (for review, see Teixeira and Reed 2013). Cyclin degradation, as well as degradation of other cell cycle related proteins, is crucial for one-way progression through the cell cycle. The two E3 ubiquitin ligases are thus considered key components of the core cell cycle machinery (Inagaki and Umeda 2011) and are conserved in algae (Huysman et al. 2014). Indeed, isolation

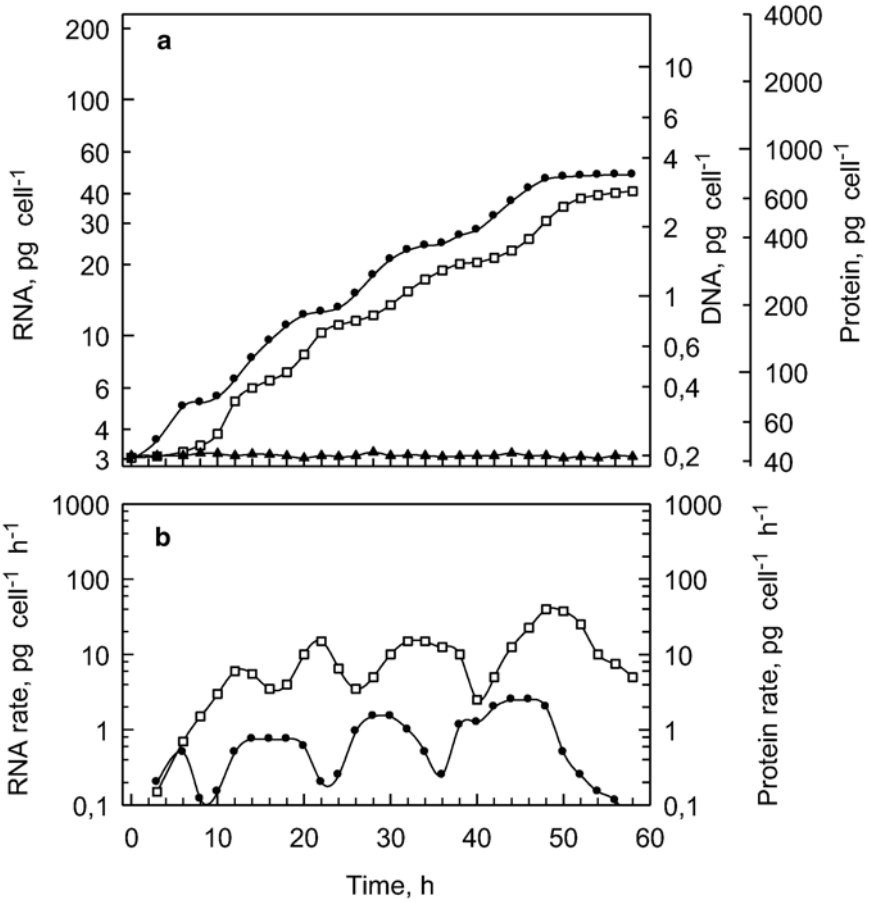

Fig. 21 The course of growth processes (RNA and protein accumulation) in synchronous populations of *Desmodesmus* (*Scenedesmus*) *quadricauda* grown at high irradiance in the presence of FdUrd. Mean irradiance 85 Wm^{-2}, continuous light, temperature 30 °C; (**a**) Variation in RNA (●), protein (□), and DNA (▲) amounts per cell (log scale); (**b**) Oscillations in the rates of accumulation of RNA (●) and protein (□) (After Zachleder 1995)

of temperature sensitive mutants proved that the directed one way progression through the cell cycle, activated by anaphase-promoting factor is conserved in *C. reinhardtii* (Tulin and Cross 2014). In the red alga *Cyanidioschyzon merolae*, chloroplast DNA and nuclear DNA replications are, in contrast to the situation in *D. quadricauda*, tightly linked. The interaction is mediated by Mg protoporphyrin IX (Mg-protoIX), a molecule used by chloroplasts to signal the nucleus to modulate nuclear gene expression (retrograde signaling) (Kanesaki et al. 2009; Kobayashi et al. 2009). The signaling by Mg-protoIX activates CDKA and promotes nuclear DNA (nuc-DNA) replication. The activation of the CDKA complex is mediated through stabilization of the CDKA cyclin partner; Mg-protoIX inhibits ubiquitin E3 ligase specific for the cyclin partner and thus stabilizes the cyclin and consequently the CDK/cyclin complex (Kobayashi et al. 2011). Such a complex interaction between chloroplast and nucleo-cytosolic compartments suggests that the chloroplast and nucleus evolved to coordinate their cycles in a distinct mechanism.

Fine tuning of CDK/cyclin complex activity is ensured by protein interaction with so called CDK inhibitors and by phosphorylation of CDKs (Morgan 1995). The phosphorylation of CDK causes both its activation and inhibition based on the kinase involved in the phosphorylation and its target site. Phosphorylation within the T-loop of the CDKs, by CDK activating kinases, is crucial for CDK/cyclin complex activation (Ducommun et al. 1991; Gould et al. 1991). In contrast, phosphorylation within ATP binding sites of CDK, executed by Wee1 kinase (Gould and Nurse 1989; Jin et al. 1996), partially inactivates already active CDK/cyclin complexes. This phosphorylation ensures the inactivation of CDKs until the G2/M transition, when they are abruptly dephosphorylated by Cdc25 phosphatases, leading to the activation of CDK-cyclin complexes, triggering mitosis (Kumagai and Dunphy 1991; Russell and Nurse 1986, 1987).

Wee1 kinases are widely conserved in both algae and plants. However, the existence of Cdc25 phosphatase homologs in the plant kingdom is a matter for discussion (Boudolf et al. 2006). The putative Cdc25 homologs in algae and higher plants are highly divergent and lack a conserved N-terminal domain (Bisova et al. 2005; Landrieu et al. 2004a, b). The only *bona fide* Cdc25 phosphatase in the plant kingdom is that of *O. tauri* (Khadaroo et al. 2004) - so far the only plant Cdc25 homolog able to complement a *cdc25* mutation in *Schizosaccharomyces pombe* - which may do so due to the presence of the N-terminal domain.

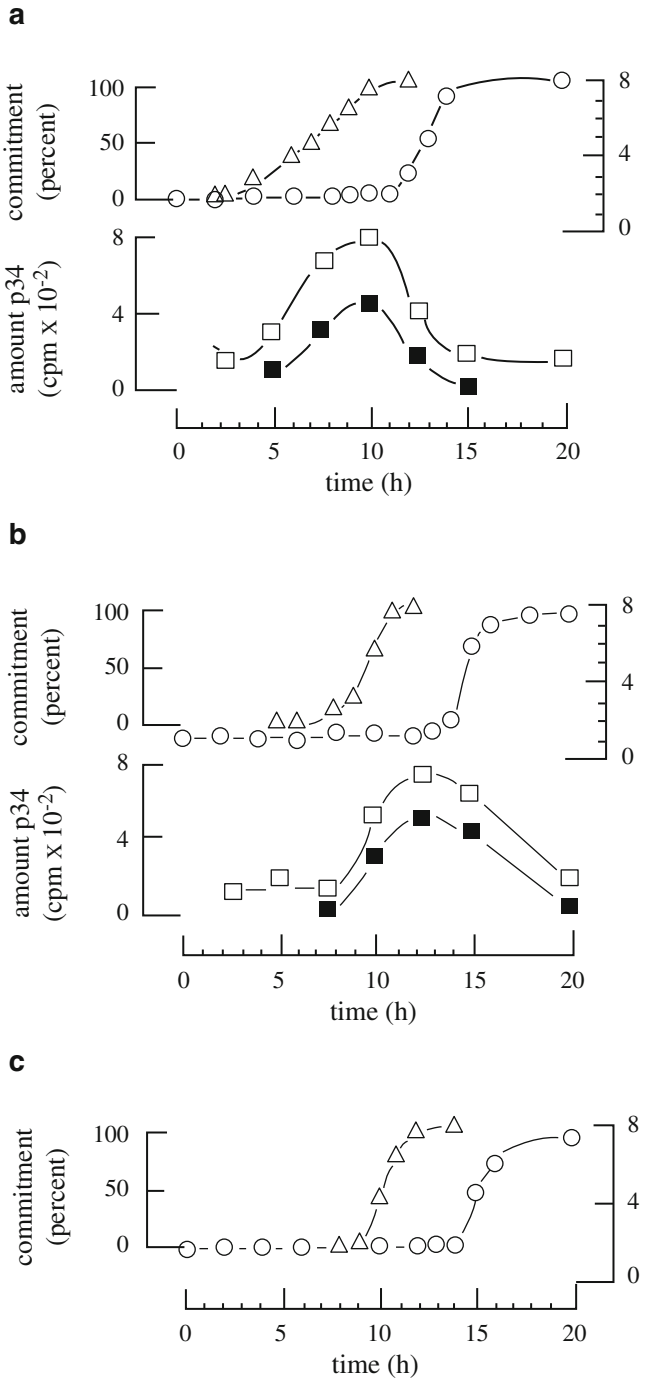

Fig. 22 Division timing and changes in the amount and phosphorylation of p34. (**a**) Very early division was caused by a reduction in phosphate to 100 µM. (**b**) Early division was caused by fast growth due to illumination at 200 µmol photons m^{-2} s^{-1} photosynthetically active radiation (PAR). (**c**) Later division was caused by slower growth due to a lower light intensity of 100 µmol photons m^{-2} s^{-1} PAR. During the period illustrated, cells were in continuous light. Commitment point to division (Δ) was in progress by 5 h, 8 h, and 10 h in cultures **a**, **b**, and **c**, respectively, and cell number (○) increased 6 h later. Total p34 (□) increased concomitantly with commitment point to division, and 2.5 h later a high incidence of mitosis correlated with the appearance of slower migrating phosphorylated forms of p34 (■) that are quantified in (**a**, **b**) (After John et al. 1989)

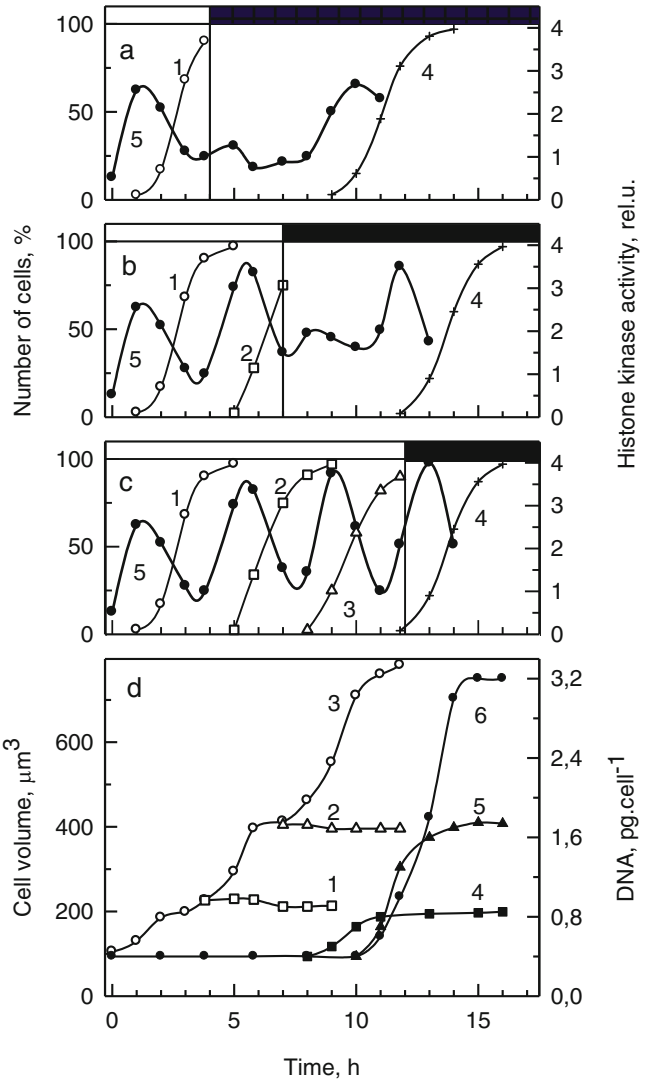

Fig. 23 Time course of commitment points to individual sequences of reproductive events, termination of these events, cell volume enlargement, and histone Hl kinase activity in synchronized populations of *Chlamydomonas reinhardtii*. The cultures were grown at a high irradiance and placed in the dark after the first (**a**), the second (**b**), and the third (**c**) commitment point to trigger a sequence of reproductive events. *Panels* **a**–**c**. Curves *1–3*: the percentage of cells that reached the commitment points for the first, second, and third sequence of reproductive processes, respectively. Curve *4*: the percentage of the cells that released daughter cells. Curve *5*: the activity of histone Hl kinase. *Panel* **d**. Curves *1–3*: the mean cell volume in subpopulations that were put in the dark after 4, 7, and 12 h, respectively. Curves *4–6*: the concentration of DNA per cell in subpopulations that were put in the dark after 4, 7, and 12 h, respectively. Light and dark periods are marked by the *lines* above *panels* **a**–**c** and separated by *vertical lines* (After Zachleder et al. 1997)

CDK inhibitors (CKIs) represent the most diversified group of all core cell cycle regulators. While the function of such proteins has been conserved, the sequence conservation is very limited among fission and budding yeasts, animals and higher plants (Inagaki and Umeda 2011; Mironov et al.

1999; Wang et al. 1997). Higher plant CKIs are represented by two families of genes, Kip-related proteins (KRPs) with limited homology to animal CKIs, and plant-specific SIAMESE (SIM) and related proteins; each of the protein families seems to have distinct functions in cell cycle regulation. Interestingly, no homologs of CKIs have, so far, been identified in *C. reinhardtii* nor in other algal species (Bisova et al. 2005; Huysman et al. 2010; Robbens et al. 2005). However, proteins with functions attributed to CKIs are most probably present in algal cells but not identified due to sequence divergence.

CDK/cyclin activity is remembered and perpetuated by phosphorylation of its substrates and their subsequent actions. Transcription of many genes required for cell cycle progression in G1/S transition and DNA replication is controlled by binding of a heterodimer of transcription factor E2F and its dimerization partner (DP) (Inzé and De Veylder 2006; van den Heuvel and Dyson 2008). The activity of the E2F-DP dimer is controlled by interaction with the negative regulator, retinoblastoma protein, Rb. During G1 phase, Rb is hyperphosphorylated by the CDK/cyclin complex, leading to a release of active E2F/DP dimer, transcriptional activation and S phase entry (Shen 2002). The Rb/E2F pathway thus represents the best characterized substrate for the CDK/cyclin complex. Genes comprising the Rb/E2F pathway are the best characterized cell cycle genes in *C. reinhardtii*. In mutant *C. reinhardtii* containing a deletion of the Rb homolog, encoded by the *MAT3* gene, commitment point is attained at a smaller critical cell size, and when dividing, they divide excessively, giving rise to tiny daughter cells (Umen and Goodenough 2001). Thus, the Rb homolog encodes a sizer involved in the regulation of cell cycle progression in response to attainment of critical cell size. A genetic screen to isolate suppressors of *mat3-4* mutation uncovered other members of Rb/E2F pathway, E2F and DP, which were both able to suppress the *mat34* size mutation (Fang et al. 2006). *DP1* mutants have larger daughter cells than wild type, while *e2f* shows a similar daughter cell size. This implies the canonical Rb/E2F pathway in *C. reinhardtii* regulates cell cycle entry in response to attainment of critical cell size. It should be noted that *C. reinhardtii* represents a unique model to study the relationship between growth and cell cycle regulation, due to its multiple fission cell cycle. Cells dividing by multiple fission are, in general, less prone to change daughter cell size in response to changes in growth rates (Rading et al. 2011), and this is quite common for yeasts. Cultures of *C. reinhardtii*, and other algae dividing by multiple fission, will, after prolonged dark incubation under different growth rates and stable temperature, produce daughter cells of very uniform cell sizes ranging from a cell size just below the commitment point, to a cell size roughly half of that. Although several components of the sizing pathway were unraveled in *C. reinhardtii*, the most interesting question remains: "What is the signal that "turns on" the sizing control"? Since *C. reinhardtii* represents an excellent genetic system, the answer to this question will most probably come from another mutant screen. Recently, a hint on the processes preceding "the sizing control" came from an unexpected organism, the red alga *C. merolae*. There E2F phosphorylation status is linked by as so-far unknown mechanism to circadian rhythm and represents a pre-requisite for the sizing control mediated by Rb phosphorylation (Miyagishima et al. 2014).

6 The Role of Light and Temperature

The cell cycle of algae starts with a period in which cells increase in size (pre-commitment period) until they reach a critical cell size and a key point of the cell cycle, commitment point, is attained. From this point, the cells are committed to divide and processes of DNA replication-division sequence are triggered. The following period (post-commitment period), during which daughter cells will be eventually formed, can be traversed without an external energy supply, and without further growth of the cells. However, if sufficient energy is supplied during this period, the cells, dividing by a C_n type of cell cycle, are able to attain another commitment point/s, leading to a higher number of daughter cells.

It is also characteristic of algae with the C_n cell cycle type that after each commitment point, growth processes that continue overlap concomitantly with running processes of triggered DNA replication-division sequences, as schematically illustrated in Fig. 3 (Sect. 2). Here we will describe the effects of light intensity and temperature, the major effectors of growth rate, on individual parts of the algal cell cycle.

6.1 Light Intensity

Most studies on the effect of light intensity were carried out on synchronized cultures of *Chlorella*, *Desmodesmus*, *Scenedesmus* and *Chlamydomonas*, as early as the 1960s (Lorenzen 1957; Nelle et al. 1975; Pirson and Lorenzen 1966; Pirson et al. 1963; Šetlík et al. 1972; Tamiya 1966; Tamiya et al. 1953; Wanka 1959, 1962, 1967; Wanka and Aelen 1973). Both the growth processes represented by an increase in RNA, protein and cell volume, and the reproductive processes, including DNA replication and nuclear division, are performed in several steps, each of which is approximately a doubling of the preceding one. With increasing light intensity, the duration of the steps in RNA and protein synthesis leading to doubling of their content per cell shortens and their number increases (Fig. 24); the same is true for an increase in cell volume (Fig. 25). In algae with a

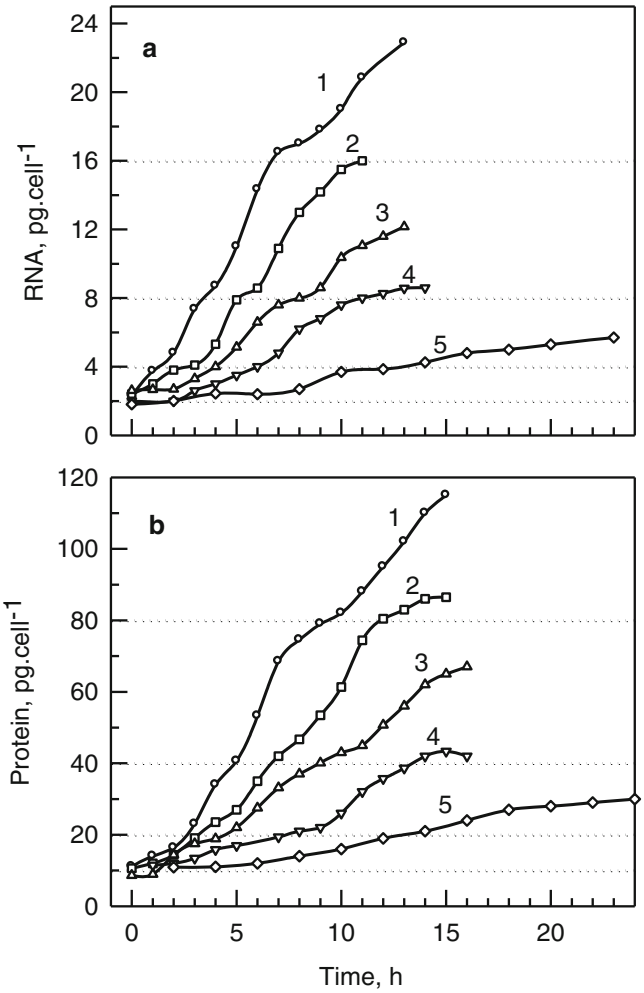

Fig. 24 Synchronous populations of *Scenedesmus armatus* grown at various mean irradiances showing (**a**) variation in RNA and (**b**) protein content per cell. Batch cultures grew for several cell cycles under alternating light (L) and dark (D) periods. In the analyzed cell cycles, the dark period has been omitted. Curve *1*: 100 W m^{-2}, 15:10 h LD; curve *2*: 72 W m^{-2}. 14:10 h LD; curve *3*: 44 W m^{-2}, 13:10 h LD; curve *4*: 17 W m^{-2}, 15:10 h LD; curve *5*: 10 W m^{-2}, 20:10 h LD. *Horizontal dotted lines* indicate doublings of initial value (After Tukaj et al. 1996)

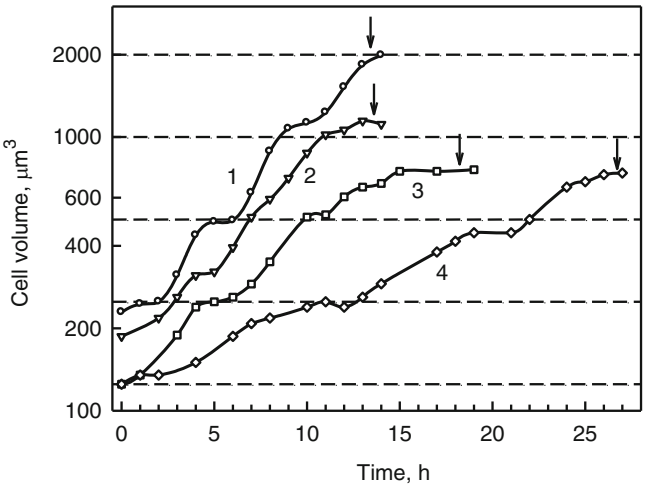

Fig. 25 Changes in mean cell volume during the cell cycle in synchronized populations of *Scenedesmus armatus* grown at different (mean) light irradiances. Curve *1*: 105 W m^{-2}; curve *2*: 85 W m^{-2}; curve *3*: 50 W m^{-2}; curve 4: 20 W m^{-2}, 15:10 h LD at 30 °C; *arrows* – the time when division of cell started; *dashed line* – doubling values for cell size levels (After Vítová and Zachleder 2005)

Scenedesmus-type cell cycle, individual doublings of DNA, e.g. individual DNA replication-division sequences, are also separated in time in Fig. 26.

The timing of individual commitment points and cellular divisions is dependent on light and temperature in synchronized cultures of *Chlamydomonas eugametos* (Zachleder and van den Ende 1992). The time interval required for attainment of the first commitment point shortened markedly (from 28 to 6 h, with increasing light irradiance from 7.5 to 70 W m^{-2}, respectively) (Fig. 27). Shortening of all consecutive pre-commitment periods with increasing light intensity caused the number of commitment points attained to increase from two to four (in 25 % of population even to five). The mother cells divided into 16 or 32 daughter cells (Fig. 27a). This was confirmed by detailed studies in distantly related *Chlamydomonas reinhardtii* (Vítová et al. 2011b). Increased growth rates (see course of cell volume in Fig. 28a–g), led to shortening of the pre-commitment periods and an increase in their number from 1 to 4. At the end of the cell cycle, cell volume was proportional to the number of daughter cells; these increased from 2 at the lowest light intensity (Fig. 28a), to 16 at the highest light intensity (Fig. 28g). The growth rates were solely dependent on mean light intensities and were not affected by dark period. When grown in continuous light, the length of the cell cycle shortened with increasing light intensity (increasing growth rate), from about 73 h at the lowest growth rate (Fig. 29a) to 15 h at the highest growth rate (Fig. 29c). Furthermore, the same dependency on mean light intensity for setting the growth rate and the length of the cell cycle was seen if the daughter cells from an asynchronous culture were separated by sedimentation or gentle centrifugation (Vítová et al. 2011b) (Fig. 30). This supports the view that rules for regulation of cell cycle length are the same in asynchronous cultures as in cultures synchronized by light/dark regime.

General rules for regulating the lengths of pre- and post-commitment phases of the algal cell cycle are the following:

1. The length of the pre-commitment period depends on irradiance, suggesting that a finite amount of photosynthetic work must be completed before the cell becomes committed. This supports the early idea that the main (if not the only) factor determining the timing of commitment point is growth rate, which is set by the rate of photosynthesis (Spudich and Sager 1980). Until reaching the "threshold

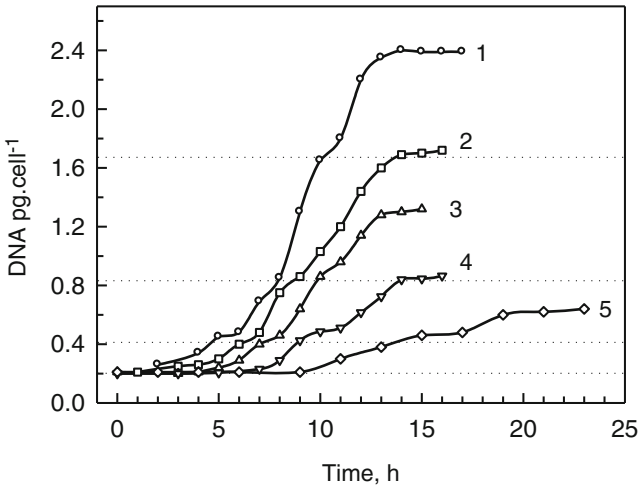

Fig. 26 Variations in DNA per cell in a synchronous population of *Scenedesmus armatus* grown at various mean irradiances. Batch cultures grew for several cell cycles under alternating light (L) and dark (D) period. In analyzed cell cycle the dark period has been omitted. Curve *1*: 100 W m^{-2}, 15:10 h LD; curve *2*: 72 W m^{-2}, 14:10 h LD; curve *3*: 44 W m^{-2}, 13:10 h LD; curve *4*: 17 W m^{-2}, 15:10 h LD; curve *5*: 10 W m^{-2}, 20:10 h LD. *Horizontal dotted lines* indicate doublings of initial value (After Tukaj et al. 1996)

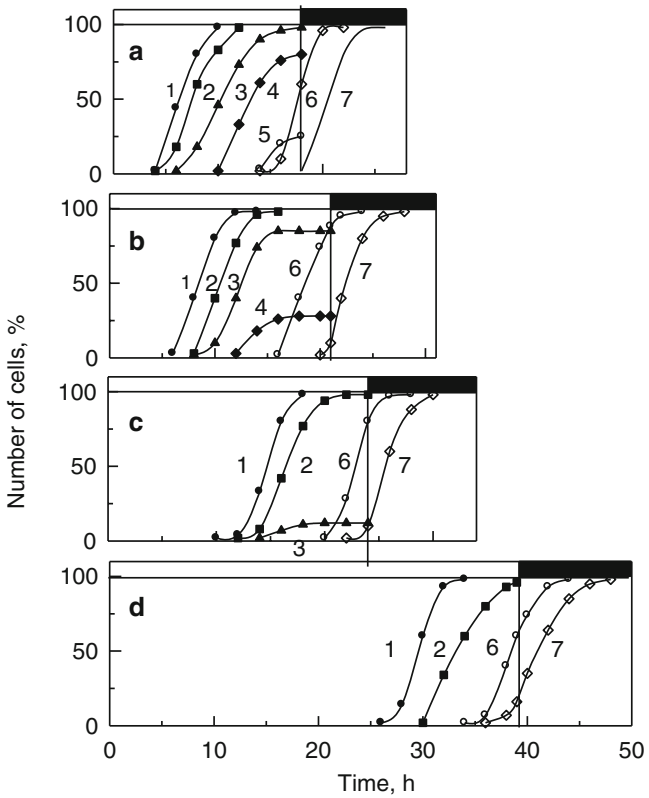

Fig. 27 Time courses of commitment points to nuclear and cellular divisions and termination of these processes in synchronous populations of *Chlamydomonas eugametos* grown at various light irradiances. (**a**) 70 W m^{-2}; (**b**) 35 W m^{-2}; (**c**) 15 W m^{-2}; (**d**) 7.5 W m^{-2}. Curves *1*, *2*, *3*, *4*, *5*: percentage of the cells that attained commitment points for the first, second, third, fourth and fifth nuclear divisions, respectively, curve *6*: percentage of the cells in which the first protoplast fission occurred, curve *7*: percentage of the cells that released their daughter cells. Light and dark periods are indicated by *white and black strips* above *panels* and separated by *vertical lines* (After Zachleder and van den Ende 1992)

photosynthetic work" required for attaining commitment point, the pre-commitment period can be interrupted by dark periods affecting its length within wide limits. As illustrated in Fig. 31 for *Desmodesmus (Scenedesmus) quadricauda* (Šetlík and Zachleder 1983) and in Fig. 32 for *Chlamydomonas reinhardtii* (Vítová et al. 2011b), if the synchronized population is darkened in the pre-commitment period for a certain interval of time, the only result is the postponement of commitment points and all post-commitment events for an equal interval of time. On the other hand, the pre-commitment period in a population growing at an irradiance well below saturation may be markedly shortened by inserting a comparatively short (2 h) interval of saturating irradiance (Figs. 33c, d). This treatment has no effect on the course of post-commitment events.

2. The time between commitment point and daughter cell release at a given temperature remains approximately constant at different irradiances (Figs. 27, 28, and 29). In sharp contrast to growth, the processes in the DNA replication-division sequence (post-commitment period) are independent of the simultaneous supply of external energy to the cell. The timing of the first commitment point determines, in principle, the timing of daughter cell release. This depends entirely on the growth rate (and hence on irradiance) and whether, in the period after the first commitment point, another commitment point will be attained before the cells divide. If this occurs, a new DNA replication-division sequence is initialized and a higher division number will be reached within the time between the first commitment point and daughter cell release.

6.2 Temperature

Most biological reactions vary with temperature such that with every 10 °C increase in temperature, the reaction rate approximately doubles; this is expressed as a temperature coefficient (Q_{10}) of about 2. It could be therefore assumed that the same rule will apply for processes involved in the regulation of cell cycle events. This was verified practically more than half a century ago by (Morimura 1959), who provided the first information on the effect of different temperatures on synchronized cultures of *Chlorella ellipsoidea* (Fig. 34). The basic rule has been repeatedly verified in other species of algae: A decrease in temperature decreases the algal cell growth rate and consequently, the cell cycle is prolonged in a manner inversely proportional to the temperature. The question remaining is how temperature affects individual phases of the cell cycle. Particularly in cell cycle type C_n, where, at high growth rates, a complex overlapping of sev-

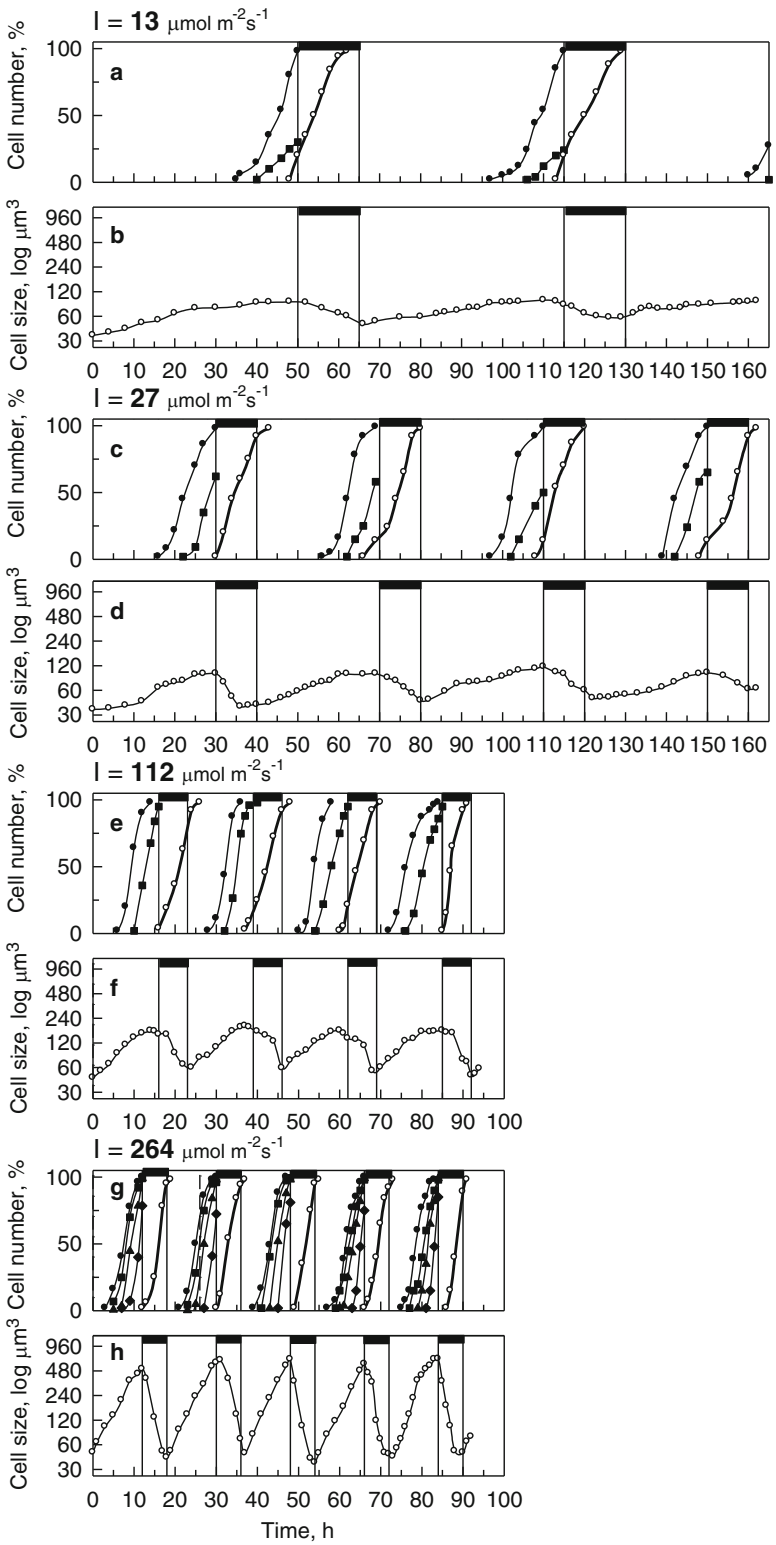

Fig. 28 Time courses of individual commitment points to cell division and daughter cell release (**a**, **c**, **e**, **g**) and changes in a mean cell volume (**b**, **d**, **f**, **h**) in synchronized populations of the alga *Chlamydomonas reinhardtii* grown at different mean light intensities (**I**) under **alternating light and dark periods**. Full symbols: percentage of the cells, which attained the commitment point for the first (*circles*), second (*squares*), third (*triangles*) and fourth (*diamonds*) protoplast fission, respectively; open symbols: percentage of the cells, which released their daughter cells. Dark periods are marked by *black stripes* and separated by *vertical solid lines* (After Vítová et al. 2011b)

eral sequences of growth (pre-commitment phases) and DNA replication-divisions (post-commitment phases) occurs, as shown in preceding chapters). The comparison between the effect of light and temperature can be seen in Figs. 35 and 36. As discussed above, with increasing light intensity, the cell cycle shortens due to shortening of pre-commitment periods. Post-commitment periods are independent of light intensity (Fig. 35). Due to this distinct effect of light, only variations in pre-commitment phases determines the final length of the cell cycle, and thus the

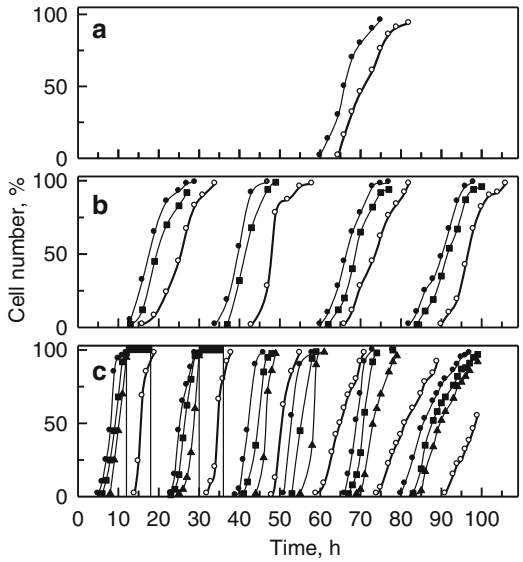

Fig. 29 Time courses of individual commitment points to cell division and daughter cell release in synchronized populations of *Chlamydomonas reinhardtii* **continuously illuminated** and grown at different mean light. Full symbols: percentage of the cells, which attained the commitment point for the first (*circles*), second (*squares*) and third (*triangles*) protoplast fission, respectively; *open symbols*: percentage of the cells, which released their daughter cells (After Vítová et al. 2011b)

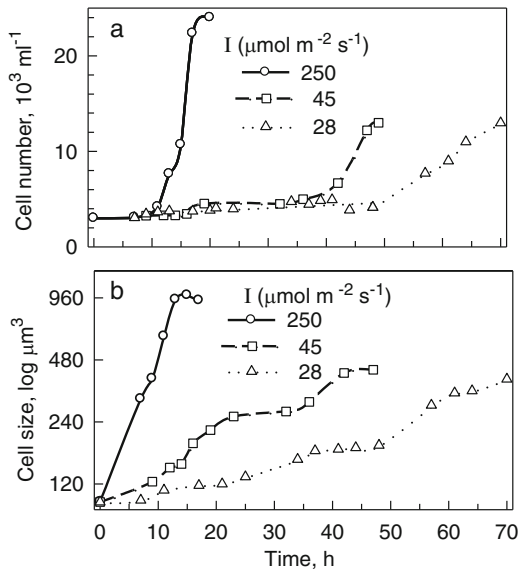

Fig. 30 Time courses of daughter cell release (**a**) and variation of the cell size (**b**) in populations of *Chlamydomonas reinhardtii* **synchronized by size-selection** and grown continuously illuminated at different mean light intensities (**I**) (After Vítová et al. 2011b)

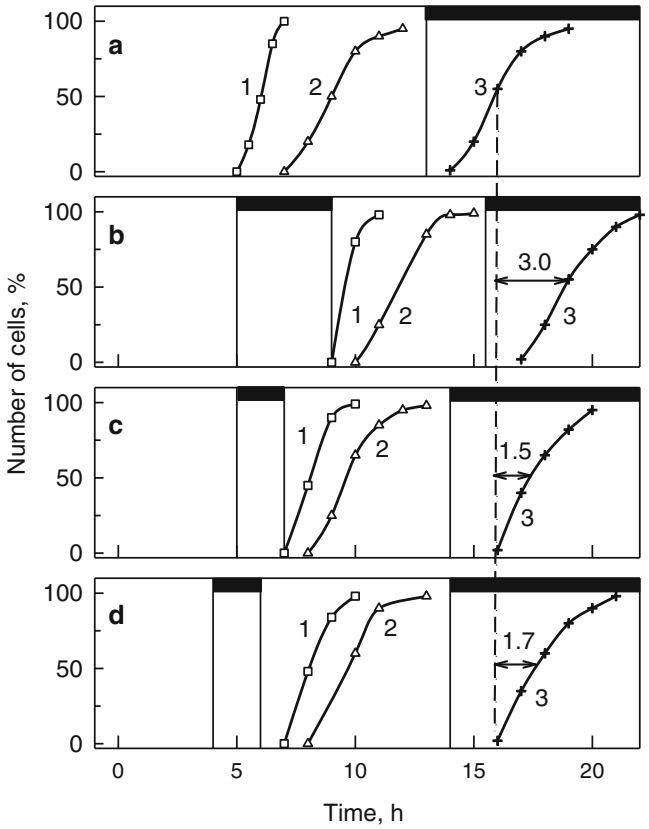

Fig. 31 Influence of interrupting illumination in the precommitment period on the timing of commitment points and of cell division in a synchronous culture of *Desmodesmus* (*Scenedesmus*) *quadricauda*. Batch cultures started from a common inoculum were grown at 30 °C, incident irradiance 80 W m^{-2}. The mid-point of cell division in a control culture (*panel* **a**) is indicated by the *vertical dashed line*. The delay of cell division caused by interrupting the light period for 4 h (*panel* **b**) and 2 h (*panels* **c**, **d**) are marked by *horizontal arrows* with figures indicating the delay in hours. Duration of post-commitment period, the distance between curve *1* and *3* remains approximately constant. *White and black strips* above the *panels* indicate the intervals of light and dark (After Šetlík and Zachleder 1983)

relative position in the cell cycle varies markedly with light intensity (see insert in Fig. 35). This is in sharp contrast to the effect of temperature.

Changes in temperature, similarly to light intensity, affect growth rate and the cell cycle shortens with increasing temperature (Fig. 36). However, temperature affects all processes in the cell, not only growth, as was the case for light intensity. When the temperature coefficients (Q_{10}) for the duration of the cell cycle, pre- and post-commitment periods were determined, the values were close to 2, indicating a doubling in the rate of metabolism with a temperature increase of 10 °C (Vítová et al. 2011a). This is well above the values expected for temperature-compensated processes and suggests metabolic dependency of both pre- and post-commitment periods solely on temperature. Consequently, the lengths of both pre- and post-commitment phases are affected by temperature in the same way, and the relative position in the cell cycle does not change substantially with temperature (see insert in Fig. 36d).

Under constant temperature, the length of the post-commitment period is rather constant and is independent of growth rate. Although this is clearly the case, there are conditions under which the length of the post-commitment period

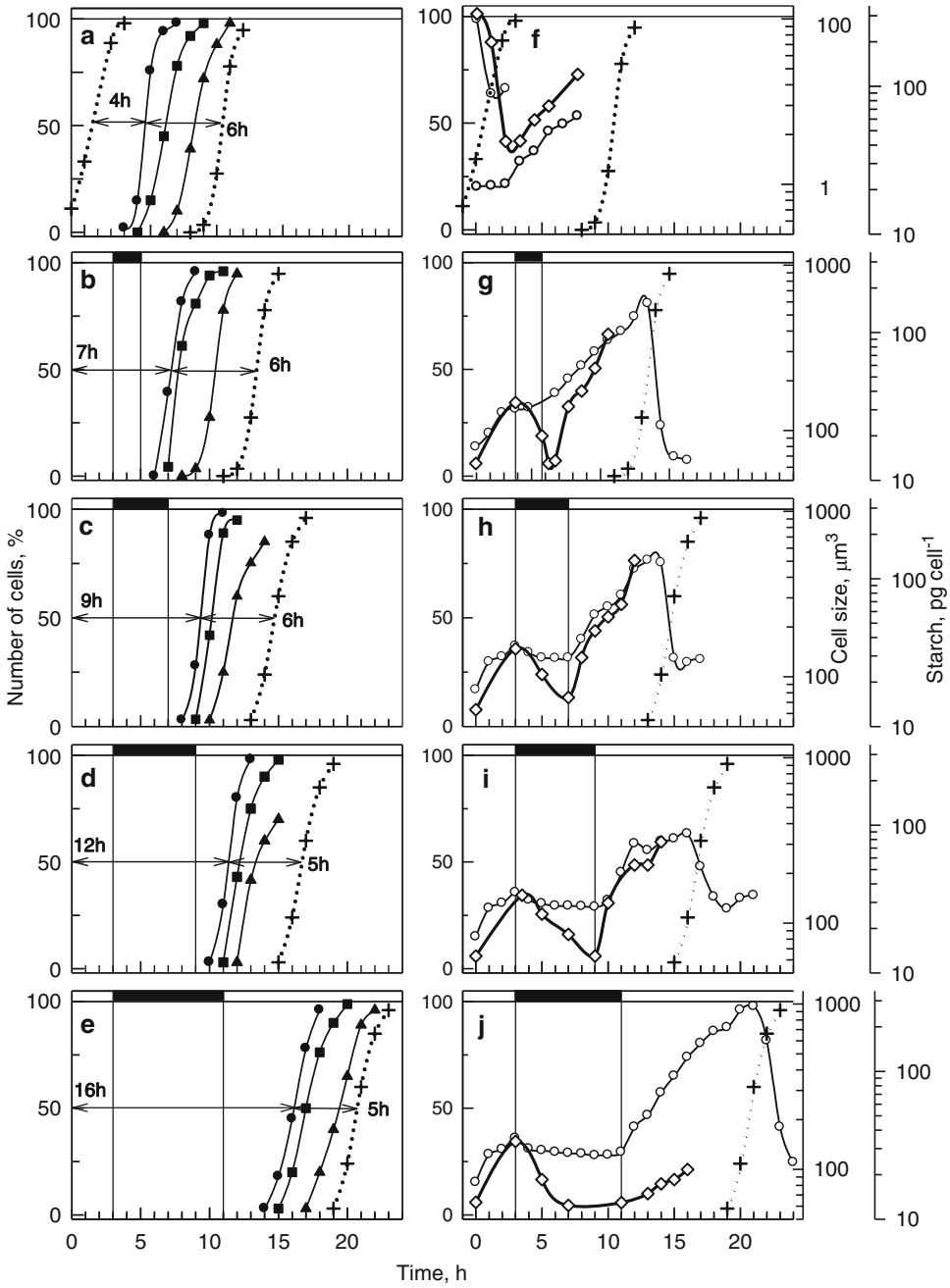

Fig. 32 Time courses of individual commitment points to cell division and the course of daughter cell release (**a**–**e**) and changes in mean cell volume and starch level (**f**–**j**) in synchronized populations of *Chlamydomonas reinhardtii* grown at constant light irradiance (I = 264 µmol photons m^{-2} s^{-1}) and different illumination regimes. **a**–**e** *Solid lines*, full symbols: percentage of the cells, which attained the commitment points for the first (*circles*), second (*squares*) and third (*triangles*) protoplast fission, respectively; *dotted line*, *crosses*: percentage of the cells that released their daughter cells. The pre- and post-commitment phases are marked by *horizontal arrowed lines* connecting midpoints of the phases, numerals indicate their duration in hour. (**f**–**j**) *Solid thick lines*, *diamonds*: starch amount; *thin solid lines*, *circles*: cell size; *dotted lines*, *crosses*: percentage of cells that released their daughter cells. Dark periods are marked by *black stripes* in panels and separated by *vertical solid lines* (After Vítová et al. 2011b)

will be prolonged, even at stable temperatures, such as in cultures of *Chlamydomonas eugametos* grown at very high light intensities (Zachleder and van den Ende 1992). This is probably caused by the insertion of additional DNA replication-division sequence/s after additional commitment points were attained. This would result in prolongation of the period from the first commitment point to the final division due to more processes being required to be completed. The prolongation is longer, the higher number of commitment points attained, and can be seen particularly well in *Chlamydomonas*, dividing into 32 daughters with 5 commitment points attained within one cell cycle (Zachleder and van den Ende 1992) (Fig. 37).

The cell cycle, comprising pre- and post-commitment periods, is quite complex and is affected by a combination of light and temperature. At low light intensities, light availability limited growth, even at higher temperatures. Consequently, the length of the pre-commitment period did not change at various temperatures, giving the impression that the length of the cell cycle was temperature-independent. On the other hand, at low temperatures (below 20 °C), growth processes were so slow that even low light intensities

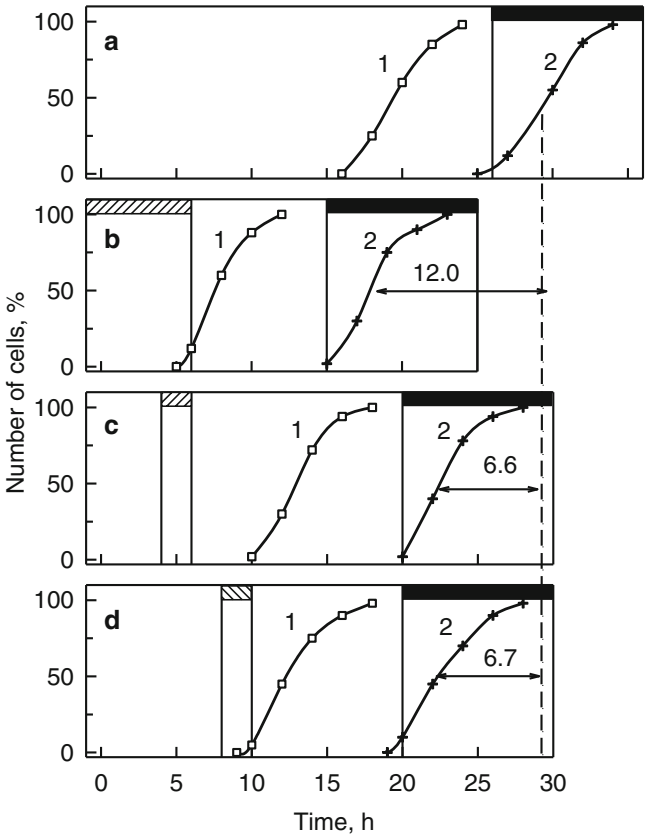

Fig. 33 Influence of a short time increase in irradiance during the pre-commitment period on the timing of commitment points and cell division in a synchronous culture of *Desmodesmus* (*Scenedesmus*) *quadricauda*. Batch cultures started from a common inoculum and were grown at 30 °C and incident irradiance 45 W m^{-2} (*panel* **a**). The shortening of the cell cycle caused by increasing irradiance to 80 W m^{-2} for 6 h (*panel* **b**) and 2 h (*panels* **c**, **d**). *Dashed*, *white* and *black* sectors of the strip above the panels indicate the periods of high irradiance, of low irradiance and of darkness, respectively (After Šetlík and Zachleder 1983)

were sufficient to saturate their photosynthetic demands. So the length of the cell cycle appeared to be light-insensitive. This constant duration of the cell cycle under a certain range of growth conditions found by some authors in *Chlamydomonas reinhardtii* (Donnan and John 1983) and *Chlorella* (Lorenzen 1980; Lorenzen and Albrodt 1981) led them to postulate the existence of a timer (pacemaker, Zeitgeber), or circadian rhythms (Goto and Johnson 1995; Lorenzen and Schleif 1966), which are triggered at the beginning of the cell cycle, causing division of cells after a constant time period, regardless of the growth rate. The finding in *Desmodesmus* (*Scenedesmus*) *quadricauda*, as well as those published for *Scenedesmus armatus* (Tukaj et al. 1996), *Chlamydomonas eugametos* (Zachleder and van den Ende 1992) and *Chlamydomonas reinhardtii* (Spudich and Sager 1980), and recent ones in the same species (Vítová et al. 2011a, b), clearly refute any role of any type of timer, endogenous oscillator or circadian rhythm in determining the length of their cell cycle, as well as cell cycle phases. Rather, the cell cycle duration is set by the combination of durations of pre- and post-commitment periods that are differentially sensitive to light and temperature.

As a crucial conclusion from the effect of light and temperature on the cell cycle, it should be kept in mind that the cell cycle is not a "clock" since its length is, to great extent, varied by external conditions. This was succinctly summarized by (Tyson and Novak 2008): "*Under constant favorable conditions, cells progress through the DNA replication – division cycle with clocklike regularity, but the cell 'cycle' is not a 'clock'.*"

Even more, the cell cycle is in fact not a "cycle"; it only appears to be one. This was lucidly put by Cooper: "*The word 'cycle' implies that at cell division something is initiated, the cell cycle. Actually, nothing starts at cell division but it is merely the end of a sequence of events which start with the accumulation of some initiation potential, and which was followed in succession by the initiation of DNA synthesis, the preparations for cell division following termination of DNA synthesis, and the final cell division. The final cell division is the end of the process and the beginning of nothing*" (Cooper 1979, 1984). This might not be so clear when studying a classical cell cycle, binary fission, but it is in striking agreement with cell cycle regulation in the C_n cell cycle type. In this, everything overlaps with everything else: growth after the last commitment overlaps with preparation for DNA replication and/or DNA replication of the just committed DNA replication-division sequence, and with preparation for and/or the running of nuclear division of the previously started DNA replication-division sequence. Clearly, all the processes are happening at the same time and the cell is neither in G1 nor G2 phases; in fact it is, at the same time, in G1, G2, S and M phases simultaneously. From this point of view, the generally accepted semantic distinction of individual cell cycle phases is bringing more confusion than help and might be limiting our understanding of cell cycle organization and regulation.

7 Chloroplast Cycle

As discussed above, light condition (duration of light interval and light intensity) is one of the most important factors affecting growth of phototrophic algae. The chloroplast, the photosynthesizing organelle, is mostly considered only as a supplier of energy and material, however it also has its own chloroplast division cycle that it is affected by light (Zachleder et al. 2004). Chloroplasts can occupy about 50 % of the total cell volume, chloroplast RNA is about 30 % of total RNA (Chiang and Sueoka 1967a) and about 3–15 % of cellular DNA (Zachleder et al. 1989, 1995). Thus, a great

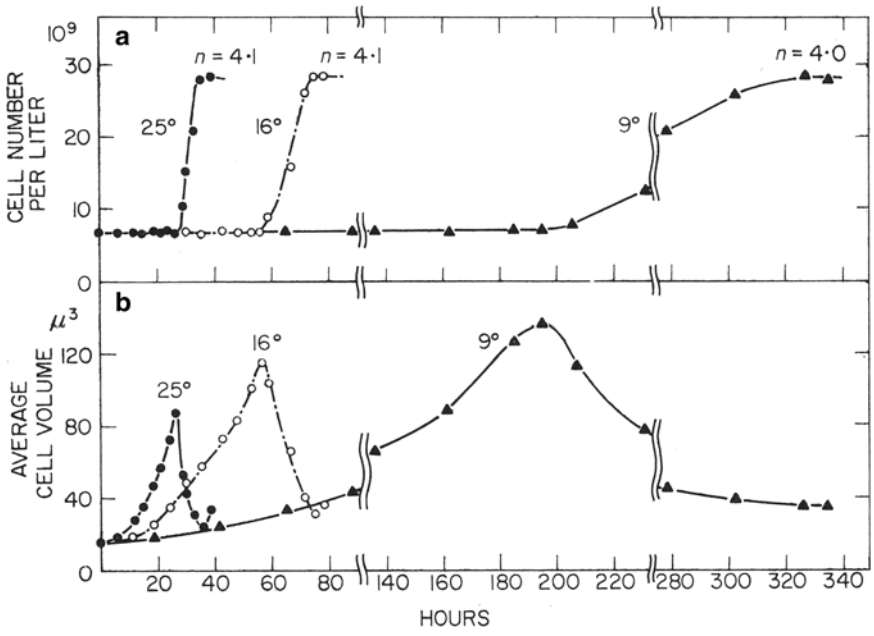

Fig. 34 Effect of temperature on the cell cycle of Chlorella ellipsoidea (under the condition of light saturation 10 kilolux). Temperatures 25, 16 and 9 °C. (**a**) Number of cells at indicated temperatures. Number of daughter cells per one mother cell expressed as division number n above the curves; (**b**) Changes in average cell volume at indicated temperatures (After Morimura 1959)

deal of the chloroplast photosynthetic capacity, as well as cellular synthetic machinery, are utilized for growth and reproduction of the organelle itself, implying that coordination of the chloroplast and the nucleo-cytosolic compartment in growth and reproduction is essential for survival of autotrophic algae.

7.1 Regulatory Relationships between Chloroplast and Nucleo-cytosolic Compartments

Chloroplasts, as photosynthetic organelles, clearly support the metabolism of the entire cell grown under autotrophic conditions via production of energy and assimilation of carbon. On the other hand, since the majority of chloroplast proteins are nuclear encoded, the high growth rate of the chloroplast requires corresponding activity in the cytoplasm. Indeed, the assembly of functional chloroplast ribosomes depends on a supply of proteins made by nuclear-encoded DNA-dependent RNA polymerase. Many essential chloroplast structures consist of supramolecular complexes involving both proteins synthesized in the chloroplast and cytoplasm (Ohad 1975; Parthier 1982). The "selfish" chloroplast, therefore, has to keep the cytoplasmic protein-synthesizing machinery running at a rate to satisfy its demands. This interdependence creates a system of feedback controls that coordinate and even couple growth and development of chloroplast and nucleo-cytosolic compartments. This is, reflected by strict proportionality in the increase in chloroplast and cytoplasmic ribosomes during the course of the *Chlamydomonas* cell cycle (Wilson and Chiang 1977). The timing and extent of reproductive processes in chloroplast and nucleo-cytosolic compartments are also dictated by the supply of energy and organic compounds produced by photosynthesis, which in turn, is strictly regulated by light conditions (irradiance and the illumination regime) (Šetlík et al. 1972; Zachleder and Šetlík 1982). The content of pt-DNA, and the number of nucleoids in which pt-DNA is located, vary markedly during the chloroplast cycle and are strictly regulated by external conditions. The number of nucleoids increases during the cell cycle, reaching a maximum at the time of chloroplast division. The rate of increase, as well as the final number of nucleoids, is strictly controlled by light intensity (Figs. 47, 48 and 49). The higher the light intensity, the more nucleoids are formed, and the rate of nucleoid divisions is strictly light-dependent (Figs. 48 and 49). This reflects the trophic effect that light intensity has on growth and consequently, on chloroplast (plastid) DNA (pt-DNA) replication and nucleoid division. Daughter cells, divided in the dark, have a lower number of nucleoids, as compared to the cell from the same culture but divided in light. This is similar to the overlapping nuc-DNA replication-division sequences in the nucleo-cytosolic compartment, as well as to other prokaryotes, and it clearly supports Cooper's continuum model of the cell cycle (Cooper 1979, 1984).

Clearly, the growth of the chloroplast is a prerequisite for cellular growth, which again is a prerequisite for attaining commitment point and allowing nuc-DNA replication-division sequences. This leads to the hypothesis that the chloroplast plays a decisive role in the regulation of cell cycle processes (Chiang 1975; Šetlík and Zachleder 1983). There are two reasons to support this notion:

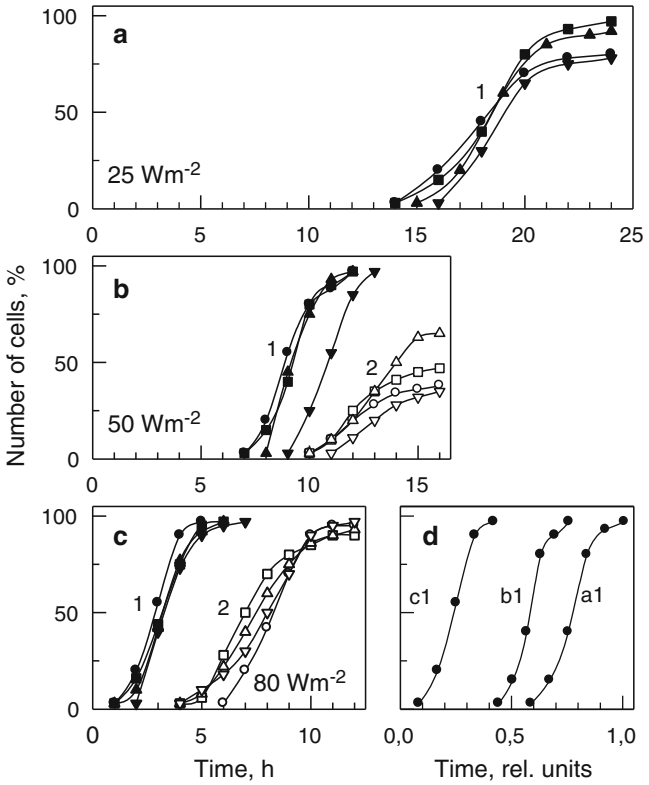

Fig. 35 The effect of light intensity on time courses of attainment of commitment points to cellular divisions in synchronous populations of *Desmodesmus* (*Scenedesmus*). Light irradiance (**a**) 25 W m^{-2}, (**b**) 50 W m^{-2}, (**c**) 80 W m^{-2}. Temperature 30 °C was used for all variants. Curves *1* (*solid symbols*): The percentage of cells that attained the commitment point to divide into four daughter cells (quadrupled coenobia), curves *2* (*open symbols*): the percentage of cells that attained the commitment point to divide into eight daughter cells (octuplet coenobia). Curves for *4* subsequent cycles (*circles*, *squares*, *triangles*, and *diamonds* respectively) are illustrated in all variants. (**d**) ***a1***, ***b1***, ***c1***: The *curves* correspond to the average of curves *1* from the cultures presented in variants (**a**–**c**). The length of the cell cycle is divided into ten units irrespective of its actual duration (After Vítová and Zachleder 2005)

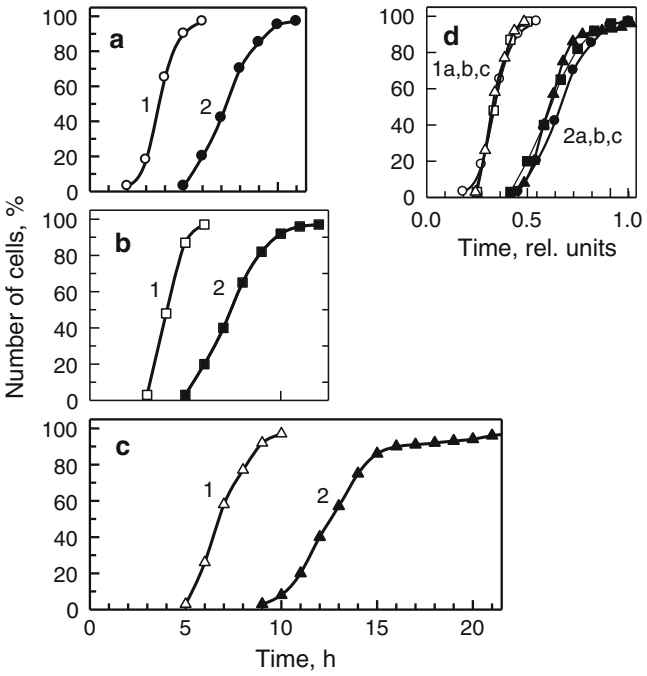

Fig. 36 The effect of temperature on time courses of attainment of commitment points to cellular divisions in synchronous populations of *Desmodesmus* (*Scenedesmus*) *quadricauda*. Temperature (**a**) 35 °C, (**b**) 30 °C, (**c**) 25 °C. Light irradiances 100 W m^{-2} was used for all variants. Curves *1* (*open symbols*): The percentage of cells, which attained the commitment point to divide into four daughter cells (quadruplet coenobia) (open symbols in **d**), curves *2* (*solid symbols*): the percentage of cells, which attained the commitment point to divide into eight daughter cells (octuplet coenobia) (*solid symbols* in **d**). *D*: The *curves* are derived from corresponding curves in *panels* **a**–**c**. *Triangles* are valid for **a**, *squares* for **b** and *circles* for **c**. The length of the cell cycle is divided into ten units irrespective of its actual duration (After Vítová and Zachleder 2005)

1. Chloroplast DNA replication precedes the corresponding reproductive events in the nucleo-cytosolic compartment.
2. The number of pt-DNA replications is equal to the number of nuc-DNA replications (and the corresponding nuclear and cell division), i.e. two processes occurring much later in the cell cycle (Dalmon 1970; Dalmon et al. 1975; Chiang 1971; Chiang and Sueoka 1967b; Iwamura et al. 1982). One experimental approach to study mutual regulatory relationships between reproductive events in the chloroplast and nucleo-cytosolic compartments was to follow the course of growth and reproductive processes within one compartment, while some of these processes were prevented in the second one. Such experiments should answer some of the questions concerning mutual relationships between chloroplast and nucleo-cytosolic compartments and can be divided into the following simple hypothesis-driven experiments:
   (1) How are growth and reproductive processes in chloroplast and nucleo-cytosolic compartments affected by blocking chloroplast DNA-replication?
   (2) How far are the reproductive processes in the nucleo-cytosolic compartment controlled by, or dependent upon, chloroplast reproductive events?
   (3) What is the course of growth and reproduction in the chloroplast under conditions of blocked DNA replication?

The outcome of the experiments, namely those with 5-fluorodeoxyuridine (FdUrd) and nalidixic acid described below, indicate that there is no regulatory mechanism directly coordinating the two cycles, and the relationship is based solely on the trophic role of the chloroplast on the nucleo-cytosolic compartment; there is a continuum of processes that depend on each other but do not require a specific regulating mechanism.

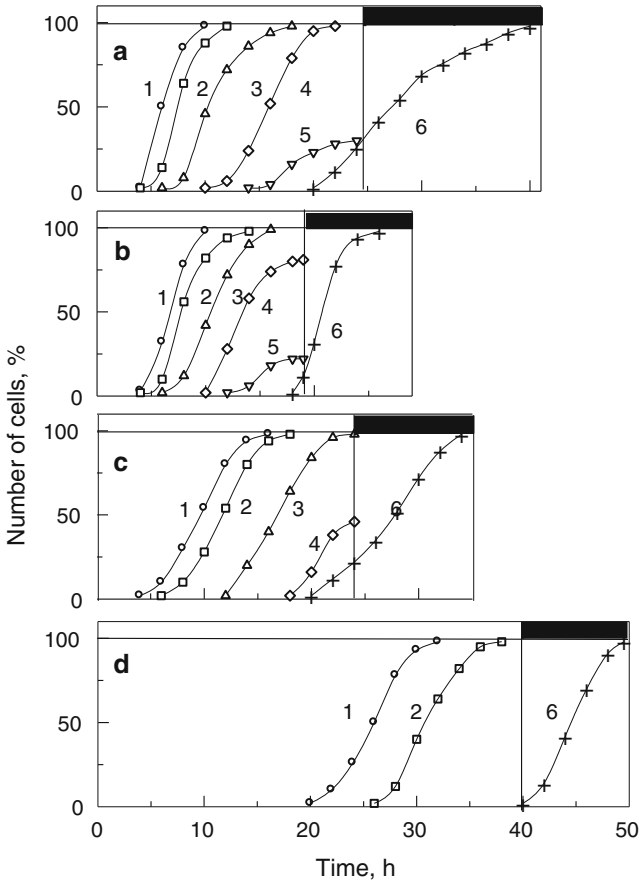

Fig. 37 Time courses of commitment points to nuclear and cellular divisions and termination of these processes in synchronous populations of *Chlamydomonas eugametos* grown at various temperatures. (**a**) 35 °C; (**b**) 30 °C; (**c**) 25 °C; (**d**) 20 °C. Curves *1*, *2*, *3*, *4*, *5*, percentage of the cells that attained commitment points for the first, second, third fourth and fifth nuclear divisions, respectively. Curve *6*, percentage of the cells that released their daughter cells. Light and dark periods are indicated by *white and black strips* above *panels* and separated by *vertical lines* (After Zachleder and van den Ende 1992)

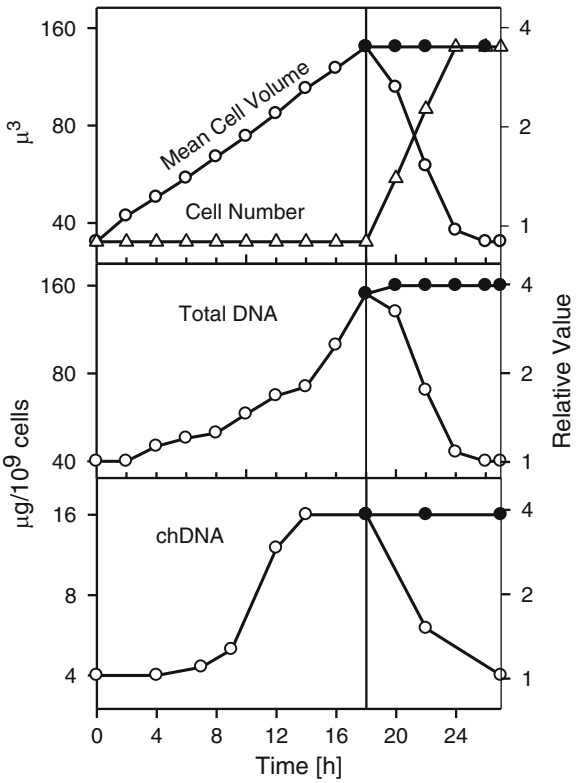

Fig. 38 Synchronous growth of the *Chlorella ellipsoidea* and synthetic patterns for total cellular and chloroplast DNAs formed during the cell cycle. *Hollow and solid bars* in the upper part of the figure represent the light- and dark-phases, respectively. Mean cell volume was calculated by dividing the packed cell volume (PCV) by the cell number per unit volume of culture, this latter unit being shown only as values relative to the value at T_0. The *solid circles* represent values estimated after assuming that no cellular division occurred after T_{18}. They express the net change in each of the variables during the dark-phase (After Iwamura et al. 1982)

7.2 Chloroplast DNA

For the study of pt-DNA and nuc-DNA, the crucial experiments were with algae having a Chlamydomonas-type cell cycle, i.e. multiple reproduction events that occurred clustered at the end of the cell cycle, immediately following each other (see Fig. 3, Chap. 3). In such cell cycles, it is relatively easy to distinguish between the course of chloroplast (pt-DNA) and nuclear (nuc-DNA) replications since they occur at different times during the cell cycle. Moreover, due to different GC contents of pt-DNA and nuc-DNA molecules in *Chlamydomonas reinhardtii*, they can be separated by fractionation and differential centrifugation (Iwamura 1962, 1966, 1970; Iwamura and Kuwashima 1969; Sueoka et al. 1967). The first experiments were carried out on slowly growing *C. reinhardtii*, with mother cells dividing only into two daughter cells (cell cycle type C_1) (see Fig. 16, Chap. 4) (Chiang and Sueoka 1967b). A distinct chloroplast DNA replication occurred earlier in the cell cycle, preceding for several hours nuclear DNA (nuc-DNA) replication. Chiang and his collaborators (Grant et al. 1978; Chiang 1971, 1975; Kates et al. 1968) later confirmed this finding for the same species dividing into more than two daughter cells (cell cycle type C_n). Multiple rounds of pt-DNA replication occurred in the middle of the growth phase of the cell cycle, and were always equal to the number of nuc-DNA replications (and the corresponding number of nuclear and cell divisions) that occurred at the end of the cell cycle. Later, a similar course of pt-DNA was also observed in the synchronized algae, *Chlorela ellipsoidea* (Fig. 38) (Iwamura et al. 1982), where pt-DNA quadrupled during the synthetic period, corresponding to the four chloroplasts present in the mother cells prior to cell division into four daughter cells (Iwamura et al. 1982). While the replication of pt-DNA was performed early in the cell cycle in *C. reinhardtii* (Chiang and Sueoka 1967a, b; Chiang 1975), leading to an increase in nucleoid size, nucle-

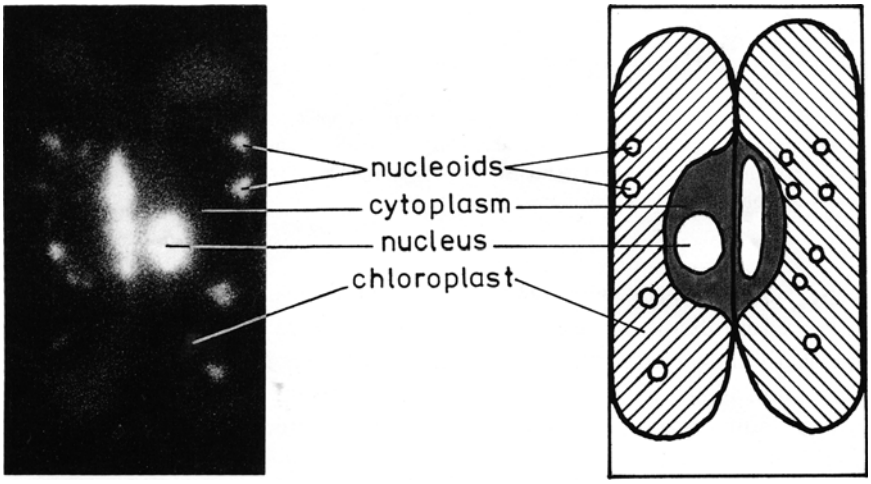

Fig. 39 Fluorescent image of *Desmodesmus* (*Scenedesmus*) *quadricauda* cells stained with DAPI. *Left side*: the photomicrograph of two cells in quadruplet daughter coenobia. *Right side*: a schematic drawing of the cell structures seen in the DAPI stained cells. Note: Chloroplasts are seen as red structures due to the autofluorescence of chlorophyll but are not clearly shown in the *black* and *white* photograph. Very faint fluorescence of the rest of the cell makes the cytoplasm and the outline of cell obscure (After Zachleder and Cepák 1987a)

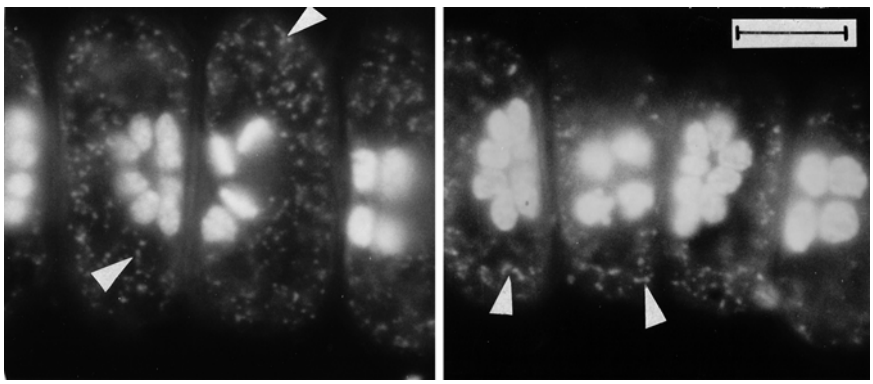

Fig. 40 Fluorescence photomicrographs of DAPI-stained cells of *Desmodesmus* (*Scenedesmus*) *quadricauda* at the division phase into four or eight cell nuclei. Chloroplast nucleoids are small, even sized; many are dumbbell shaped (see *arrows*) indicating intense division. *Bar* = 10 μm (After Zachleder et al. 1975)

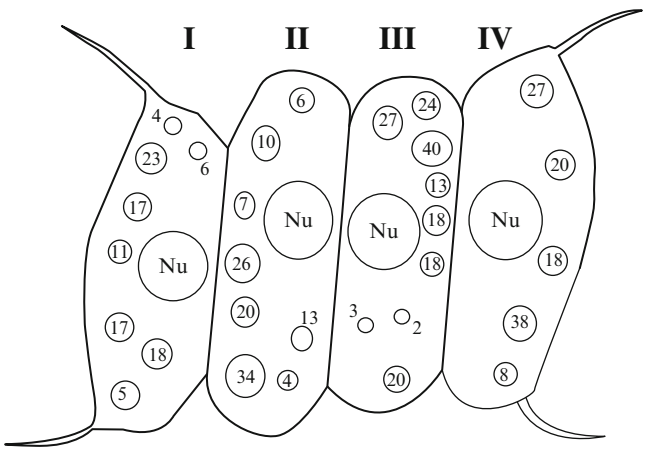

Fig. 41 Schematic drawing of the localization and size of nucleoids and cell nuclei (Nu) in cells of *Desmodesmus* (*Scenedesmus*) *quadricauda*. *Numerals* indicate DNA amount ($\times 10^{-16}$ g) in individual nucleoids (After Zachleder et al. 1995)

oid division was restricted to the very end of the cell cycle. This however, seems to be a specific case since in the closely related *Volvox carteri* and *Volvox aureus*, each pt-DNA rep1ication was followed by the division of nucleoids (Coleman and Maguire 1982). The xantophycean alga *Bumilleriopsis* displays the consecutive Scenedesmus-type of cell cycle, i.e. multiple reproduction events occur consecutively during the whole cell cycle (see Fig. 3, Chap. 3). The alga has numerous chloroplasts dividing during the growth phase of the cell cycle; their division rate traces a curve similar to the rate of pt-DNA synthesis in the algae mentioned above (Hesse 1974).

Similarly as it was found in *Desmodesmus (Scenedesmus) quadricauda* (Zachleder 1995), the recent study of the course of the pt-DNA in a synchronous culture *C. reinhardtii* (Kabeya and Miyagishima 2013) proved that the chloroplast DNA is replicated independently of the timing of chloroplast division and the cell cycle. Chloroplast DNA replicates under the continuous light condition correlated with chloroplast and cell sizes increase to keep the proper DNA content per cell/chloroplast volume.

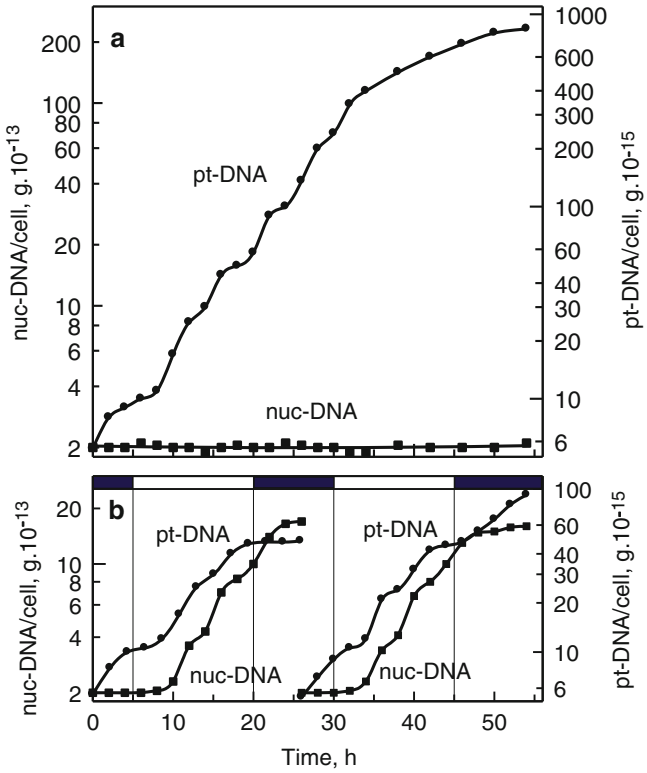

Fig. 42 Variations in chloroplast and nuclear DNA per cell in the FdUrd treated (**a**) and control (**b**) synchronized populations of the *Desmodesmus* (*Scenedesmus*) *quadricauda*. The values for pt-DNA are the mean values from about 30 cells in which total pt-DNA was calculated as the sum of pt-DNA in individual nucleoids. Values for cell-nuclear DNA were obtained by biochemical assay. The FdUrd treated culture (**a**) was continuously illuminated. Time intervals including sequences of dark and light periods in control culture (**b**) are indicated by *black and white bars* at the *top* of *panel* (After Zachleder et al. 1996)

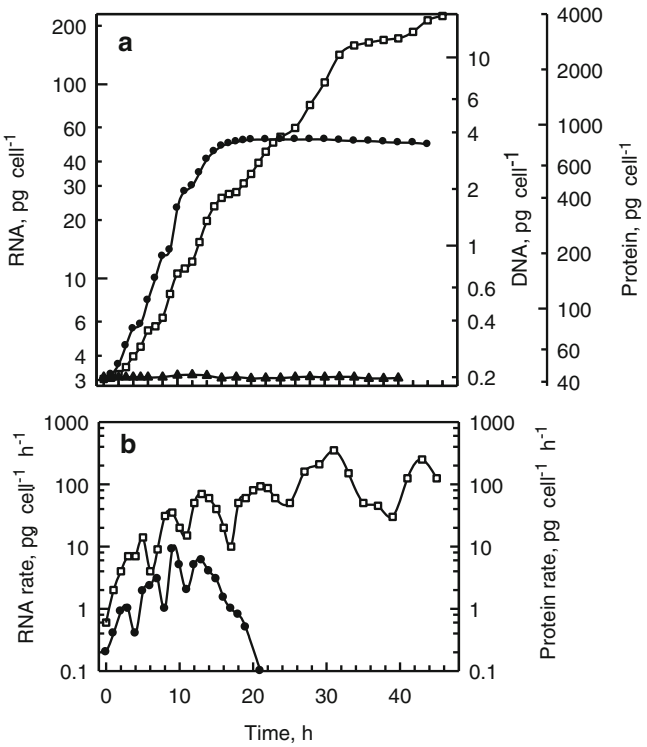

Fig. 43 The course of growth processes (RNA and protein accumulation) in synchronous populations of *Desmodesmus* (*Scenedesmus*) *quadricauda* grown at high irradiance in the presence of FdUrd. Mean irradiance 85 W m^{-2}, continuous light, temperature 30 °C, (**a**) Variation in RNA (●), protein (□), and DNA (▲) amounts per cell; (**b**) Oscillations in the rates of accumulation of RNA (●) and protein (□) (After Zachleder 1995)

Taken together, DNA replication is initiated at each doubling of chloroplast size and it is thus linked with chloroplast growth in a manner characteristic of prokaryotic cells.

Early experiments characterized the bulk of pt-DNA synthesis, however, the structure formed by pt-DNA was, at the time, unknown. This was significantly changed by the introduction of the fluorescent dye, DAPI (4;6-diamidino-2-phenylindole), which can bind with DNA (James and Jope 1978; Williamson and Fennel 1975) and thus made it possible to view such tiny structures as compactly organized DNA-protein complexes (Zachleder and Cepák 1987a, b, c), called nucleoids (Kawano et al. 1982; Kuroiwa et al. 1981; Nagashima et al. 1984) (a term will be used from now on) or chloroplastic nuclei (Kuroiwa et al. 1982, 1991; Nemoto et al. 1990, 1991) (Fig. 39).

Staining with DAPI also simplified research into pt-DNA replication in algae with a Scenedesmus-type cell cycle, where the rounds of replication of nuc-DNA overlap with the replication of pt-DNA and thus cannot be easily followed as for the Chlamydomonas-type cell cycle. *D. quadricauda* cells possess a cup-shaped (often lobed) chloroplast; 2–16 nucleoids can usually be observed in daughter cells at the beginning of the cell cycle. During the cell cycle, the nucleoids divide intensively by binary fission, forming many tiny ones in mother cells at the end of the cell cycle (Fig. 40). In *Desmodesmus quadricauda*, dividing into eight daughter cells, three consecutive steps in the replication of chloroplast DNA occurred over the cell cycle. The first step was performed shortly after release of the daughter cells (even in the dark), the second and third steps occurred consecutively during the cell cycle. While the first round of pt-DNA replication occurred earlier than nuclear DNA, the other two steps overlapped the first two steps in nuc-DNA replications (Fig. 42b).

Once the DNA-containing structures (cellular, chloroplast and mitochondrial nuclei) are stained with DAPI, fluorescence intensity can be used to estimate DNA content during the mitotic cycle in synchronized populations of the chlorococcal algae. An example of such an estimate is presented in Fig. 41, where the size, localization and DNA content of individual pt-nuclei are schematically drawn; the DNA content per nucleoid inside a single chloroplast varied between 2 and 40×10^{-16} g pt-DNA.

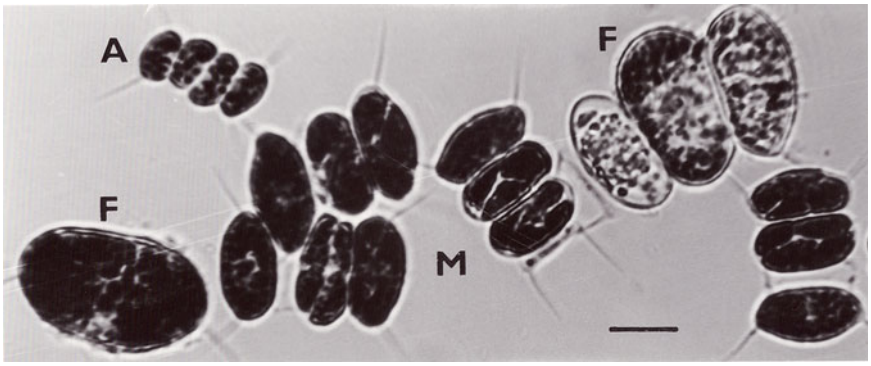

Fig. 44 Comparison of mother cell size of *Desmodesmus* (*Scenedesmus*) *quadricauda* at the end of the control cell cycle with a cell grown for 48 h in the presence of FdUrd. The cells from control and FdUrd treated cultures were mixed together and stained with Lugol solution to visualize starch content. Mother cells in four/celled coenobia (***M***) are dividing their protoplasts and releasing four-celled daughter coenobia (***A***); FdUrd treatment produced a giant cell (***F***) Bar = 10 μm (After Zachleder 1995)

7.2.1 Inhibition of Nuclear DNA Replication

One of compounds used to uncouple chloroplastic and cytoplasmic processes in *Desmodesmus quadricauda* is 5-fluorodeoxyuridine (FdUrd), a specific inhibitor of thymidylate synthase. Replication of nuc-DNA was specifically blocked by FdUrd, leading to a blockage of nuclear division/s and cytokinesis/es. Under the same treatment regime however, growth of the chloroplast was accompanied by continuous intensive replication of pt-DNA while nuc-DNA replication was completely inhibited (Fig. 42a). Growth processes continued for at least one cell cycle (24 h) in the case of the nucleo-cytosolic compartment, and even longer than 48 h in the chloroplast compartment. The first process affected by the treatment was RNA in the nucleo-cytosolic compartment, while bulk protein synthesis continued (Fig. 43) simultaneously with chloroplast growth. Eventually, the FdUrd-treated cells grew to a giant size (Fig. 44) with only a single nucleus containing its initial content of nuclear DNA.

Due to the blocking of nuc-DNA replication, all consequent energy and carbon consuming processes, such as nuclear division/s and cytokinesis, were inhibited, leading to a massive accumulation of unspent and continuously synthesized starch grains in a growing giant chloroplast (see, Fig. 53, Chap. 8). Once the inhibitor was removed, the cell rapidly underwent all committed processes of the DNA replication-division sequence. Even in the case of *D. quadricauda*, this would lead to cell cycle progression resembling that of *Chlamydomonas* (Zachleder et al. 2002).

7.2.2 Inhibition of Chloroplast DNA Replication

As is discussed above, inhibition of nuclear DNA effectively blocked all consecutive processes of the DNA replication-division sequence, e.g. nuclear and cellular division/s. In contrast, the chloroplast cycle, i.e. replication of chloroplast (plastid) DNA (pt-DNA) and division of nucleoids, was not affected by the absence of reproductive processes in the nucleo-cytosolic compartment, leading to the production of giant cells with a single nucleus and its initial DNA content. In spite of inhibition of the DNA replication-division sequence, growth was not substantially affected, even in the nucleo-cytosolic compartment.

Similarly to blocking nuc-DNA replication by FdUrd, due to its prokaryotic nature, pt-DNA in algae can be inhibited by prokaryotic-specific DNA inhibitors such as nalidixic acid (1-ethy1-1,4-dihydro-7-methy1-4-oxo-1,8-naphtyridine-3-carboxylic acid) (NAL), an inhibitor of DNA gyrase. NAL inhibits chloroplast DNA synthesis with no effect on nuc-DNA synthesis in higher plants (*Nicotiana tabacum*) (Heinhorst et al. 1985), in green flagellates (*Euglena*) (Hashimoto and Murakami 1982; Pienkos et al. 1974), green algae (*Chlamydomonas reinhardtii*) (Robreau and Le Gal 1974), and the unicellular red algae (*Cyanidioschyzon merolae*) (Itoh et al. 1997).

The application of NAL to synchronized cultures of *D. quadricauda* inhibited both pt-DNA replication (Fig. 45b) and nucleoid division (Fig. 45c) (Zachleder et al. 2004). Chloroplast growth was not defective because the chloroplast nucleoids usually contain multiple DNA molecules so that growth is not limited by DNA template, even if pt-DNA replication is blocked (Zachleder et al. 2004). Interestingly, chloroplast division was not affected and was performed in coordination with cellular division. The divided chloroplasts had reduced numbers of nucleoids and, in some cases, only one or even no nucleoid was observed in daughter cell chloroplasts. Similarly, no nucleoids were present under some conditions in the red alga *Cyanidioschyzon merolae* (Itoh et al. 1997), the green flagellate *Euglena gracilis* (Hashimoto and Murakami 1982) and part of the chloroplast population in *Acetabularia* (Dasycladales) (Woodcock and Bogorad 1970).

The rate of growth processes in the nucleocytoplasmic compartment was about half that in the control, probably due to reduced accumulation of rRNA and ribosomes. However, all processes ran normally with the CPs reflecting the slower

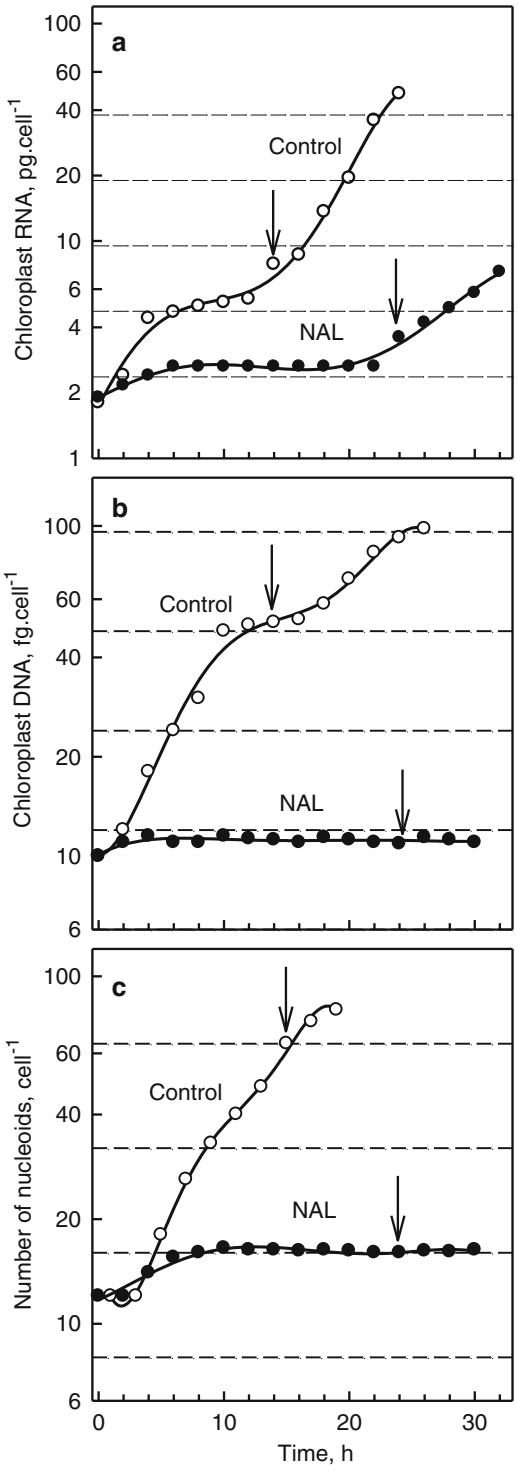

Fig. 45 Changes in chloroplast RNA (**a**), chloroplast DNA (**b**), and the number of chloroplast nucleoids (**c**) per initial cell during the cell cycle in synchronized populations of *Desmodesmus* (*Scenedesmus*) *quadricauda* grown at an irradiance of 100 W m^{-2} in NAL-free medium (Control *open symbols*) or in the presence of nalidixic acid (NAL *solid symbols*) from the beginning of the cell cycle. *Horizontal dashed lines* mark doubled values (After Zachleder et al. 2004)

growth rate with no affect on the nuclear DNA replication-division sequence. The control cells, as well as those grown in the presence NAL, divided into the same number of daughter cells, but in the case of NAL-treated cells, with reduced numbers of nucleoids per chloroplast (on average 2, range 1-4) (Fig. 46b). The existence of chloroplast division, even in the presence of NAL, indicates that it is, at least to some extent, independent of pt-DNA and nucleoid replication and is directed by the "master" cell division (Figs. 47, 48 and 49).

7.3 Chloroplast RNA

7.3.1 Effect of Light on RNA Synthesis

Changes in the availability of light to individual cells of *C. reinhardtii* cause fluctuations in photosynthetic capacity, levels of starch, rates of respiration, and the acquisition of enzyme activities (Donnan et al. 1985; Donnan and John 1983). Other processes affected by light availability are the accumulation of chloroplast and cytoplasmic RNA Both cytoplasmic and chloroplast RNA accumulate exponentially when grown in light but the rate of accumulation and final content of rRNA per cell is strictly light intensity-dependent. Time courses of cytoplasmic and chloroplast RNA synthesis are more or less parallel (Fig. 50a, b).

7.3.2 Inhibition of Chloroplast-Protein and RNA Synthesis

Chloroplast protein synthesis can be blocked by a specific inhibitor, chloramphenicol, leading also to a cessation in chlorophyll (Fig. 51) and chloroplast ribosomal RNA (chl-RNA) synthesis (Fig. 52). Under these conditions, autotrophic growth of algal daughter cells is completely prevented due to the inability to use light and CO_2 as an external source of energy and carbon via photosynthesis. Thus, glucose must be used as a source of energy and carbon in order to study the relationship between chloroplast and nucleo-cytosolic compartments and the cells grow heterotrophically. Under heterotrophic conditions, chl-RNA synthesis (Fig. 52), chlorophyll synthesis and starch synthesis in the chloroplast (Fig. 51a), were not affected in the presence of glucose. In spite of the presence of glucose, chloramphenicol, however completely stopped the synthesis of chlorophyll (Fig. 51) and chl-RNA (Fig. 52) regardless if grown in the light or dark (Fig. 52, empty and full symbols, respectively). Surprisingly, in the presence of chloramphenicol, starch synthesis was not affected and continued as in heterotrophically grown control cultures (Fig. 52, G and G+CAP, dashed line). In spite of a severe limitation in chloroplast growth processes

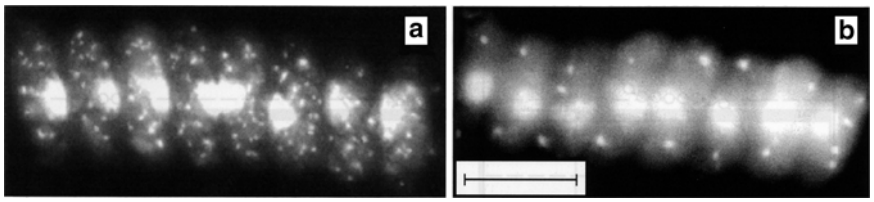

Fig. 46 Fluorescence photomicrographs of DAPI daughter coenobia *Desmodesmus* (*Scenedesmus*) *quadricauda* liberated from control (**a**) and NAL-treated (**b**) mother cells; intensive fluorescence of DAPI-stained structures is seen as *white spots*; the largest spots represent the nuclei; nucleoids are seen as *small spots* localized in chloroplast. Bar = 10 μm (After Zachleder et al. 2004)

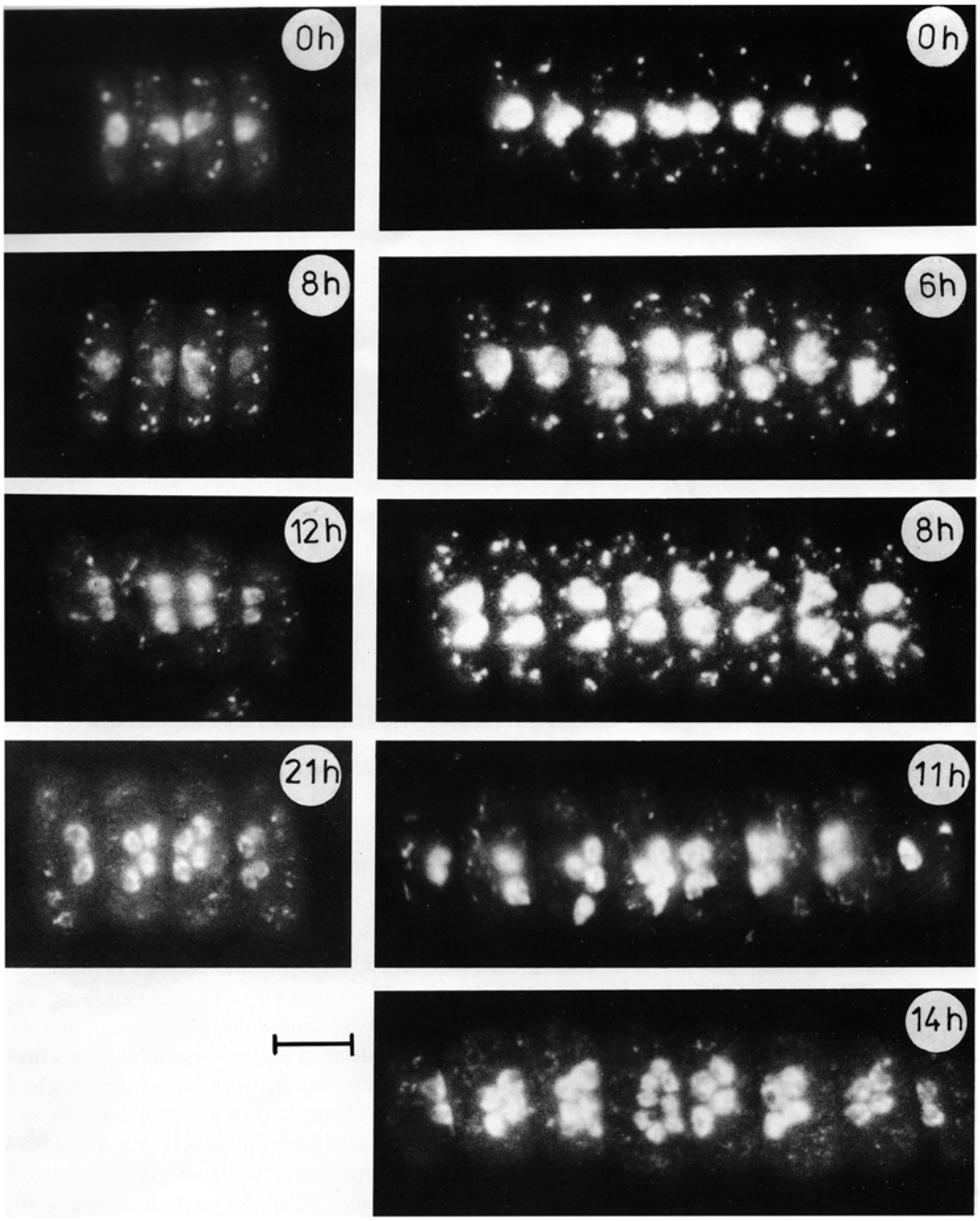

Fig. 47 Fluorescent microphotographs of typical DAPI-stained coenobia in synchronous populations of *Desmodesmus* (*Scenedesmus*) *quadricauda* grown at low and high irradiances. The age of the cells in hours, from the beginning of the cell cycle, is indicated at the-*top right corner* of individual microphotographs. *Left column*: The cells in quadruplet coenobia grown at the mean irradiance 30 Wm^{-2}. *Right column*: The cells in octuplet coenobia grown at the mean irradiance 100 W m^{-2}. Intensive bluish fluorescence of DAPI-stained structures can be seen as *white spots* in the black and white photographs. *Big spots* represent nuclei. Nucleoids are seen as small spots distributed seemingly at random in the chloroplasts. *Bar* = 10 μm (After Zachleder and Cepák 1987b)

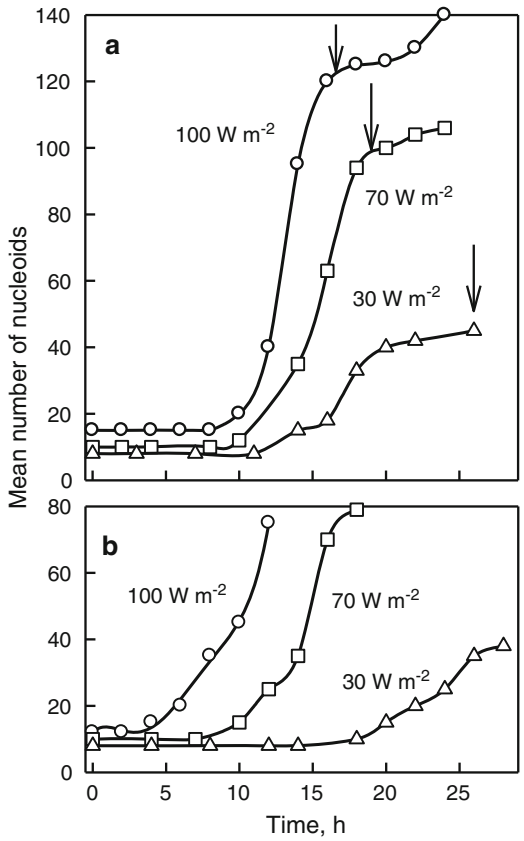

Fig. 48 Variations in the mean number of chloroplast nucleoids in synchronous populations of *Desmodesmus* (*Scenedesmus*) *quadricauda* grown under continuously illumination at high (100 Wm^{-2}), moderate (70 Wm^{-2}), and low (30 Wm^{-2}) irradiance. *Arrows* indicate the moment when 50 % of the population has divided. (**a**) The first cell cycle: Daughter cells were released during dark period of the preceding cell cycle. The moment of the illumination is taken at the beginning of the cell cycle. (**b**) The second cell cycle: Daughter cells were released in light. The times when 50 % the population has released the daughter cells are taken as the beginning of the cell cycle. Only parts of the second cell cycles at three irradiances are illustrated (After Zachleder and Cepák 1987b)

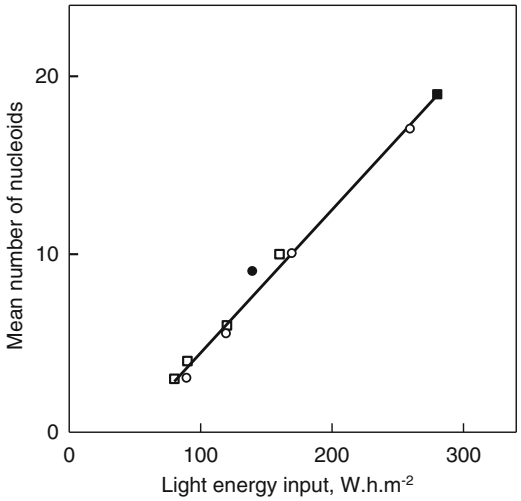

Fig. 49 Relationship between the total amount of light energy obtained by one daughter cell of *Desmodesmus* (*Scenedesmus*) *quadricauda* and the number of chloroplast nucleoids. Light energy (**E**) was calculated from the light intensity (**I**), interval of illumination (**t**) under which mother cells grew and number of daughter cells formed (**n**) according to the formula: $\mathbf{E = I.\ t/n}$. □ daughter cells in four-celled coenobia from mother cells grown at 25 Wm^{-2} PAR, daughter cells in four-celled coenobia (■) and in eight-celled coenobia (●) from mother cells grown at 75 Wm^{-2} PAR, ○ daughter cells in eight-celled coenobia from mother cells grown at l30 Wm^{-2} PAR (After Zachleder and Cepák 1987a)

due to inhibition of proteosynthesis, events in nucleo-cytosolic compartment were not substantially influenced. Growth processes in the nucleo-cytosolic compartment were not affected by the presence of chloramphenicol, as illustrated by the time course of cytoplasmic ribosomal RNA (cyt-RNA) (Fig. 52) regardless if kept in the presence of glucose in the dark or in light. The time course of the DNA replication-division sequence was the same as in the control culture (Fig. 51b). The cells were able to complete the entire cell cycle and release four small daughter cells.

Ribosomal RNA is a crucial component of growth since it is required for building ribosomes. Furthermore, a threshold level of nuc-RNA is a prerequisite for DNA replication (Singer and Johnston 1979; Zachleder and Šetlík 1988). DNA replication in the chloroplast often preceded replication events in the nucleus, and the number of chloroplast DNA replications seemed to be predictive of the final number of DNA replications in the nucleus. It was suggested that the chloroplast might play an important role in regulating cell cycle events in photoautotrophic organisms (Chiang 1975; Chiang and Sueoka 1967b; Šetlík and Zachleder 1984). Experiments with uncoupling DNA replication in the chloroplast and nucleus by blocking nuc-DNA replication by FdUrd suggested that there is no mechanism blocking the chloroplast cycle when the nuclear DNA replication-division sequence is blocked. On the other hand, experiments with nalidixic acid indicated that inhibition of chloroplast growth will not directly block the nuclear cycle processes. Finally, the effect of growth inhibition of the chloroplast had a mere trophic effect, suggesting a growth dependency of the nuclear cycle on the chloroplast but no supporting the existence of any exclusive coordination mechanism.

7.3.3 Role of Starch

Algal cells develop fully active chloroplasts, even after long-term cultivation (15 years) in the dark under heterotrophic

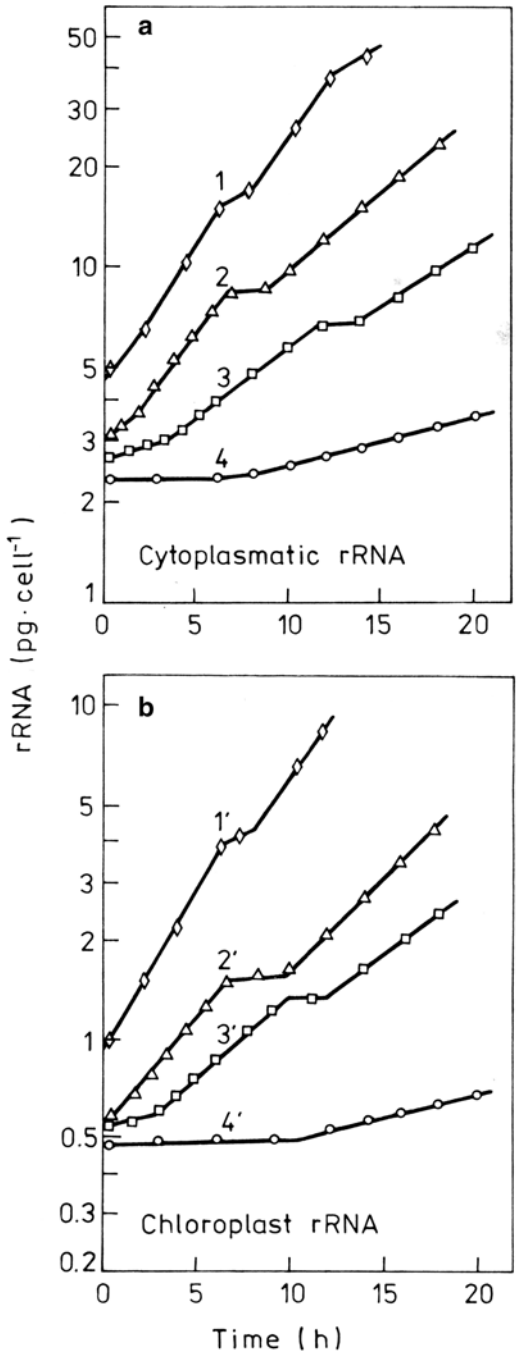

Fig. 50 Accumulation of cytoplasmic (**a**) and chloroplast (**b**) RNA during the second cell cycle in a synchronous population of *Desmodesmus* (*Scenedesmus*) *quadricauda* grown and continuously illuminated at four different mean irradiances. Curves *1*:150 W m^{-2} ; curves *2*; 75 W m^{-2}; curves *3*: 40 W m^{-2}; curves *4*: 20 W m^{-2} (After Cepák and Zachleder 1988)

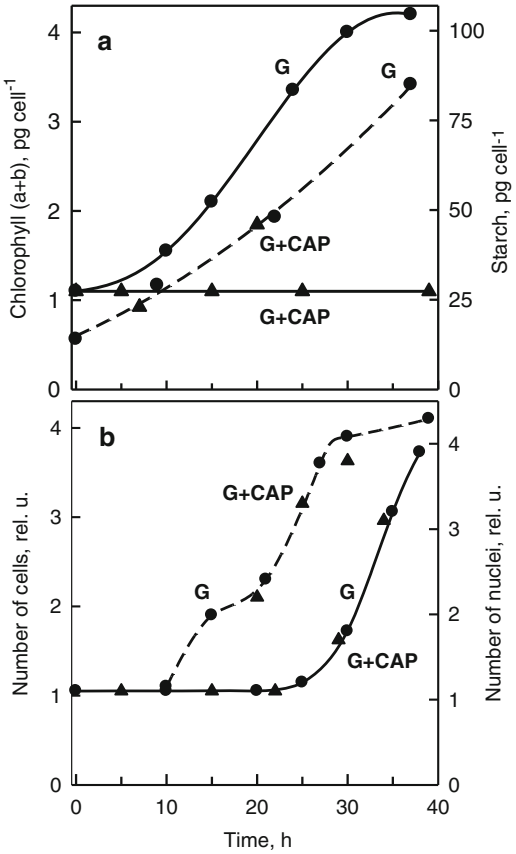

Fig. 51 Accumulation of chlorophyll (*solid line*) and starch (*dashed line*) (**a**) and the time course of the nuclear (*dashed line*) and cellular division (*solid line*) (**b**) in synchronous populations of *Desmodesmus* (*Scenedesmus*) *quadricauda* grown in dark in the presence of 1 % glucose (**G**, ●) or in the presence of glucose and chloramphenicol (**G+CAP**, ▲) (After Zachleder et al. 1990)

conditions (Iwamura et al. 1982). This indicates that certain functions of the chloroplast must be preserved to enable normal progress through the cell cycle. One candidate is starch synthesis. It is not affected by light, even in the presence of chloramphenicol, if a substitute source of external energy (glucose) is provided. Accumulation of starch remains active under a wide range of restrictive conditions such as limitation by nitrogen (Ballin et al. 1988), sulphur (Šetlík et al. 1988) or phosphorus (Zachleder et al. 1988), or under benzopyrene inhibition (Zachleder et al. 1983), when most other synthetic processes were more or less inhibited. Chloroplastic starch reserves are used exclusively for performing the nuc-DNA replication-division sequence in *Chlorella* (Wanka

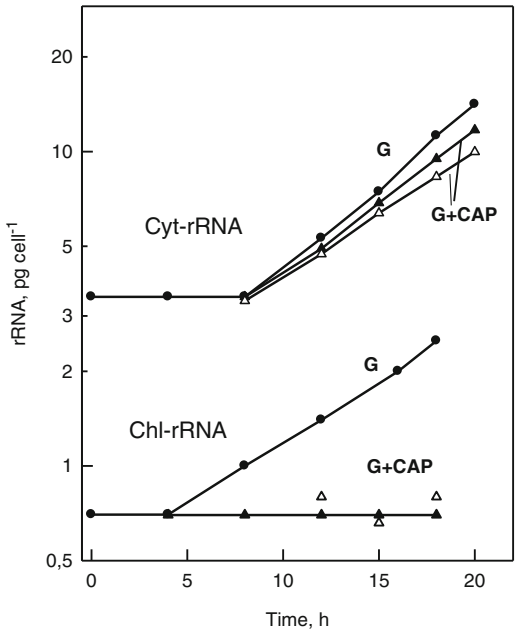

Fig. 52 Accumulation of cytoplasmic and chloroplast RNA in synchronous populations of *Desmodesmus* (*Scenedesmus*) *quadricauda* grown in presence of glucose in dark (**G**, ●). glucose and chloramphenicol (**G+CAP**) in dark (▲) or in light (Δ) (After Zachleder et al. 1990)

1968) and *Chlamydomonas* (Spudich and Sager 1980; Vítová et al. 2011b) by cells grown both in the light and dark. Furthermore, sufficient starch reserves are required for attainment of commitment points (Spudich and Sager 1980; Vítová et al. 2011b). This indicates the amount of starch or the rate of its degradation might be the process connecting/coordinating the DNA replication-division sequences in chloroplast and nucleo-cytosolic compartments.

8 Energy Reserves

The amount of sun light, the sole source of energy for growth and division of phototrophically grown algae, varies during the day in addition to day/night alternation. While growth processes can easily adapt to variations in energy supply by changing their rate, the variation or even absence (during nights) of an external energy source it is extremely challenging for vital high energy demanding reproductive processes, DNA replication, nuclear and cellular division. Producing energy reserves for reproductive processes is therefore essential in order to compensate for differences in the rate of energy production. Algae have, in principle, two main types of energy reserves: starch (and other polyglucans) and lipids. Both components serve both as an energy source and as a supply of carbon allowing the cells to become completely independent of a direct supply of energy and carbon from photosynthetic activity.

Surprisingly, with the exception of a few early papers (Ballin et al. 1988; Duynstee and Schmidt 1967; Šetlík et al. 1988; Zachleder et al. 1988), little research has focused on this crucial process. The recent boom in energy reserve research in algae, motivated by the idea that algae could serve as a high-yielding source of energy-rich components for bioethanol production from starch or biodiesel from lipids has, however, resulted in a considerable body of information on starch and lipids in algae.

Starch is produced in most green algae as a primary store of carbon and energy. Other algae produce different types of polyglucans such as chrysolaminarin in diatoms, floridean starch and glycogen in red algae and paramylon in euglenophytes (Hildebrand et al. 2013). Lipids are the primary carbon and energy store in algal species from eustigmatophytes like *Nannochloropsis*, (Rodolfi et al. 2009) and *Trachydiscus* (Řezanka et al. 2010) apparently unable to produce glucans, but are also produced under specific conditions by other algae. Depending on the conditions, some strains of *Chlorella* or *Parachlorella* can overproduce starch (Fig. 53a) (Brányiková et al. 2011), while some strains of the same species overproduce lipids instead (Fig. 53b) (Li et al. 2013). A diatom *Odontella aurita* is industrially used to produce omega-3-fatty acids, while it also produces chrysolaminarin (Xia et al. 2014). A detailed description of this topic is out of the scope of this Chapter and would duplicate recently published reviews (Ahmad et al. 2011; Brennan and Owende 2010; Cheng and Timilsina 2011; John et al. 2011; Lam and Lee 2011; Mata et al. 2010; Rodolfi et al. 2009; Singh and Olsen 2011; Přibyl et al. 2013; Zachleder and Brányiková 2013).

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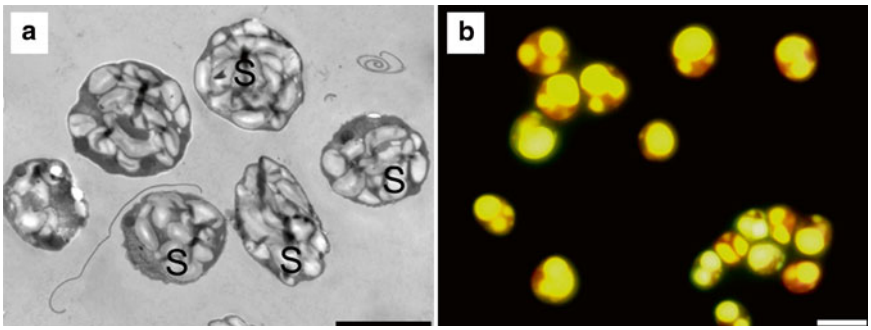

Fig. 53 Electron photomicrographs of *Chlorella vulgaris* grown in sulfur free mineral medium (**a**) S large starch bodies filling most of the cell volume. Scale bar = 10 µm (After Brányiková et al. 2011). (**b**) Overproduction of lipids during batch cultivation of *Parachlorella kessleri* in nitrogen free medium. Lipid bodies were stained using Nile Red (*yellow*); autofluorescence of chloroplasts is seen in red. Scale bar = 10 µm (After Li et al. 2013)

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Biosynthesis of the Cell Walls of the Algae

David S. Domozych

1 Introduction

"Algae" constitute a diverse array of photosynthetic eukaryotes that are derived from multiple evolutionary origins and today occupy most of modern earth's photic zones. These organisms are of profound importance to many food chains as well as planet-wide gas exchange dynamics and mineralization processes (Graham et al. 2009). Algal cells, like virtually all forms of life, are covered by an extracellular matrix (ECM) that contributes significantly to cell-cell adhesion, cell expansion control, the sensing of environmental stressors, defence, reproduction and morphogenesis. Algal ECM also contributes prominently to human activities and has been harnessed for extraction of biochemicals that are used in the pharmaceutical, food and biofuel industries (Radakovits et al. 2010; Craigie 1990).

The algal ECM exhibits many architectural designs that include multi-shaped scales, highly mineralized organic shells and crystalline glycoprotein coverings (Domozych 2012; see Table 1). In most taxa of the late divergent green, brown and red algae, the ECM is typically characterized by ***cell walls*** that consist of microfibrillar networks embedded in matrices of diverse polysaccharides and proteins. Approximately 450–500 million years ago, one group of green algae, the charophytes or "Charophycean Green Algae" (CGA or the Streptophyta; Leliaert et al. 2012; Timme et al. 2012), invaded terrestrial ecosystems and ultimately gave rise to modern land plants in arguably the most important event in the history of life on the planet. The cell walls of these early invaders and their extant relatives changed the biosphere profoundly (Niklas 2004) and became a basis for modern human cultural evolution (e.g., agriculture, wood, paper and textiles).

Currently, most of our knowledge of the structure and function of cell walls is centered on research dealing with those found in land plants and for good reason. Polymers of these cell walls constitute the largest source of annual renewable biomass on the planet. Likewise, the synthesis and maintenance of a cell wall is "front and center" in the physiology of a plant cell. A very large percentage of a land plant's photosynthetically-derived organic product and as much as 30 % of its genetic machinery are employed for the construction and maintenance of wall architecture (Popper et al. 2008, 2011). Algal cell walls and other ECM materials also contribute significantly to the biomass of specific habitats and though no specific information is yet available, one might reasonably speculate that the cell wall of an alga requires similar investment of the cell's genetic and photosynthetic machinery.

Our current understanding of the developmental processes and regulatory controls required for the production of algal cell walls is not nearly as well resolved as that of land plants. In those land plant taxa that have been investigated thoroughly (e.g. *Arabidopsis*), a highly coordinated interaction of multiple subcellular systems functioning in response to multiple genetic prompts and environmental stresses that utilize complex signal cascades is required for competent synthesis, secretion and remodelling of the cell wall. For algae, recent research, especially that employing the advanced tools of molecular biology biochemistry, cell biology, immunology and high throughput/rapid screening methodologies such as chemical genomics (e.g. Mravec et al. 2014; Sørensen et al. 2011; Michel et al. 2010) is beginning to yield new and valuable insight into the structure of algal cell walls and more importantly, their developmental dynamics. In this chapter, a review of some of these recent research efforts dealing with algal cell wall developmental dynamics in algae is provided.

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Table 1 Overview of extracellular coverings and their chemical constituents

Algal group	Components of extracellular coverings
Chlorophyta (green algae)	Scales, theca, cell wall
	Cellulose, mannans, xyloglucans, xylans, 1,3 ß-glucans, mixed linkage glucans, xylogalactorhamnans, rhamnoxyloglactogalacturonans, Kdo and 5-O-Kdo polymers, arabinogalactan proteins, extensin
Rhodophyta (red algae)	Cell wall
	Cellulose, xylans, mannans, sulfated mixed linkage glucans, sulfated galactans
Phaeophyta (brown algae)	Cell wall
	Cellulose, alginates, fucoidans
Haptophyta	Coccoliths
	Acidic polysaccharides, proteins, calcite
Dinophyta	Amphiesma
	Cellulose
Bacillariophyta	Frustule
	Mannose and 1,3 ß-glucans, multiple proteins, silica

2 Cell Walls: An Architectural Paradigm with Much Fine Tuning

The cell wall of plants consists of a framework of fibrillar polysaccharides that is embedded in a matrix composed of neutral and charged polysaccharides, various proteins and in some cases, polyphenolics like lignin (Sarkar et al. 2009; Fry 2000). This structural design is also found in cell walls of late divergent taxa of the charophytes (Fangel et al. 2012; Domozych et al. 2012; Sørensen et al. 2010, 2011; Popper et al. 2011). Previous interpretations of the phylogeny of the charophytes (Becker and Marin 2009; Graham 2009) suggested that the ancestors of modern day charophytes were capable of making the successful transition to land because specific features of their physiology and biochemistry allowed for a degree of pre-adaptation to life on land. Sørensen et al. (2011) further proposed that the ability of the charophytes to produce cell walls with specific polymer arrays was a critical aspect of this pre-adaptation for this terrestrial invasion. It is also important to note that the general architectural design of a fibril/matrix cell wall is also found within many taxa of the Chlorophycean green algae, the red algae (Rhodophyta), the brown algae (Phaeophyta) and Xanthophyceae (Fig. 1). Though the specific polymeric composition of both the fibrillar and matrix components may vary, it seems quite clear that the design of a matrix-reinforced fibrillar composite (e.g., like reinforced concrete or fiberglass) was selected by a broad phylogenetic spectrum of algae as the most efficacious covering for survival in a wide range of habitats, each with specific challenges.

The microarchitecture of the cell walls of algae and plants is not only complex, it is dynamic. The polymers therein are secreted/woven, incorporated and interconnected in precise networks that are closely regulated by genetic and environmental cues. Subsequent biochemical modulations alter these polymeric networks during expansion, development and in response to environmental stress. Production of the cell wall requires the temporally and geographically coordinated activities of the endomembrane system, cytoskeletal network, plasma membrane and the wall itself.

3 The Structural Framework of Microfibrils: Cellulose, Mannans and Xylans

The ß-1,4-glucan polymer, cellulose, is the major fibrillar and load-bearing component of the cell walls of plants and many algal taxa (Popper et al. 2011). Cellulose is found in unbranched and insoluble "crystalline" strands called microfibrils that are products of inter-and intrapolymer hydrogen bonding of hydroxyl groups among adjacent β-1,4-glucan chains. Microfibrils are highly resistant to digestion and often have a tensile strength that is greater than some steels (Niklas 1992), i.e., advantageous features for a macromolecule that serves as the load-bearing framework of the cell wall. The infrastructure of the cellulose network in a cell wall and its functional role are also products of the complex and interconnected matrix of other polysaccharides and proteins that surrounds the microfibrils (Cosgrove 2014).

The identification of the pathway and components of cellulose biosynthesis of photosynthetic eukaryotes is deeply grounded in algal-based studies (Giddings et al. 1980; Roberts and Roberts 2007; Roberts et al. 2002). The biosynthetic machinery for making cellulose in these organisms is believed to be an evolutionary product of lateral gene transfer derived from an ancestral bacterial endosymbiont (Niklas 2004). Cellulose is most often synthesized in the plasma membrane and is subsequently deposited or incorporated into the cell wall (McFarlane et al. 2014; Lerouxel et al.

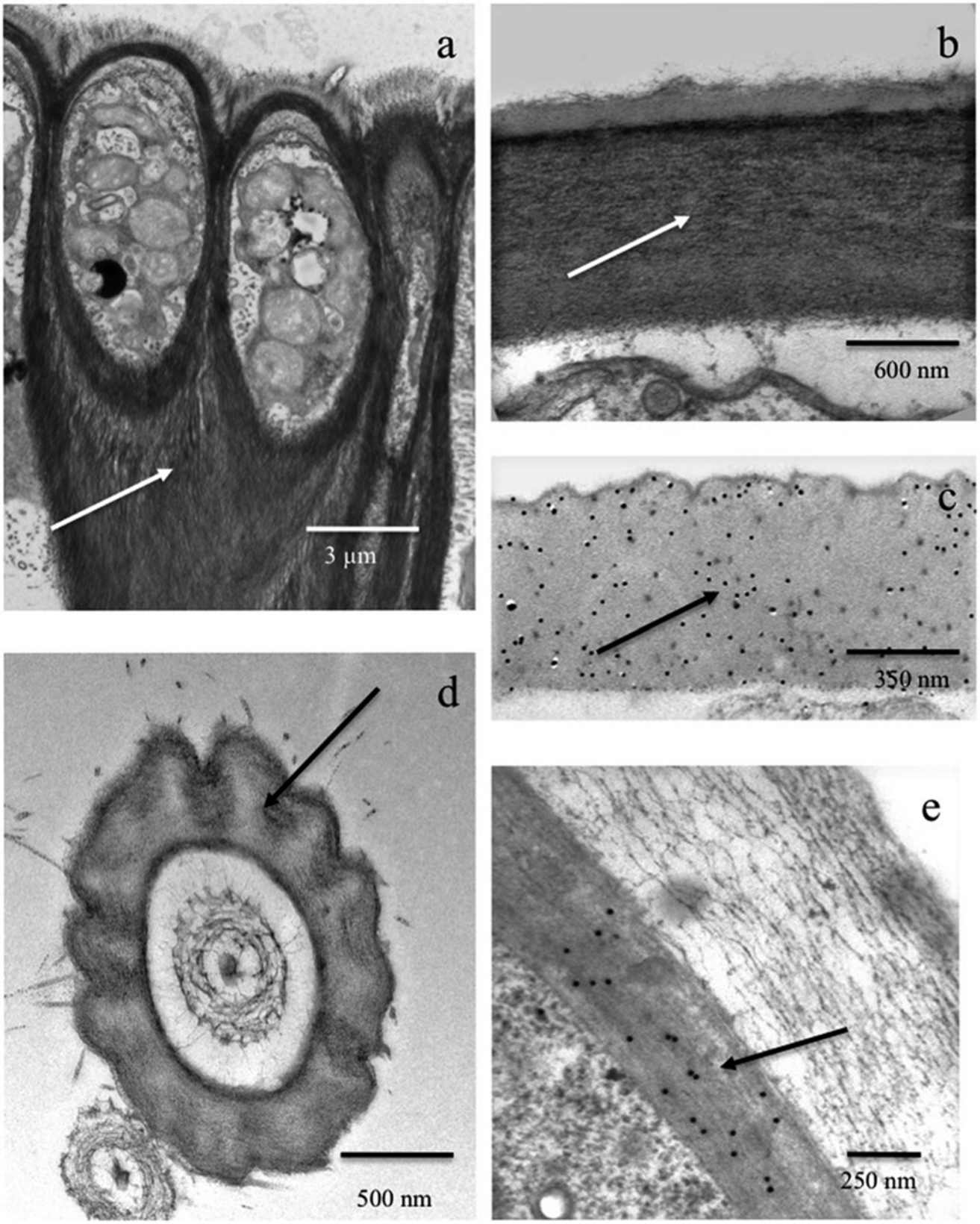

Fig. 1 Overview of the diversity of cell walls in the algae. (**a**) The thick fibrillar walls (*arrow*) of the epidermal cells of the "button" of the brown alga, *Himenthalia*. (**b**) The thickened wall (*arrow*) of an internodal cell of the CGA, *Chara corallina*. (**c**) Anti-mannan (LM21) labelling of the cell wall (*arrow*) of the green alga, *Codium fragile*. (**d**) The thickened cellulosic wall (*arrow*) of a hair cell of the green alga, *Chaetosphaeridium*. (**e**) Anti-MLG labelling of the cell wall (*arrow*) of the desmid, *Pleurotaenium trabecula*. All images are TEM

2006). The enzyme complex that is responsible for cellulose synthesis is the cellulose synthesizing apparatus or CESA which has been classified in one *super* gene family containing nine cellulose synthase-like (Csl) families and one cellulose synthase (CESA) family (Yin et al. 2009). CESA synthesis occurs (i.e., synthesized or initially recognized) in the Golgi apparatus (Gutierrez et al. 2009). It is transported to the plasma membrane in Golgi-derived vesicles where it is incorporated into terminal complexes (TCs) that fabricate cellulose microfibrils (Somerville 2006; Guerriero et al. 2010). TCs contribute both to the polymerization of the glucan chains and the assembly of these chains into microfibrils (Roberts and Roberts 2007). Cellulose is most likely produced on the interior of the plasma membrane and ultimately moves to the outside via porin-based pores (Guerriero et al. 2010). Cellulose biosynthesis requires nucleotide sugars and is often associated with sucrose synthase, endo-ß1-4 glucanses and annexins (Baroja-Fernández et al. 2012; Davies 2014). Freeze fracture/etch-transmission electron microscopy (TEM)-based imaging has demonstrated that in land plants and most of the late divergent taxa of the charophytes, the TCs are arranged in hexameric rosettes of 25–30 nm. Each of the six components of the rosette contains six CESA units, each of which produces one ß1-4 glucan chain. The 36 glucan chains produced by a single rosette, associate via H-bonds to form a microfibril of approximately 2–4 nm in width and thickness. However, new imaging technology has shown that microfibrils may be considerably smaller, consisting of 24 or 18 chains formed by rosettes containing three CESA components (Cosgrove 2014). The verification of the role of TCs in cellulose biosynthesis was provided through labelling of TCs with a monoclonal antibody (mAb) specific for CESA using the green alga, *Micrasterias* (Nakashima et al. 2006).

In many algal groups, microfibril size may also be much greater than that of land plants. For example, in some red algae, microfibrils may measure from 25 to 68 nm (Tsekos 1999; Saxena and Brown 2005; Roberts and Roberts 2009). This size variation and the deposition/layering of cellulose microfibrils in the wall architecture are directly related to variations in the architecture of the TC. Chlorophycean green algae possess linear TCs that contain three rows of particles (Roberts and Roberts 2007) while in brown algae, single linear TCs with up to 100 nm subunits yield distinct ribbon-like microfibrils (Tsekos 1999). The red alga, *Erythrocladia subintegra* (= *Sahlingia subintegra*),[1] produces single linear terminal complexes that measure 180 by 35 nm arranged in four rows of 30–140 particles, while in the genera, *Radicilingua* and *Laurencia*, two rows of three particles each have been observed. These smaller complexes though could represent microfibril terminating synthesizing complexes. In the Xanthophytes, distinct stacked or diagonally-arranged linear TCs have been described (Mizuta and Brown 1992).

During different phases of the cell or developmental cycles of plants and algae, cellulose microfibrils are often deposited in specific layers or lamellae. Cellulose microfibrils have great strength under tension but are relatively weak against shear and compressive forces. Therefore, the production of cellulose in multiple layers allows the cell to resist forces both internal and external from various directions (Anderson et al. 2010). The orientation of cellulose microfibrils in the layers of the cell wall also correlates closely with the orientation of cortical microtubules found just underneath the plasma membrane (Taylor 2008). In land plants, this microfibril-microtubule orientation has been demonstrated using live-cell imaging (Crowell et al. 2011; Taylor 2008; Paradez et al. 2006; Lerouxel et al. 2006). Here, microtubules guide the trajectories of GFP-labelled TCs in the plasma membrane that, in turn, orient the deposition of microfibrils. The exact molecular mechanisms behind the function of the microtubules are not yet fully resolved. They may play a role in controlling the velocity of the cellulose synthesis machinery in the plasma membrane, determine the density of TCs in the plasma membrane or target the insertion of CESA (Wolf et al. 2012; Gutierrez et al. 2009). However, in older epidermal cells of *Arabidopsis* roots, it has been shown that cellulose deposition and microtubule orientation may sometimes be independent of each other (Sugimoto et al. 2000). The interaction of cortical microtubule networks and cellulose has also been explored in algae. In the desmid, *Penium margaritaceum*, a band of cortical microtubules found in the main pre-division expansion zone is arranged in the same orientation as that of the innermost layer of cellulose (Fig. 2; see also Ochs et al. 2014). If the microtubule network is altered with the pharmacological agent, oryzalin, the cellulose-based inner wall layer thins considerably causing the cell to swell (Domozych et al. 2014b). Actin networks also influence cellulose synthesis including regulating the rate of CESA vesicle delivery to the plasma membrane in land plants, most likely through its role in cytoplasmic streaming (Cai et al. 2011). The actin machinery may also control the amount of time CESA is situated in the plasma membrane (Sampathkumar et al. 2013). Finally, actin-based transport may be responsible for recycling of CESA via clathrin-mediated endocytosis to the trans Golgi network/early endosome (Bashline et al. 2013).

In red algae, limited data suggests that cellulose synthase enzyme complexes are also synthesized in the Golgi apparatus. Microfibrils are synthesized at the plasma membrane and are deposited in variably oriented layers. No direct correlation between cortical microtubule arrangement and

[1]Wherever possible the currently accepted names for species are used. The name used in the paper cited is also indicated. For details of names see chapter "Systematics, Taxonomy and Species Names: Do They Matter?" of this book (Borowitzka 2016).

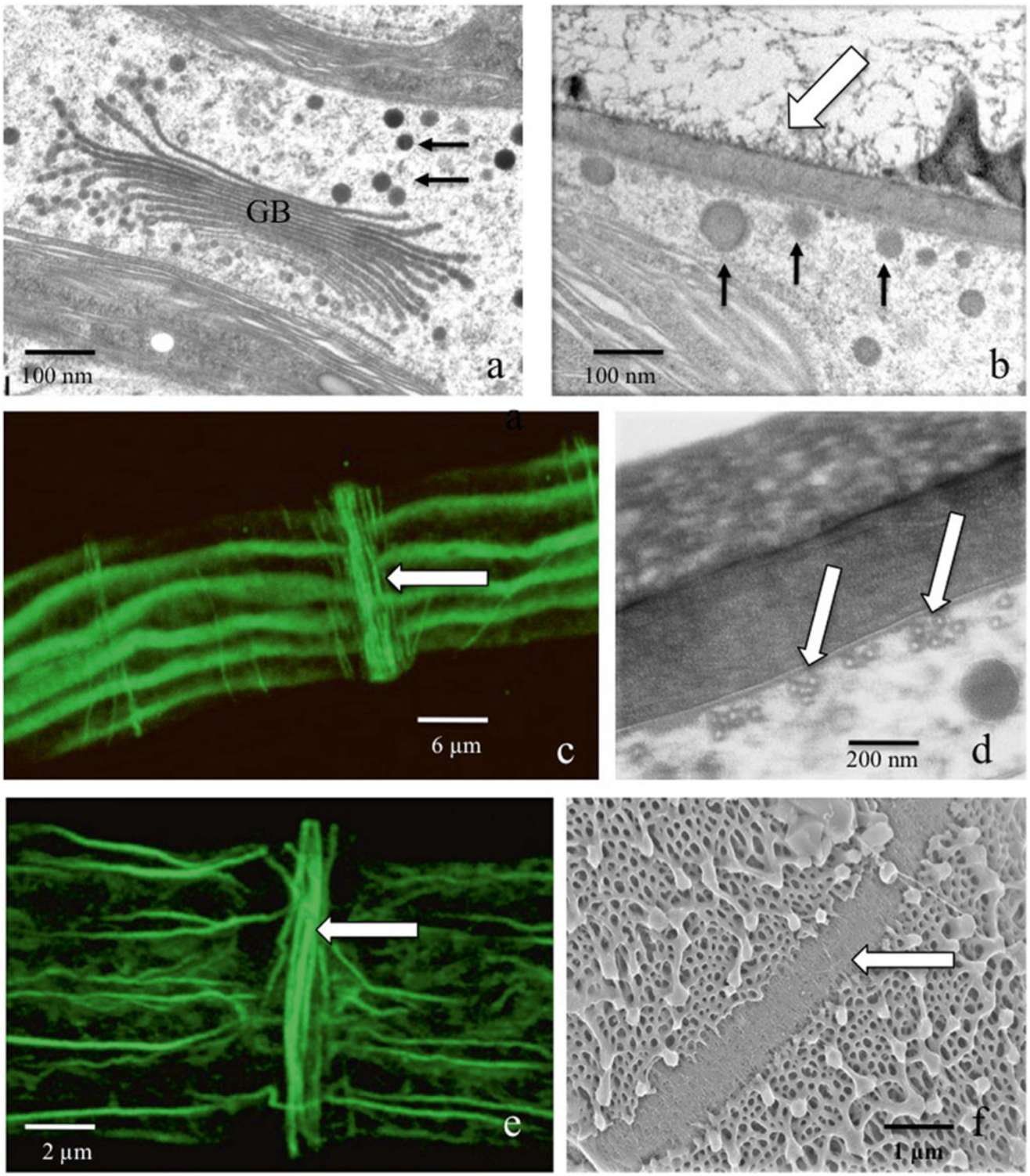

Fig. 2 The coordinated activities of multiple subcellular systems during wall formation in *Penium margaritaceum.* (**a**) The Golgi Body (GB) is the site of synthesis of HG. The HG is carried to the expansion zone via small vesicles (*arrows*). (**b**) The vesicles (*small arrows*) move to the expansion band (*large arrow*) and release the HG to the apoplast. (**c**, **d**) The expansion band is highlighted by a cortical band of microtubules (*arrows*). **c** = anti-tubulin. Confocal laser scanning microscopy (CLSM); **d** = TEM. (**e**) CLSM imaging of rhodamine phalloidin labeling of a transient cortical actin band (*arrow*) that underlies the expansion band. (**f**) FESEM Imaging of the expansion band highlighting the zone where the HG outer layer has yet to form

microfibril deposition in the cell wall has been described but it is very likely that actin plays a key role in cellulose deposition (Tsekos 1999). In some brown algae, TC activity and microfibril production are controlled most likely by actin and not by microtubules (Bisgrove and Kropf 2001).

Cellulose may also be part of other algal extracellular coverings that are not considered as typical cell walls. Thecate dinoflagellates (Pyrrhophyta, Dinophyceae) produce an extracellular covering called the amphiesma. This highly complex structure consists of an outer continuous membrane i.e., the plasma membrane, an outer plate, a single membrane bound compartment that contains a cellulose-based theca and an inner pellicle (Morrill and Loeblich 1983; Kwok and Wong 2003; Höhfeld and Melkonian 1992). The theca is processed through the Golgi apparatus and an associated network of Golgi-derived vesicles. The amphiesma is often shed during development via a process called ecdysis (Pozdnyakov and Skarlato 2012). While much remains to be resolved about the amphiesma and the cellulosic theca, it has also been shown (Kwok and Wong 2010) that cellulase activity was critical for coordination of cell cycle activity when amphiesma development occurs.

Many algal taxa produce microfibrillar wall materials that are not cellulosic (Popper et al. 2011). In coenocytic Chlorophycean green algae, microfibrils consist of ß-1,3 xylans, ß-1,4 mannans and heteropolymeric fibrillar polysaccharides (Fernandez et al. 2010; Fig. 1). In red algae, microfibrillar ß1-4 mannans, ß1-3 or ß1-4 linked xylans have been described (Craigie 1990; Rinaudo 2007). To date, little detailed information is available about their biosynthesis.

Few detailed studies are available today concerning the mechanisms and regulatory controls of cellulose synthesis in algae. However, new efforts and technologies offer much promise. For example, genomic sequencing of key taxa of several algal groups is rapidly progressing and the development of transformed cell lines expressing fluorescent protein-CESA complexes for cell wall studies of algae have recently made significant progress (Sørensen et al. 2013; Timme et al. 2012; Coelho et al. 2012; Vannerum et al. 2010; Harris et al. 2010). New developments in high resolution light, electron and atomic force microscopy coupled with new cellulose-specific probes (Wallace and Anderson 2012) should soon provide great insight into cellulose production in algae. Finally, "chemical genetics"-based screening of large arrays specific bio-active molecules including cellulose synthesis-affecting agents have provided new tools for investigating the specifics of the cell all biosynthetic machinery (Xia et al. 2014; Brabham and DeBolt 2013; Zabotina et al. 2008) that may also be applied to algae (Worden et al. 2015). All of these innovations will soon resolve many questions about cellulose in algae and contribute significantly to current and future efforts to harness the algal cell wall polysaccharides for biofuel production.

4 The Matrix Polysaccharides of Algae

While cellulose and other fibrillar polysaccharides represent the critical load-bearing framework of the cell wall, other polymers form a matrix that is necessary for diverse cell wall functions including cell expansion regulation, physical defence, adhesion and absorption. In plants and many green algae, hemicelluloses (e.g. xylans, xyloglucans, mannans) and pectins dominate the wall matrix and have been the most well-studied. These polysaccharide groups have been historically classified based on extraction characteristics. Pectins for example, are typically extracted with chelators and/or hot water/acid while hemicelluloses are extracted by strong bases. Further extraction with specific polymer-degrading enzymes also complements extraction efficacy and enhances subsequent chemical analyses. Recently though, it has been demonstrated that at least some of these matrix polymers along with wall proteins form extensive macromolecular modules that are directly linked to each other (Tan et al. 2013). This results in a far more complex image of cell wall architecture that will need to be elucidated if we are to understand the intricacies of wall function. Additionally, a diverse array of matrix polysaccharides that are not "typical" pectins or hemicelluloses have been found in algal cell walls, including highly complex polymers of red and brown algae. While detailed characterization of some of these polymers has been performed (e.g. agar, carrageenan), very little is known about other similar ECM constituents.

4.1 Hemicelluloses

Hemicelluloses represent a chemically-diverse group of polysaccharides that associate with cellulose microfibrils thus creating a cohesive network in the structural framework of the cell wall. Hemicelluloses from plants include xyloglucans, xylans, mannans and mixed linkage glucans (MLG; ß1-3,1-4 glucans). Hemicelluloses are synthesized by genes from the large Csl group containing eight families in land plants (Ulvskov et al. 2013; Burton and Fincher 2009; Yin et al. 2009). Recent work with higher plants has shown that these polymers are synthesized in the Golgi apparatus via the activity of several glycosyl transfereases and nucleotide conversion enzymes. These polymers are then transported to the plasma membrane and secreted to the cell wall (Driouich et al. 2012). Here in the apoplast, they associate with cellulose microfibrils and may subsequently be remodelled by endotransglycosylases such as xyloglucan endotransglucosylase (XET)/hydrolase (XTH; Franková and Fry 2013) and side chain-modifying enzymes. Recently, hemicellulose-like polysaccharides have been identified in several algal groups. In the charophytes, mannans, xyloglucans, xylans, and MLG

have been described (Sørensen et al. 2011; Fig. 1) while xyloglucan epitopes were specifically noted in antheridium development in *Chara corallina* (Domozych et al. 2009b). The presence of MLGs in algae is notable because it was long thought that MLGs were only found in the cell walls of grasses. Presently, little is known about the biochemical similarity of these polymers to those of land plants or their biosynthetic pathways.

"Hemicellulosic"-like polysaccharides of great complexity are found in other algal groups as well. In "ulvophycean" green algae, the hemicelluloses consist of complex xyloglactoarabinans, glucuroxylorhamnans and rhamnoxylogalactogalacturonans (Lahaye and Robic 2010; Ray and Lahaye 1995). The walls of some brown algae contain fucoglucuronxylans (Kloareg and Quatrano 1988) while in red algae, glucomannans and sulfated MLG have been identified (Rinaudo 2007).

4.2 Pectins

Pectins constitute a class of galacturonic acid (GalA)-containing polysaccharides that form a major domain of the matrix of the cell walls of plants and many algae (Palin and Geitmann 2012; Caffall and Mohnen 2009; Sørensen et al. 2011; Popper et al. 2011). In plants, pectins have diverse functions in the wall including cell-cell adhesion (e.g. middle lamella of multicellular plants), absorption and pathogen-induced signalling. They also have a major role in regulating the physicochemical properties of the cell walls including the regulation of turgor-driven expansion (Wolf and Greiner 2012; Wolf et al. 2012; Yapo 2011). The most common type of pectin is homogalacturonan (HG) which is made of α-1,4,-linked galacturonic acid (GalA). HG may be methyl- or acetyl-esterified at the C-6 or C-2 position of a GalA. Highly esterified HGs are synthesized in the Golgi apparatus and transported by vesicles to the plasma membrane for release to the apoplast. Here, HGs can be enzymatically de-esterified, most notably by pectin methylesterase (PME; Senechal et al. 2014; Wolf and Greiner 2012). The release of the methyl group exposes the negative charge on the C-6 of the GalA residues that then allows for binding to cations. Cross-linking of adjacent HG chains may occur when binding with divalent cations like calcium (Ca^{2+}). This results in the formation of a stable gel, a feature that is strain stiffening which in turn, affects the physical properties of the wall (Braybrook et al. 2012). Pectins also comprise other GalA-containing polysaccharides, some of which are the most complex biopolymers on the planet (Cosgrove 2005). In land plants, these include substituted pectins like rhamnogalacturonan-II (RG-II), xylogalacturonan and apiogalacturonan (Caffall and Mohnen 2009). Rhamnogalacturonan-I or RG-I is another class of pectin that is made of a disaccharide backbone repeat unit of α-1,4-GalA-α-1,2-L-(rhamnose) Rha. The Rha residues often contain branch points of ß-1,4-galactan, branched arabinans and other arabinogalactan side chains (Harholt et al. 2010).

Pectins have also been identified in the cell walls of many of the charophytes and other green algae. The desmid, *Micrasterias*, possesses a primary cell wall that contains HG with medium levels of methyl-esterification (Eder and Lutz-Meindl 2008; Meindl 1993). The outer layer of this wall also contains comparatively lower levels of methyl-esterified HG than the inner portion. The desmids, *Netrium digitus* (Eder and Lutz-Meindl 2010) and *Closterium acerosum* (Baylson et al. 2001) also possess HG in their cell walls, most notably found at points of expansion at the cell surface. A very distinct pectin architecture is found in the desmid, *Penium margaritaceum* (Fig. 2). This alga only possesses a primary cell wall that contains a distinct Ca^{2+}-HG "lattice" that constitutes the outer wall layer (Domozych et al. 2007a, 2009a). The lattice is embedded in an inner layer of cellulose microfibrils. The interface between these two wall zones, the median layer, contains HG and most likely, RG-I (Domozych et al. 2014a), perhaps serving as the physical connection of pectin with the cellulose microfibrils. The vegetative thallus of the charalean taxon, *Chara corallina*, contains cellulose microfibrils that are tethered by HG that in turn, is cross-linked by Ca^{2+} (Proseus and Boyer 2012). Other charophytes also have pectins (Sørensen et al. 2011) but their detailed biochemistry has yet to be resolved. RG-I and HG have also been described in the Chlorophycean green alga, *Oedogonium* (Estevez et al. 2008). In taxa of the some ulvophyceaen green algae, no "typical" pectins (e.g., HG, RG-I) have not been found but sulfated uronic acid-rich polymers contribute to the cell wall matrix (Ciancia et al. 2012; Percival 1979).

In land plants, pectin synthesis begins in the Golgi apparatus. These are subsequently carried in vesicles to specific sites of the cell surface or cytokinetic cell plate (Caffall and Mohnen 2009; Harholt et al. 2010). At least 16 gene families are responsible for pectin synthesis/modulation and several have recently been identified in the charophytes (McCarthy et al. 2014). Atmodjo et al. (2013) recently proposed a new hypothetical model for pectin synthesis called the domain synthesis model. Here, an oligosaccharide or polysaccharide primer is initially synthesized. The primers are then elongated by "elongation enzymes" to make particular pectin domains like HG or RG-I. The domains are then transferred en bloc to the expanding polysaccharide in the cell wall.

In algae, pectin synthesis and secretion is best known in the charophytes. In *Micrasterias*, pectins are initially synthesized in the Golgi apparatus via "dark" vesicles that arise from the peripheries of the medial-to-trans loci of a Golgi body and are moved to the cell surface (Lutz-Meindl and Brosch-Salomon 1999; Brosch-Salomon et al. 1998; Holzinger 2000). These vesicles also carry cell arabinogalactan proteins

or AGPs, which may provide geographic evidence that pectins form large macromolecular complexes with AGP and other polymers like xylan (Tan et al. 2013). The pectins are transported to cell expansion zones where they are incorporated into the growing primary cell wall. In a study employing Electron Energy Loss Spectroscopy (or EELS) of thin sections of HG-carrying vesicles, notable levels of Ca^{2+} were also detected. This has led to the suggestion of a regulatory role of Ca^{2+} in the controlling gelation qualities of the HG in the secretory apparatus/process (Eder et al. 2008). The directed secretion of the HG vesicles to specific cell surface sites is most likely mediated by actin (Pflugl-Haill et al. 2000). A Rab GTPase, MdRABE1, may also be involved in this spatial targeting of vesicles to particular surface sites (Vannerum et al. 2012). In *Micrasterias* and *Netrium*, once secreted, the pectin modulating enzyme, PME, most likely modifies the pectin (Eder and Lutz-Meindl 2010).

In *Penium margaritaceum*, extensive cell wall expansion takes place in a 1 μm-wide band at a narrow band at the central isthmus prior to cell division (Fig. 3). The inner cellulose layer is deposited first and is soon penetrated by domains of HG-RG-I. Further high-esterified HG is then deposited (Domozych et al. 2009a, 2011, 2014a) where it is subsequently de-esterified as it is displaced toward the two polar zones. Ca^{2+}-complexing then occurs leading to the formation of the distinct HG lattice. After cytokinesis, residual wall expansion continues at a narrow band at the polar tip of each of the daughter semicells. These events can be easily observed and quantified using live cell immunofluorescence labelling with monoclonal antibodies that have specificity toward various HG epitopes (Domozych et al. 2011). Pectin and cellulose deposition at the isthmus-based expansion zone is also defined by a cortical band of microfilaments and microtubules positioned in the cortical cytoplasm around the nucleus. These cytoskeletal bands are thought to act like preprophase bands that define the future plane of cell wall expansion and cell division (Ochs et al. 2014).

Pectin secretion and subsequent incorporation into the cell wall has also been described in *Chara corallina* (Proseus and Boyer 2007) where a "pectin cycle model" has been proposed (Proseus and Boyer 2008, 2012). As newly synthesized pectin is secreted into the cell wall at specific expansion zones, it removes Ca^{2+} from the pre-existing pectins. This results in a loosening of the tension in the wall and wall expansion that is driven by turgor. Ca^{2+} then enters the wall from the growth medium to re-establish cross-links and to strengthen the cell wall.

4.3 The Matrix Polymers of Brown Algae

Brown algae constitute a large and diverse assemblage of primarily marine taxa that often display complex multicellular phenotypes and in some cases, grow to sizes of 50 m or longer (e.g. *Macrocystis)*. The cell walls of most brown algae are very thick yet contain only small amounts of cellulose (e.g. 1–8 %). The biochemical microarchitecture of the wall is dominated by a thick matrix (Michel et al. 2010) that is primarily composed of anionic polysaccharides that include alginates and fucoidans (Kloareg and Quatrano 1988). Detailed biochemical analyses of these and other wall constituents and their modes of biosynthesis have recently been complemented by molecular studies as exemplified by the recent sequencing of the genome of the model taxon, *Ectocarpus siliculosus* (Coelho et al. 2012; Le Bail et al. 2011; Cock et al. 2009). Alginate polymers are comprised of repeating blocks containing two uronic acids, ß-1,4-D-mannuronic acid and α-1,4-L-guluronic acid that complex divalent cations to form gels (Michel et al. 2010). These polysaccharides may also complex phlorotannins, i.e., halogenated (e.g. iodine) and/or sulfated phenolic compounds (Berglin et al. 2004; Verhaeghe et al. 2008; Shoenwaelder and Wiencke 2000). Alginates associate with/tether cellulose microfibrils creating a structural design that is critical for maintaining wall architecture. Alginates are evolutionarily-derived from lateral gene transfer of an *Actinobacterium* and their presence in the wall is critical to the development of large multicellular and morphologically complex thalli of brown algae.

Alginates are synthesized in the Golgi apparatus and are transported by Golgi-derived vesicles to wall expansion sites at the cell surface (Nagasato et al. 2010; Nagasato and Motomura 2009; Shoenwaelder and Wiencke 2000; Callow et al. 1978). During cell division, alginates are transported to the growing septum in electron dense vesicles via actin-generated movement. In *Dictyota*, alginate synthesis and deposition at cell division are also central to the formation of plasmodesmata (Terauchi et al. 2012). Alginate biosynthesis is also controlled by developmental and environmental prompts. For example, the guluronic acid residues of the polymer are remodelled during development by mannuronate C5-epimerases (Michel et al. 2010).

Fucoidans are α-L-fucose-rich polysaccharides of considerable structural diversity. Fucoidans include highly sulfated homofucans as well as highly substituted forms containing xylose, galactose, mannose and glucuronic acid (Popper et al. 2011). These polymers are also synthesized in the Golgi apparatus and delivered in Golgi-derived vesicles to wall growth zones via actin-mediated transport. In some taxa such as *Silvetia*, fucoidans represent the dominant polysaccharides found in the septum formation during cytokinesis (Nagasato et al. 2010).

Brown algae exhibit a variety of morphogenetic strategies that include both tip-based (i.e., polar) and diffuse growth mechanisms at the cell level (Le Bail et al. 2011; Bisgrove and Kropf 2001). These are responsible for the ultimate expression of the diverse phenotypes displayed in this group including simple filaments, flattened blades and large cones.

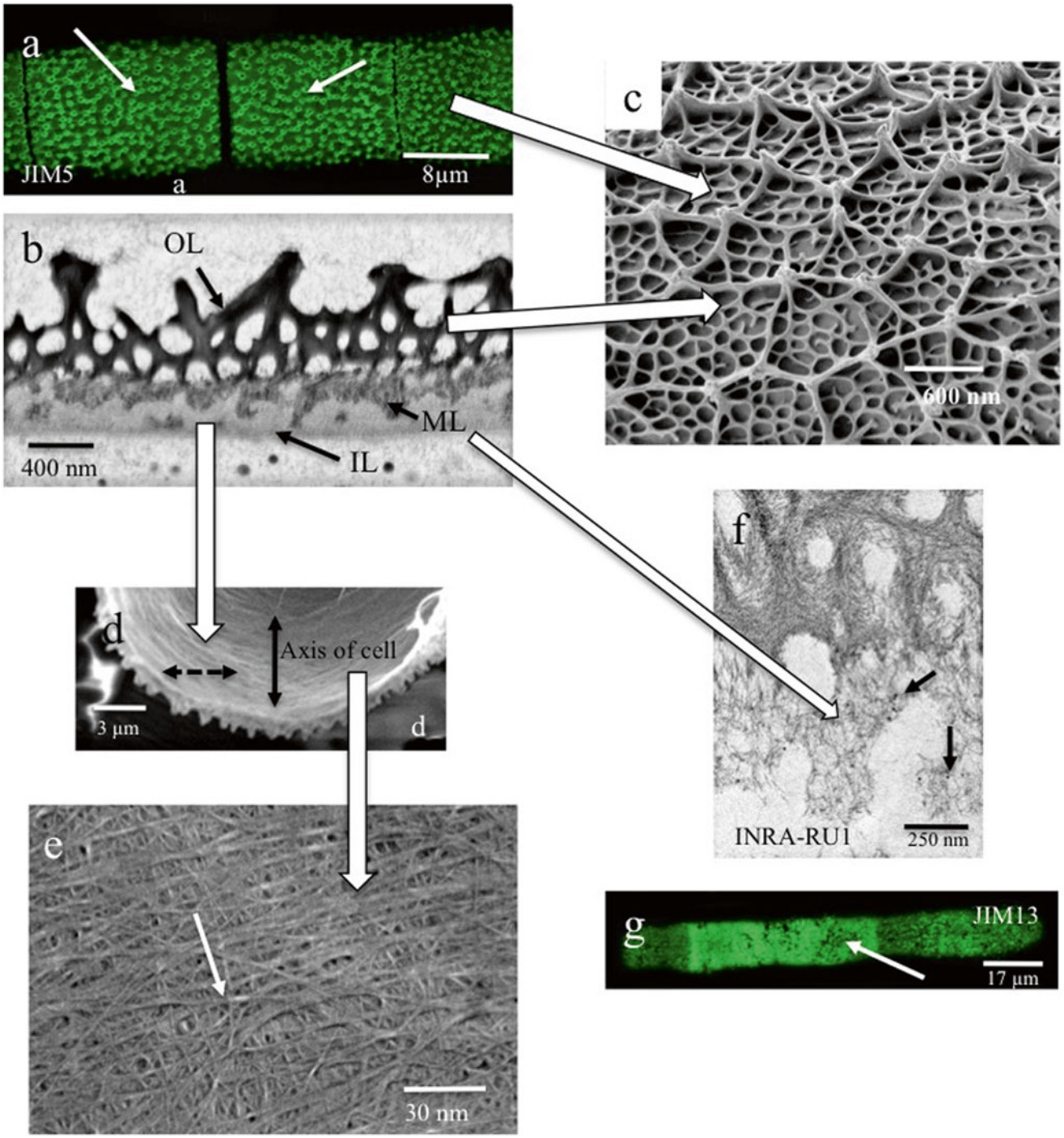

Fig. 3 The complexity of the cell wall as illustrated by the desmid, *Penium margaritaceum.* (**a**) The outer layer of the cell wall is highlighted by a distinct HG lattice as labeled by the mAb, JIM5 (*arrows*). (**b**) TEM imaging shows that the wall consists of three layers, an outer (OL) that contains most of the HG, an inner layer (IL) of cellulose and a medial layer (ML) where the HG is anchored in the cellulose. (**c**) Field emission scanning electron microscopy (FESEM) highlights the unique HG lattice. (**d**) The inner layer of the wall contains cellulose microfibrils (*dashed double arrow*) that are arranged perpendicular to the long axis of the cell (*solid double arrow*). FESEM image. (**e**) FESEM magnified view of the cellulose microfibrils (*arrow*). (**f**) TEM immunogold labelling with INRA-RU1 (antibody courtesy, M. C. Ralet, INRA Nantes, France) demonstrates that the medial layer (*arrows*) contains RG-I epitopes. (**g**) In addition to the polysaccharide constituents, the wall also contains proteoglycans such as AGP as labelled by the antibody JIM13

During cellular expansion and morphogenetic events, it has been shown that interactions of transmembrane proteins with components of the cell wall are key to development (Fowler et al. 2004). For example, tip-based expansion entails the specific coordination of cortical Ca^{2+} gradients, the targeted fusion of vesicles at the expansion tip, spatial regulation of the secretion of cell wall components and regulation of actin cytoskeleton dynamics (Katsaros et al. 2003, 2006). In the well-studied *Fucus distichus*, polar growth has been shown to be regulated by Rho GTPases that are associated with actin assembly and vesicle secretion at the growing tip (Katsaros et al. 2003; Etienne-Manneville and Hall 2002).

4.4 The Matrix of Red Algae

The cell wall matrix polymers of red algae consist primarily of sulfated polysaccharides (Popper et al. 2011; Rinaudo 2007; Painter 1983; Kloareg and Quatrano 1988; Ramus 1972). These include sulfated galactans (with sulfation levels of 20–38 %) containing a backbone of repeating galactose (Gal) and 3,6-anhydrogalactose linked ß-1-4 and ß-1-3 respectively. The geographic positioning of these galactans within the thallus is critical for the calcification process common to many red algae (Martone et al. 2010). These polymers are synthesized in the Golgi apparatus and are also found in distinct mucilage sacs that are associated with endoplasmic reticulum (Tsekos 1981). The timing and coordinated activation of components of the secretory apparatus of these matrix polysaccharides are indispensable for maintenance of the anterior-posterior axis of the thallus including the production of monospores (Li et al. 2008). When the apparatus is compromised by application of external agents such as the fungal metabolite, brefeldin A, the secretion machinery and the actin cytoskeleton are disrupted. Li et al. (2009) also demonstrated the phosphoinositide/phospholipase machinery and the establishment of specific Ca^{2+} gradients are needed for proper wall polymer secretion and cell expansion.

5 Cell Wall Proteins

While polysaccharides dominate the architecture of algal cell walls, proteins, especially highly glycosylated forms, are also common and important constituents. Hydroxyproline-rich glycoproteins (HRGPs) of the cell wall have been the most well-characterized in algae and display great chemical diversity.

Arabinogalactan proteins or AGPs are one group of highly glycosylated HRGPs that are found in or on many cell walls of plants and many algae (Nguema-Ona et al. 2012; Showalter 2001). AGPs are non-enzymatic glycoproteins that are highly glycosylated with the carbohydrate domain often constituting 90 % or more of the mass of the polymer. AGPs are characterized by alanine-hydroxyproline (Ala-HyP) repeat motif interspersed with serine (Ser). The repeating Ser $(Hyp)_4$ units in the protein are sites of *O*-glycosylation with the short arabinose side chains while larger arabinogalactosyl side chains are attached to Ser, Ala, valine (Val) or threonine (Thre). Some AGPs have glycosylphosphatidylinosotol (GPI) domains that are used for anchorage in the plasma membrane. AGPs have diverse functions including those directly associated with expansion, development and adhesion (Ellis et al. 2010). These proteins have been found in all land plant groups (Nguema-Ona et al. 2012; Lee et al. 2005) and recently, AGP epitopes have been identified in green algae most notably in the charophytes (Sørensen et al. 2011). This includes the pore complexes of the desmid *Pleurotaenium trabeculata* (Domozych et al. 2007b) and in the primary cell wall and pores of the secondary cell wall of *Micrasterias* (Eder et al. 2008). AGPs of algae appear so far to be quite different than those found in land plants. For example, ß-Yariv reagent is an important tool employed for identifying, isolating and quantifying land plant AGPs (Willats and Knox 1996). However, green algal AGPs do not appear to bind to, or be affected by this compound. Little is known about the biosynthetic machinery of AGPs in the charophytes. In *Micrasterias*, AGPs are most likely synthesized in the Golgi apparatus and then packaged and transported to the cell surface in primary wall vesicles and a group of 160–300 nm type-2 mucilage vesicles.

Extensins constitute a second group of glycosylated HRGPs found in plants and some algae. These proteins are identified by a characteristic Ser-$(Hyp)_4$ repeat motif (Lamport et al. 2011). Upon deposition to the cell wall apoplast, extensins self-assemble to form scaffolding in the cell wall that contributes to the maintenance of wall architecture, enhancing the tensile strength of wall, contributing to cell plate formation and participating in defence responses (Estevez et al. 2006). In plants, extensins are synthesized in the Golgi apparatus and are transported to the cell surface by Golgi-derived vesicles. In green algae, extensin-like proteins have been well-characterized in the volvocalean taxa of the Chlorophyceae (Ertl et al. 1992; Roberts et al. 1985; Roberts 1974) but have also been recently found in charophytes (Sørensen et al. 2011).

The unicellular volvocalean taxon, *Chlamydomonas reinhardtii*, contains 25–30 different extensin-like HRGPs in its cell wall (Sumper and Hallmann 1998) that are arranged in interlocking fibrillar and granular elements (Voight and Frank 2003). This yields a distinct "crystalline cell wall". In the colonial taxon, *Volvox*, extensin-like proteins comprise rod-shaped modules that contribute to the formation of its complex extracellular colonial matrices in which the cells are encased (Kirk et al. 1986). It is esti-

mated that the covering of *Volvox* is roughly 10,000 times larger than the cell wall of *Chlamydomonas* (Abedin and King 2010). HRGPs are also components of gametic membranes and sexual agglutinins expressed during sexual reproduction in volvocalean algae (Lee et al. 2007; Woessner and Goodenough 1994; Imam et al. 1985). *Chlamydomonas* and *Volvox* extensin-like HRGPs are also synthesized in the Golgi Apparatus and transported to the cell surface in Golgi-derived vesicles. Prolyl-4-hydroxylase is a critical enzyme in the modelling of these HRGPs during biosynthesis (Keskiaho et al. 2007). Upon release to the wall, they self-assemble into their crystalline form. During development of the cell wall in *Chlamydomonas*, the first elements to assemble during wall synthesis are long fibers that constitute the inner (W1) and outer (W7) wall layers. Other cell wall layers subsequently assemble in this initial complex (Goodenough and Heuser 1985). In the unicell chlamydomonad, *Gloeomonas kupfferi*, HRGPs of the inner wall layer are also synthesized in the Golgi apparatus but are then transported in vesicles first to the contractile vacuole. It is then released from the vacuole to the cell surface where it initiates self-assembly of the glycoprotein wall (Domozych and Dairman 1993). Disruption of the biosynthetic machinery through knockdown genetics in *Chlamydomonas* yields notably abnormal cell walls that lack the typical multilayered crystalline architecture (Goodenough and Heuser 1985; Adair and Snell 1990).

Other cell wall proteins have been identified in diverse algal groups. Extensin-like proteins have been identified in ulvophytes such as *Codium* as well as in the Chlorophycean taxon, *Oedogonium* (Estevez et al. 2008, 2009). Vannerum et al. (2011) described an expansin-like protein in the cell wall of the desmid, A glycine-rich protein was found in the cell wall of the desmid, *Closterium acerosum* (Baylson et al. 2001). In the green alga, *Botryococcus braunii*, an alga of considerable importance in the biofuel industry, a thick wall/sheath is most likely responsible for holding together the colonial complex (Weiss et al. 2012). This sheath is comprised of ß1-3 and ß1-4 glucans, deoxyhexoses, a distinct polysaccharide consisting of arabinose, galactose and a HRGP.

6 ECM: Scales and Fused Scales: Green Algae

Some primitive unicellular green algae including many prasinophytes and early divergent charophytes are covered with a single layer or multiple layers of variously-shaped scales that may number in the thousands (e.g. *Pyramimonas*, Moestrup and Walne 1979). Scales consist of 90 % polysaccharide that contains the unusual 2-keto-sugar acids, Kdo (3-deoxy-2-manno-octulosonic acid), 5-O-methyl Kdo and 3-deoxy-2-lyxo-heptulosaric acid (Becker et al. 1991). These polysaccharides may also be associated with small amounts of protein that function in the adhesion of scales to other scales or to the plasma membrane (Becker et al. 1996; Becker and Melkonian 1992). In the early divergent charophyte clade of the Mesostigmatophyceae, represented by *Mesostigma viride* (Fig. 4), the outermost layer of scales consists of approximately 800 spectacular "basket" shaped scales that contain glucose, 3-deoxy-lyxo-2-heptulosaric acid and two protein components (Domozych et al. 1991). Biosynthesis and deposition of scales in green algae is often spectacular. In prasinophytes and *Mesostigma*, scale synthesis occurs in the Golgi apparatus. Here, various scale types may be observed forming and maturing as they are moved from cis-to-trans cisternae. Vesicles most often move to and fuse with a vacuole-like scale reservoir where the scales are arranged in layers before deposition onto the cell and/or flagellar membranes (Moestrup and Walne 1979; Domozych 1991).

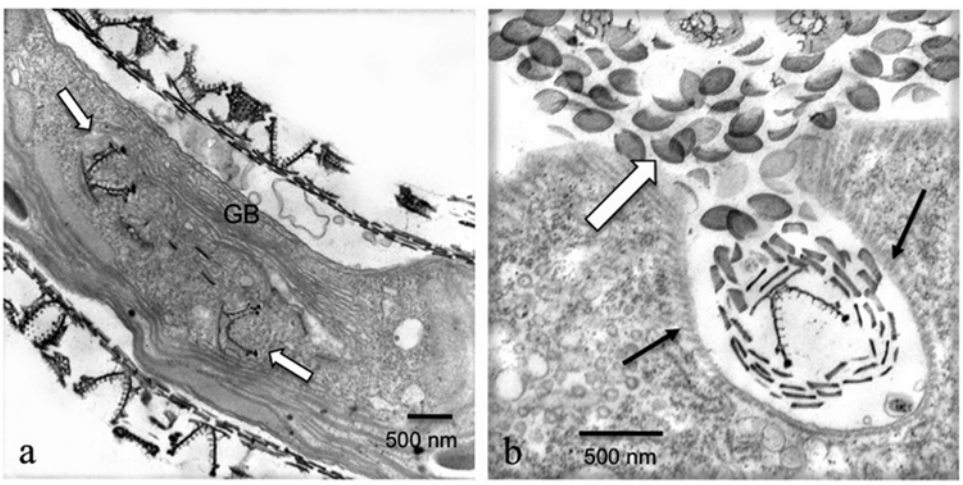

Fig. 4 Scale formation in the early divergent CGA, *Mesostigma viride*. (**a**) The scales are synthesized in the Golgi body (GB; *hollow arrow*). (**b**) The scales are first deposited in an anterior scale reservoir (*solid arrows*) and are then released externally (*hollow arrow*) to the surface

Other green algal flagellates like *Tetraselmis* and *Scherffelia*, produce a special cell wall-like covering, called a theca that consists of approximately 50 % Kdo and 15 % GalA (Vierkotten et al. 2004; Becker et al. 1995). It is thought that the theca is a product of fused scales (Manton and Parke 1965; McFadden and Melkonian 1986; Domozych et al. 1981). Thecal precursors are synthesized in the Golgi apparatus and are transported to the anterior region of the cell by Golgi-derived vesicles. Here, they are deposited on the cell surface where they coalesce to form the theca. During this secretory event, pre-theca materials fuse to form to the complete theca by the addition of long Kdo chains substituted with sulfated GalA disaccharides that are cross- linked by Ca^{2+} (Becker et al. 1994).

7 Scales of Haptophytes

The extracellular coverings of haptophytes or prymesiophytes are typically characterized by a "coccosphere" that consists of multiple plate-like coccoliths covering the cell surface (Marsh 1999). Coccolith production represents a major biogeochemical event planet-wise as it is represents up to 89 % of the total burial flux of calcium carbonate in ocean sediments (Mertens et al. 2009). A coccolith is made of an organic base plate to which calcite crystals are complexed (Mackinder et al. 2010). The nucleation and growth of the calcite crystals are controlled by acidic polysaccharides and proteins of the organic base plate (Hirokawa et al. 2005; Kayano et al. 2010; Shroeder et al. 2005). The construction of the coccocolith in many taxa (e.g. *Emiliania*) begins in the Golgi apparatus and proceeds to a Golgi-derived coccolith vesicle (CV). The distal portion of this vesicle is comprised of a reticular body that consists of a dynamically changing membranous mass. This mass contains organic material derived from both the Golgi body and endoplasmic reticulum. The reticular body then becomes closely associated with the contractile vacuole as calcite crystals start to form. This event ultimately leads to secretion of the coccolith (Kayano et al. 2010; Taylor et al. 2007; Marsh 1999; Brownlee and Taylor 2004). This distinct biosynthetic and secretory machinery demonstrates the important regulatory role of the reticular body in supplying the organic and inorganic materials necessary for calcification of the coccolith (Drescher et al. 2012). For example, the reticular body may provide a significant surface area that could accommodate the large number of Ca^{2+}- and perhaps bicarbonate ion pumps needed for coccolith formation. This, in turn, would provide a supersaturated solution of cations for calcite nucleation and expansion. Coccolith formation also employs other subcellular components. For example, V- and P-type Ca^{2+}-stimulated ATPases are involved in coccolith biosynthesis (Corstjens and González 2004; Corstjens et al. 2001; Araki and Gonzalez 1998). Additionally, both microtubules and microfilaments play significant roles in intracellular coccolith formation as well as transport (Langer et al. 2010).

8 Diatom Frustules

Diatoms (Bacillariophyceae) represent a wide ranging group of algae of profound importance to aquatic food chains, global photosynthetic yields and biogeochemistry (Graham 1993). Diatoms are surrounded by a distinct "crystalline" covering called a frustule (Crawford and Schmid 1986). This covering is highlighted by intricate networks of girdle and valve bands and consists of silica that is complexed to an organic matrix. The matrix consists of a complex network of mannose- and ß,1-3glucose-containing polysaccharides and distinct sets of proteins that regulate the synthesis of the frustule (Tesson and Hildebrand 2013; Kröger 2007). The components of the frustule are made in a membrane-bound intracellular compartment known as the silica deposition vesicle, or SDV. The SDV directly contributes to the morphogenesis of the frustule during its formation. Subsequently, the frustule is exocytosed to the cell surface. The formation of the frustule also requires the cytoskeletal network (Tesson and Hildebrand 2010). Recently, considerable interest in the production of the diatom frustule has emerged in the nanotechnology world (Saade and Bowler 2009; Kröger and Poulsen 2008).

9 Conclusions

We are in an infancy stage in our understanding of the structure and development of the cell walls and related coverings of the vast assortment of algae. However, the recent application of new technologies in algal cell wall research and the expansion of this research to screen wide arrays of algal taxa are revolutionizing our understanding of these coverings. Central to these advancements are genome and transcriptome sequencing, high throughput immunoprobing, high resolution optical and electron microscopy and atomic force microscopy. Likewise, several algae have recently become valuable model systems for cell wall-based studies (e.g. *Ectocarpus, Micrasterias, Penium*) especially research dealing with the biosynthesis, cell expansion/morphogenesis and polymer structure/function. All of these new activities have yielded valuable new insight into the physiology, cell biology and molecular genetics of alga as well as algal evolution and biogeography. As important, this new information will be of great significance to the expansion and enhancement of algal cell wall components to food additives, for ecoremediation and as initial substrates in the production of biofuels.

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Part II

The Fundamental Physiological Processes

Photosynthesis and Light Harvesting in Algae

Anthony W. Larkum

1 Introduction

Photosynthesis provides the major energy supply and carbon input to the biosystems of the Earth. It may also be almost as old as life itself, dating back at least 3.7 billion years (Ga) (Czaja et al. 2013). Only two major primary photosynthetic pigments are recognized, **chlorophyll** (Chl) and **bacteriochlorophyll** (BChl) (Scheer 1991). There are only eight major light-harvesting systems (Larkum 2006). The photosynthetic systems of Cyanobacteria, photosynthetic protists (algae) and land plants are built around chlorophyll *a* (Chl *a*), although now chlorophyll *d* (Chl *d*) may also be considered as a major pigment (Schliep et al. 2013). These organisms are all capable of splitting water and forming oxygen in the process, in **oxygenic photosynthesis** (Fig. 1). This is all the more remarkable because anoxygenic photosynthesis carried out by anoxygenic photosynthetic bacteria employs a number of BChls (although Heliobacteria employ γ-bacteriochlorophyll which, while it is a bacteriochlorophyll – i.e. it possesses a bacteriochlorin ring – is close to chlorophyll in structure (Scheer 1991).

It might be concluded from this logic that BChl preceded Chl on the early Earth. However, this is by no means certain and has been challenged (Larkum 2006, 2008; Mulkidjanian et al. 2006); it is quite possible that the early photosynthetic organisms possessed Chl, or perhaps Chl + BChl and had many similarities to cyanobacteria. A possible scenario sees the pro-cyanobacterial organisms developing towards oxygenic photosynthesis over the billion years up to the Great Oxidation Event (GOE) at 2.45 Ga, alongside anoxygenic photosynthetic bacteria. Then, as water splitting became ubiquitous, anoxygenic photosynthetic bacteria were outcompeted by their more efficient relatives and were pushed to the borders of habitable ecosystems, where they were forced to exist on low light, much of it relegated to the infrared region. It was at about this time, viz. the GOE, that Cyanobacteria would first be recognizable, although pro-cyanobacteria would have existed long before this.

It is therefore just as remarkable that these two great realms of the photosynthetic world do not share any similar light-harvesting systems, despite the fact that both groups have developed a wide, but not fully comprehensive, set of such pigment systems, to absorb energy from sunlight. In the Cyanobacteria, algae and land plants, the light-harvesting systems are mainly based on the chlorophylls, with the notable exception of the phycobiliproteins (Toole and Allnutt 2003). The proteins interacting with the chlorophylls are surprisingly few: the CAB proteins, the relatives of the inner antennae complex (CP43 and CP47), and the novel peridinin chlorophyll complex (PCP) (see Fig. 2). The cyanobacteria, red algae and cryptophyte algae also possess phycobiliproteins. In addition, the carotenoids have a large role to play in all photosynthetic systems, although again there is little overlap between the carotenoids found in anoxygenic and oxygenic photosynthesis. For other reviews in this area the reader is referred to Larkum and Barrett (1983), Larkum and Howe (1997), Larkum (2003), and Falkowski and Raven (2007).

2 The Photosynthetic Pigments of Cyanobacteria and Algae

We now take a closer look at those pigments which define the Cyanobacteria and algae (and the land plants, evolving as they did from streptophyte green algae). For convenience the reader is referred to Fig. 5 for an explanation of how the various algal phyla are related.

2.1 Chlorophylls

Chl *a*, and Chl *b* were isolated and their chemical structure determined in the early twentieth century, Chl *c* was

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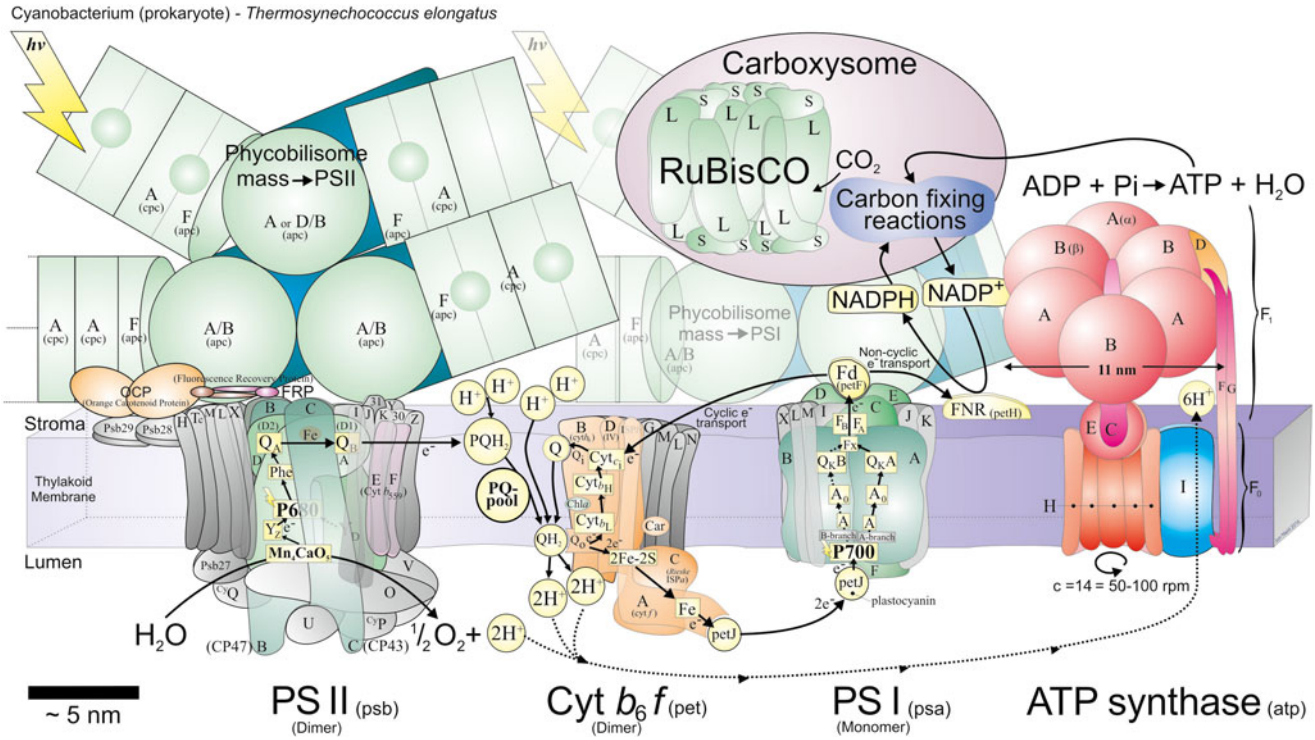


Fig. 1 A diagram of the thylakoid membrane showing the major supercomplexes (Reproduced from www.scienceopen.com and John Nield with permission)

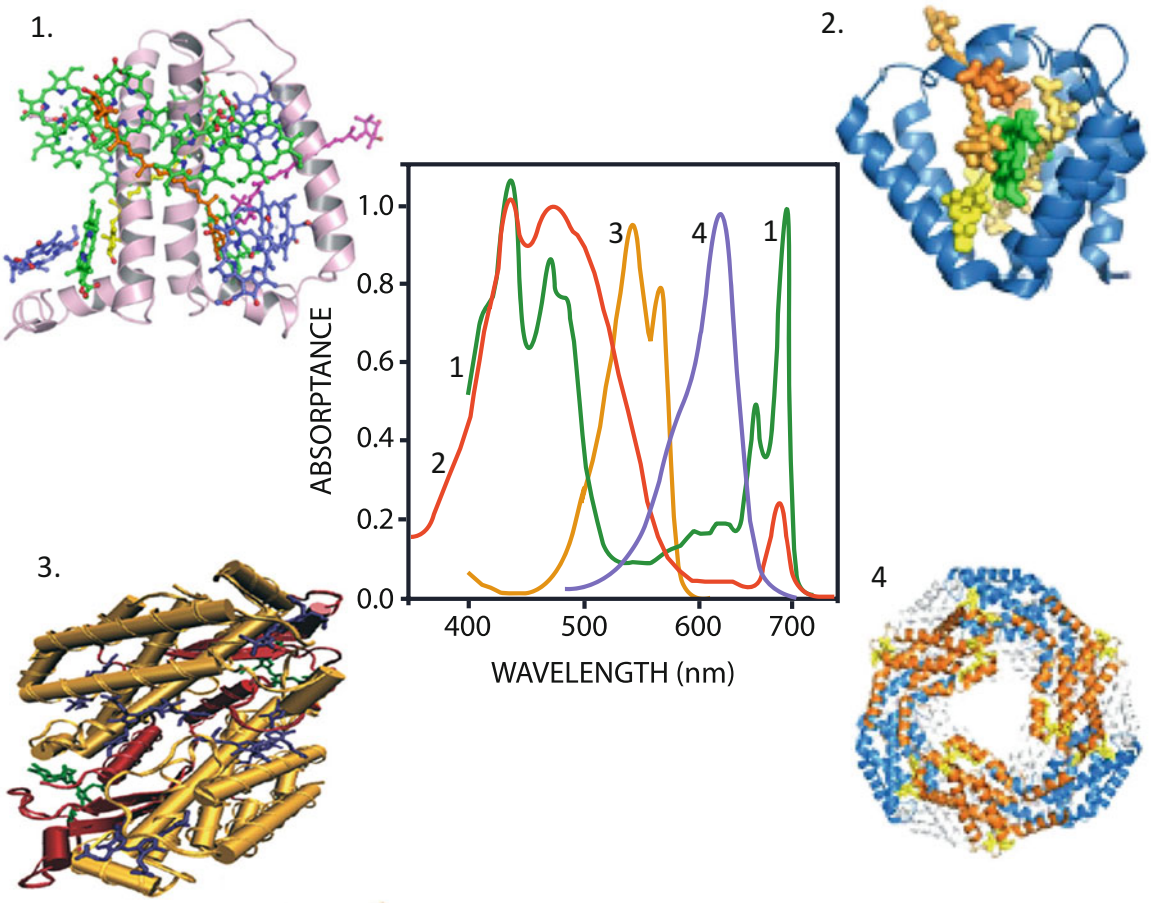
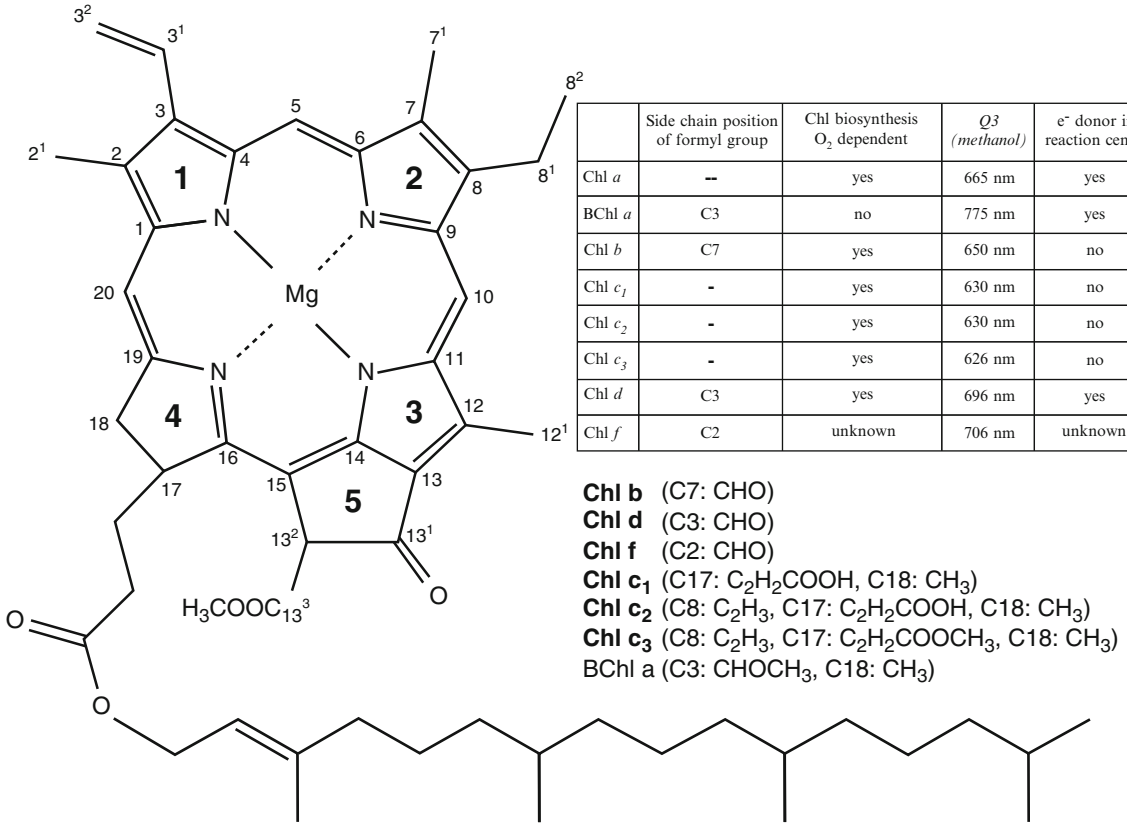


Fig. 2 Space filling diagrams of the major light harvesting complexes of eukaryotic algae and land plants. (*1*) LHCII; (*2*) Peridinin chlorophyll complex; (*3*) Phycobiliprotein subunit; (*4*) Novel phycoerythrin from *Chroomonas* (Reproduced from www.scienceopen.com with permission)



	Side chain position of formyl group	Chl biosynthesis O_2 dependent	*Q3 (methanol)*	e^- donor in reaction centre
Chl *a*	--	yes	665 nm	yes
BChl *a*	C3	no	775 nm	yes
Chl *b*	C7	yes	650 nm	no
Chl c_1	-	yes	630 nm	no
Chl c_2	-	yes	630 nm	no
Chl c_3	-	yes	626 nm	no
Chl *d*	C3	yes	696 nm	yes
Chl *f*	C2	unknown	706 nm	unknown

Chl b (C7: CHO)
Chl d (C3: CHO)
Chl f (C2: CHO)
Chl c_1 (C17: C_2H_2COOH, C18: CH_3)
Chl c_2 (C8: C_2H_3, C17: C_2H_2COOH, C18: CH_3)
Chl c_3 (C8: C_2H_3, C17: $C_2H_2COOCH_3$, C18: CH_3)
BChl a (C3: $CHOCH_3$, C18: CH_3)

Fig. 3 Chemical structures and in vivo absorption spectra of Chls *a*, *b*, *c*, *d* and *f*.

discovered in the late 1930s and Chl *d* in 1944 (see Larkum and Barrett 1983; Falkowski and Raven 2007), although Chl *d* was not fully recognized until its rediscovery in the cyanobacterium, *Acaryochloris marina*[1] (Miyashita et al. 1996) (Fig. 3). Chls *a* and *b* are found in higher plants, including agricultural crops, and were an obvious target. However, Chl *b* is also found in a variety of algae, most notably the Chlorophyceae (but also Euglenophyceae, chlorarachniophytes, and in the cyanobacteria, *Prochlorococcus*, *Prochlorothrix* and *Prochloron*). Chl *c* is found only in chromophytic algae (also called Chromista and alveolates); all of which occur only in algae with secondary plastids (see Sect. 3.2); this bizarre pigment, for which no real function has been agreed, is divided into Chl c_1 and Chl c_2 (Jeffrey 1989). Most algae which possess Chl *c* possess both c_1 and c_2, but two phyla, the Dinophyceae and the Cryptophyceae possess only c_2, while a small group, mainly of prymnesiophytes, possess a third type, Chl c_3 (Jeffrey 1989). In addition, the biosynthetic intermediate, Mg-2,4 divinyl phaeoporphyrin dimethyl ester (MgDVP), is found in some chromophytes, in the primitive green algae Monadophyceae and in *Prochlorococcus* and *Prochloron* (Larkum 2003, 2006).

Of all these Chls, Chl *a* has ranked supreme until recently, taking on the photochemical role in both Photosystem I (PSI) and Photosystem II (PSII). Chl *a* has a Soret peak at 436 nm (in vivo) and Q_Y peaks at 680–700 nm (in vivo) and is a good photosynthetic pigment for visible light, but needs augmentation in the blue, green and orange and near-infra red regions of the spectrum. However, the supremacy of Chl *a* has recently been challenged by Chl *d* which appears to replace Chl *a* in a photochemical role in PSI and PSII of *Acaryochloris marina* (Schliep et al. 2013). The only role known for Chl *a* in the photosystems of *A. marina* is the apparent need for one molecule of Chl *a* in PSII where it is converted to pheophytin *a* and acts as the primary acceptor. Nevertheless, Chl *a* probably dominates in most oxygenic photosynthetic situations, while Chl *d* most likely ekes out a most restricted role where mainly near-infra red (NIR) light (700–750 nm) predominates (note also that this is a region where some BChls allow for anoxygenic photosynthetic activity).

Recently a fifth major Chl was discovered (Chl *f*) (Chen et al. 2010). With a peak in vivo of 735 nm this Chl can absorb light further into the NIR than Chl *d*. However, it does not take on any photochemical role and so far has only been

[1]Wherever possible the currently accepted names for species are used. The name used in the paper cited is also indicated. For details of names see chapter "Systematics, Taxonomy and Species Names: Do They Matter?" of this book (Borowitzka 2016).

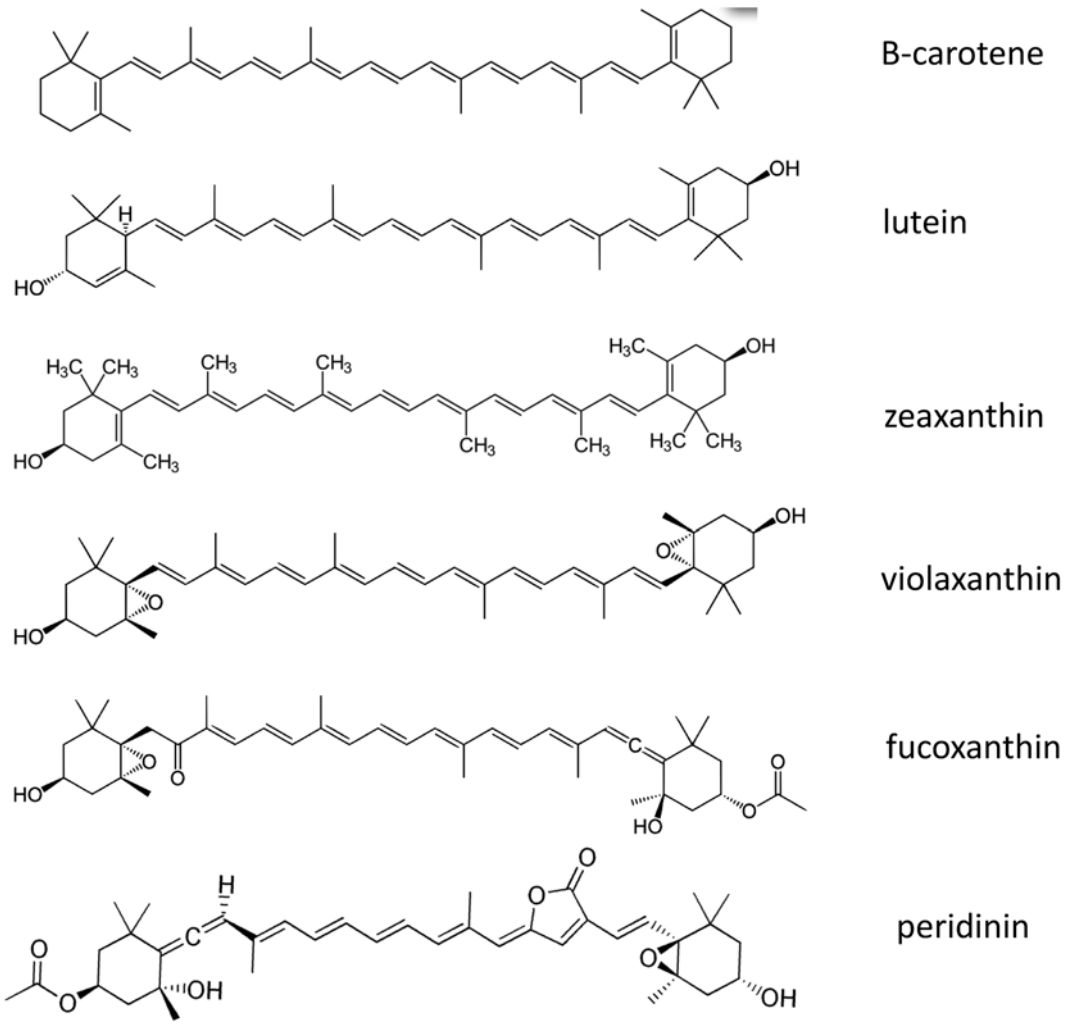


Fig. 4 Chemical structures of some of the more important carotenoids (**a**) β-carotene, (**b**) Violaxanthin, (**c**) Zeaxanthin, (**d**) Fucoxanthin, (**e**) Peridinin, (**f**) Siphonaxanthin

found in small amounts when the algae that contain it are grown under NIR (Gan et al. 2014).

2.2 Carotenoids

The carotenoids in algae are very diverse (Fig. 4) and very different from those in anoxygenic bacteria, partly because they have to exist in a highly oxidising environment, but where anoxygenic photosynthetic bacteria exist in aerobic environments the carotenoids are very different again (Takaichi 2011).

With the carotenes there is less room for differences, except in chain length, and alpha- and beta-carotenes play a similar role, but nevertheless they are often quite specific in their distribution.

With xanthophylls, there are almost endless variants that are found in photosynthesis. Many of these play a light-harvesting role, such as fucoxanthin, peridinin and vaucheriaoxanthin, which harvest light in the green region of the spectrum (500–550 nm) (Fig. 4). However there are other xanthophylls, which play other roles. For example, zeaxanthin + violaxanthin (green algae and land plants) and diatoxanthin + diadinoxanthin (chromophytic algae) take part in non-photochemical quenching in the xanthophyll cycle (see Sect. 7.1). The water-soluble keto-carotenoid 3′-hydroxyechinenone is the major carotenoid in the orange carotenoid protein (Sect. 7.3).

2.3 Phycobiliproteins

Phycobiliprotein (PBP) plays an exceptional role in light harvesting in Cyanobacteria and a small number of algal classes, notably Rhodophyceae, Glaucophyceae and Cryptophyceae. PBPs harvest light in the region of 490–650 nm

where the "so-called" green window is a region where Chl and carotenoid pigments are poorly absorbing. In fact PBPs play such a dominant role in Cyanobacteria that it is often assumed that they evolved before light-harvesting Chls, an assumption that is likely to be wrong (see above and Larkum 2006). It is much more likely that at least some Chls apart from Chl *a* evolved before the evolution of PBPs. Larkum (2006) advanced the idea that phycobilisomes only evolved as a shading response to other algae. This has the merit that PBP is an "expensive" molecule (in terms of nitrogen), is not a membrane intrinsic molecule and is specifically tailored for absorption of light where the Chl and, generally, carotenoids have poor absorption properties. PBPs are built into phycobilisomes and from which the energy is funneled into the thylakoid membrane via a specific stalk of additional molecules (see Fig. 1). It has recently been shown that passage of energy down this stalk is modulated in many cyanobacteria by a caroteno-protein, the orange carotenoid protein (OCP) (see Sect. 7.3).

3 The Evolution of Protists with Plastids (Algae)

3.1 Algae with Primary Plastids

Current evidence suggests that endosymbiosis of a cyanobacterium and an early protist organism gave rise to the ancestral line of plastids over 1 Ga ago (e.g. Price et al. 2012; Keeling 2013) (Fig. 5); there has even been some speculation as to from which group of Cyanobacteria the first endosymbionts came. A single endosymbiosis is generally held to be the most likely, because it is held to be a very unlikely event; this is called the **monophyletic hypothesis**. This hypothesis is embodied in the concept of the Archaeplastida, in which it is stated that this single endosymbiosis gave rise to the modern Glaucophyta, Rhodophyta and Chlorophyta. These three phyla all have primary plastids, i.e. plastids with only two envelope membranes, in contradistinction to the secondary plastids, which have three or four envelope membranes. Since today the primary plastids contain only a few genes (usually much less than 250) it is assumed that the original nucleus lost most of its genes, some to the host nucleus and others altogether. An alternative **polyphyletic hypothesis** states that there were several endosymbioses at that time (1–2 Ga), but that reticulate evolution, occurring over many Ma, gave rise to the three modern classes with primary plastids, i.e. the Glaucophyceae, the Rhodophyceae and the Chlorophyceae. This is the **shopping bag model** (Larkum et al. 2007; Howe et al. 2008). It has gained support recently from evidence that cyanobacterial endosymbioses have occurred much more recently than the period when primary plastids evolved, and have given rise to endosymbionts where some genes have been transferred to the host nucleus, e.g. *Paulinella* (Nowack and Grossman 2012) and other examples (Dorrell and Howe 2012). It was also supported by an analysis of slowly evolving genes (Nozaki et al. 2009).

A further point to notice is that the protistan organism(s) into which this endosymbiosis occurred, already possessed a mitochondrial endosymbiont, since all protists have been shown to possess at least a relict mitochondrion (Fehling et al. 2007) and likely a chlamydial endosymbiont too (Ball et al. 2013).

Thus a key feature of modern research is to identify the cyanobacterial organism(s) and the genes, which were transferred to the protistan host(s), and those that have survived (either in the plastid or the host DNA).

3.1.1 Glaucophytes

Glaucophyceae are protist organisms (eukaryotic algae), whose affinities are not clear. There are about a dozen species, none of which is common. *Cyanophora paradoxa* is the most studied and consists of a motile cell without a cell wall. Another species, *Glaucocystis* is immotile, although it retains vestigial flagella. It has a cellulose cell wall. *Gloeochaete* has both motile and immotile stages and it appears that its cell wall is not composed of cellulose. The structure of the flagellum root and the presence of two unequal flagella suggest links with Chlorophyta, although the presence of cortical alveoli is a difficulty with such a suggestion (Berner 1993).

The primary plastid, known as a cyanelle, has by definition two outer membranes, but in addition there is a vestigial cell wall, a peptidoglycan layer lying between the two bounding membranes. The latter layer could be the vestige of the cell wall of the cyanobacterium, which gave rise to the symbiosis.

The photosynthetic machinery of the cyanelles is the most similar to cyanobacteria of all the plastids. Only Chl *a* is present and the major light harvesting proteins are phycobiliproteins, which lie in the plastid matrix and funnel energy down to the thylakoid membrane via a stalk. There are no LH proteins in the CAB family present (Fig. 9); but, as would be expected however endosymbiosis took place there are proteins, which are relatives of the HLIP protein of Cyanobacteria (see Sect. 9), and which have one alpha-helix and the Stress Enhanced Proteins (SEP), with two helices (see Sect. 9), which occur in Glaucophyceae, Rhodophyceae and diatoms (Engelken et al. 2010; Sturm et al. 2013). The plastid genome of *Cyanophora* has a size of 139 Kb and holds 192 genes.

If the concept of Archaeplastida is upheld then Glaucophyceae, Rhodophyceae and Chlorophyceae share a common origin. Recently sequencing of the whole genome of *Cyanophora paradoxa* was accomplished (Price et al. 2012). While this work indicated many shared characteristics between these three phyla there are many differences,

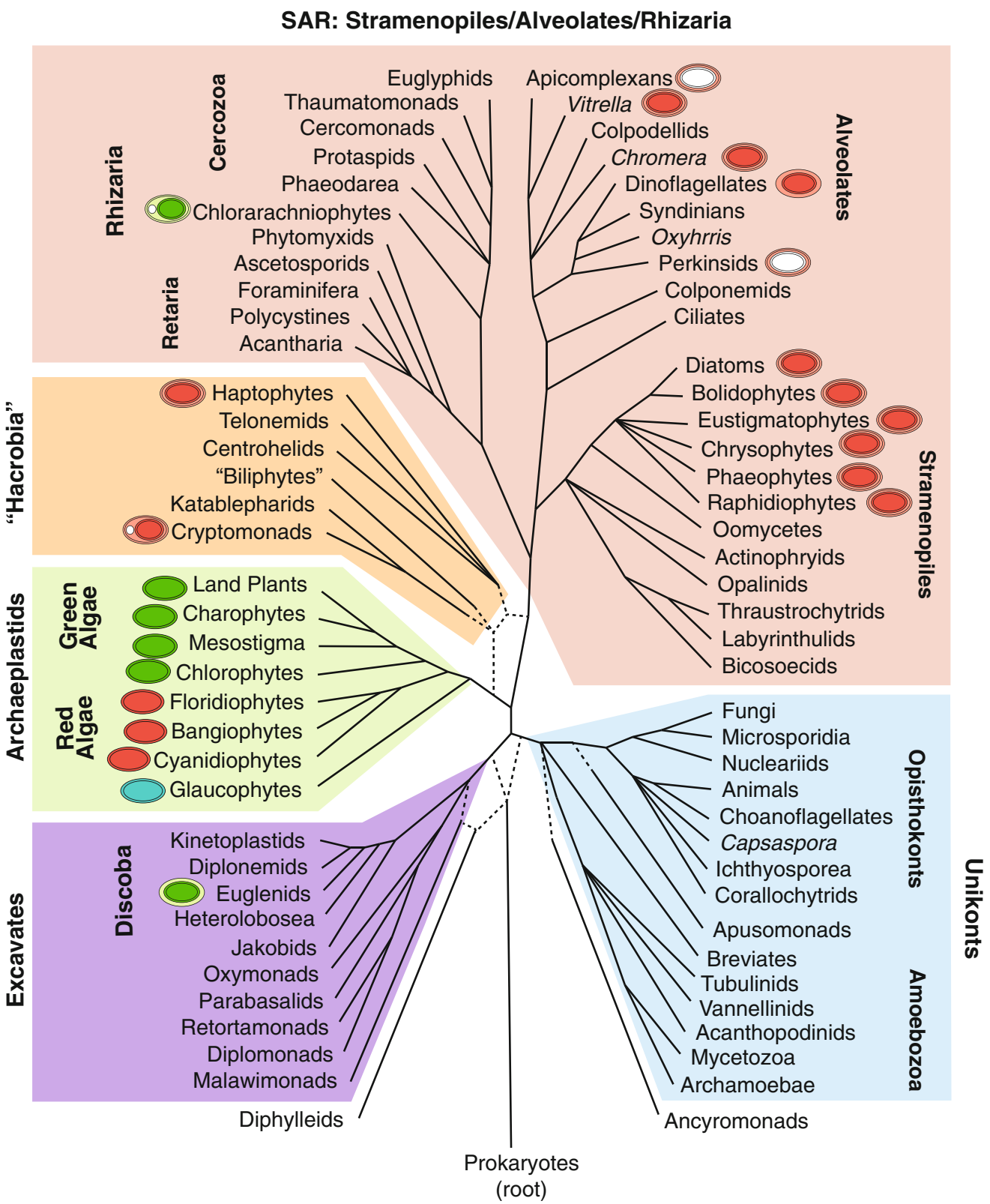


Fig. 5 The photosynthetic eukaryotic algae arranged phylogenetically, based on the arrangement of Keeling (2013) (Reproduced from Annual Review of Plant Biology with permission)

which still cannot be explained. For example the lack of any LHC genes is not easy to explain on a shared origin.

3.1.2 Rhodophyta

The Rhodophyta is a group of non-flagellate protists with affinities with Amoebozoa (Larkum and Vesk 2003; Fehling et al. 2007). The presence of two outer membranes places their plastids with the primary plastids. However, there are many features which separate them from the plastids of glaucophytes. Firstly, their phycobiliproteins show considerable evolution as compared with cyanobacteria and glaucophytes. For example, many rhodophytes have an extra pigment group, the gamma phycobiliproteins. They also have a CAB protein (see Fig. 9) with affinities with the CAB proteins of green algae and many Chl *c*-containing algae, although their only chlorophyll is Chl *a*. They also have a special form of starch known as floridean starch. Like most Cyanobacteria, the thylakoids are non-appressed and bear phycobilisomes on their outer surface.

Two whole genomes of rhodophyte algae have been sequenced: the primitive hot springs alga, *Cyanidioschyzon merolae* (Matsuzaki et al. 2004) and the advanced red alga (Floridiophyceae) *Porphyridium purpureum* (Bhattacharya et al. 2013). In addition to this, six plastid genomes are available; from *Cyanidioschyzon merulae*, from two bangiophyte algae and from three advanced red algae (DePriest et al. 2013). It should therefore be possible to trace the evolutionary inheritance of the cyanobacterial symbiont that gave rise to the red algal plastid. However, this has not been achieved to date (see Li et al. 2014).

The plastid genome, although varying between the different red algae, shows generally similar features in having a size of 150–190 Kb and a protein coding gene number of 193–234 (DePriest et al. 2013). This is a much larger size that is found in green plastids.

3.1.3 Chlorophyta

The third group of algae possessing primary plastids is the Chlorophyta. This group of protists is predominantly flagellated with two unequal flagella. The plastid outer membranes enclose many thylakoid membranes, which are closely positioned to each other over significant portions, but the term **appression** has been reserved for the grana of higher plants (Larkum and Vesk 2003). Unlike the plastids of glaucophytes and rhodophytes, the light harvesting system is based on membrane integral proteins. Many of these proteins are in the CAB family of proteins binding Chl *a* and Chl *b* (see Sect. 5). In addition there are a number of other differences: the presence of starch, the close positioning of thylakoid membranes, presence directly in the cytoplasmic compartment, and not enclosed in any other membranes. It should be noted, however, that a CAB-type gene and gene product is found in PSI of rhodophyte plastids (see above), an occurrence which might be explained either by vertical or lateral gene transfer. It should also be pointed out that eukaryotic algae in the chromophyte lineages (chomalveolates) (Fig. 5), which bear secondary plastids, have proteins in the same family as CAB proteins, but they carry Chl *c* not Chl *b* and are referred to as CAC light-harvesting proteins (see below).

The genomes of many chlorophyte plastids have been sequenced and range in size from 118 to 204 kb, with 94–107 genes.

A number of chlorophyte genomes have been fully sequenced. The size ranges from 12 to 121 Mb (Blanc et al. 2010), with *Chlamydomonas* being the largest by far. Interestingly, *Chlorella* (*Chlorella variabilis*) turns out to be a member of the Trebouxiaceae, with a history of endosymbiosis, cryptic sex and cryptic/residual flagella (Blanc et al. 2010),

3.2 Secondary and Tertiary Plastids

It is surmised that all other plastids have arisen by secondary symbiosis, followed in some cases by tertiary symbiosis. Some evidence for this comes for the nucleomorph of cryptophytes, where there are four membranes around the plastid and the nucleomorph, bearing three reduced chromosomes, lies between the two sets of outer membranes. This nucleomorph has been sequenced and indicates that the primary host was a relative of a red alga (Douglas et al. 2001). The cryptophyte plastid genome of *Guillardia theta* also bears strong similarities to rhodophyte plastids (Douglas and Penny 1999). This is interesting because the primary light-harvesting protein of cryptophytes is quite different, in the alpha subunit from the phycobiliproteins of red algae (see below). In addition they have acquired Chl *c* (see Sect. 3.2.4).

For most of the secondary plastids, largely in the chromophyte lineage (also called the chromalveolate branch), no equivalent of the nucleomorph has been found (but it is assumed that these secondary plastids evolved in a similar way by serial endosymbiosis): four outer membranes usually exist, although in the case of dinoflagellates only three are found. A case for tertiary endosymbiosis can also be made out in certain limited instances (e.g. dinoflagellates – Keeling 2013).

3.2.1 Diatoms (Bacillariophyceae) and Related Phyla including the Phaeophyceae

The LHCs of diatoms, the fucoxanthin chlorophyll proteins (FCPs) form the most recalcitrant and least understood of all the LHCs (Gundermann and Büchel 2014). While in other algae it has been relatively easy to assign LHCs to PSI or PSII, this has not been so easy in diatoms.

The LHCs of diatoms have been classified into three major groups (Caron et al. 1988; Gundermann and Büchel 2014):

Group 1, coded by Lhcr genes, is equated with a pure light-harvesting component of PSI,
Group 2, coded by Lhcf, gives rise to the major fraction of light-harvesting proteins (FCPs),
and
Group 3, coded by Lhcx, is similar to the LI818 proteins of other algae (Gundermann et al. 2013; Gundermann and Büchel 2014), which have a controlling role in non-photochemical quenching (Lepetit et al. 2013).

The levels of subpopulations of Lhcf change with light conditions: recently two groups have proposed models to describe changes to the thylakoid membranes under low and high light (Gundermann et al. 2013; Lepetit et al. 2013). Nevertheless, it is broadly true that neither state transitions nor spillover of excitation energy from one photosystem to the other have been observed in diatoms (Arsalane et al. 1994).

Diatoms have the ability to respond to rapid changes in light intensity (Wagner et al. 2006) as a result of non-photochemical quenching (NPQ) mechanisms. The situation in diatoms is atypical because they have both the diadinoxanthin cycle (DdC) and the violaxanthin cycle (Lohr and Wilhelm 1999; Goss and Jakob 2010). However, in the diatoms it has recently been shown that there are two NPQ mechanisms, one associated with antenna units attached to PSII and the other associated with antenna units that detach from PSII and remain isolated during high light conditions (Miloslavina et al. 2009). None of these NPQ mechanisms would be expected to affect the maximum efficiency of photosynthesis in diatoms, which has been shown to be high (Torres et al. 2013).

Diatoms have a unique outer wall formed of silica and arranged in two abutting frustules. The function of the frustules is most probably protection, but LH has been proposed: the frustule has a unique architecture with sculpturing giving rise to small perforations which are the right distance apart to affect coherent laser light at wavelengths of >1000 nm; as a result frustules have become a favorite tool for laser studies (Hsu et al. 2012); however, to the present time there is no evidence to suggest that there is any connection to photosynthesis and LH.

All these unknowns make diatoms one of the most intriguing systems in the field of algal light harvesting today.

3.2.2 Related Phyla

In very general terms the following Phyla fit into the diatom pattern, having, in addition to Chl *a*, Chl c_1 and c_2, diatoxanthin and diadinoxanthin and Lhc light-harvesting proteins:

Phaeophyta, Rhaphidophyta, Chrysophyta, Bolidophyta. The Eustigmatophyta also fall into this group but retain only Chl *a*.

3.2.3 Dinoflagellates

Dinoflagellates evolved within the chromalveolate group of algae and gave rise, via *Chromera* (Quigg et al. 2012) to their non-photosynthetic relatives, the apicomplexans, according to modern phylogenetic studies (Fig. 5). In the botanical system they form part of the Chromista (also known as Chromalveolates – see Glossary).

In terms of LH, dinoflagellates have a fascinating array of pigments and pigment proteins arranged in a unique thylakoid membrane and unique plastid. In addition, the molecular biology of the chloroplast is quite unique in dinoflagellates.

There are two major types of LHPs in dinoflagellates, (i) a membrane bound protein (Chl *a*, Chl c_2, peridinin protein complex, acpPC) and (ii) a water-soluble peridinin-chlorophyll protein (PCP) complex. Of these two proteins much more is known on the PCP complex, which has been resolved to a crystal structure of 1.63 Å (Wilk et al. 1999) and studied in considerable molecular detail (Hofmann et al. 1996; Schulte et al. 2009).

The gene sequence for PCP was established by Hofmann et al. (1996) and has no homology to any other known protein. The crystal structure indicates a protein with the monomer binding two Chl *a* molecules and two peridinin molecules (Hofmann et al. 1996) (Fig. 2), essentially as predicted from CD spectra by Song et al. (1976). Peridinin has an in vivo absorption spectrum extending up to 540 nm and adds greatly to the light absorption capacity of dinoflagellates in the blue-green region of the spectrum. Clearly PCP significantly augments the light-harvesting capacity of the dinoflagellate CAC proteins and does this at low nitrogen cost (Larkum 2003) and can attain concentrations of up to 50 % of the total peridinin, i.e. 50 % of the concentration of acpPC (Hofmann et al. 1996).

The functioning of PCP is still not well resolved. PCP lies in the thylakoid lumen space and is extracted as a soluble protein. However, if it is to function as an efficient LH protein, it presumably interacts efficiently with adjacent PCPs. This suggests that there may be a semi-crystalline state in vivo, which aids resonance (or even coherent) energy transfer; otherwise energy transfer would be very slow. Recent work on PCP indicates that energy transfer is efficient and is directed efficiently into the thylakoid membrane at specific sites (Reynolds et al. 2008; Hill et al. 2012). Earlier it was suggested that there were two types of PCP and that one of these high-salt PCPs might play a role in directing energy into the thylakoid membrane; however, now, the evidence is less compelling (Schulte et al. 2009).

In hermatypic corals, where coral bleaching occurs, and is triggered via the photosynthetic machinery, there is a definite

effect of coral bleaching temperatures (30–34 °C) on the interaction of PCP with the thylakoid membrane, as indicated by changes in the wavelength of fluorescence emission (Reynolds et al. 2008; Hill et al. 2012). Kanazawa et al. (2014) have suggested that energy transfer into the membrane is increased by coral bleaching temperatures in Clade C *Symbiodinium* (the most heat sensitive clade of hermatypic corals).

3.2.4 Cryptomonads (Cryptophyceae)

Cryptomonads belong to a phylogenetic group of biflagellate protists, only a part of which are photosynthetic algae and to which has recently been given the name "Macrobians" (see Keeling 2013) (Fig. 5). Previously this group would have been placed in the Chromista (Chromalveolates). In broad terms, cryptomonads have a secondary plastid that has engulfed an organism with a primary plastid allied to the red algae (Douglas et al. 2001). However, this cannot be the complete story because the plastid bears an LHC with Chl *a* and Chl c_2. In addition the plastid bears phycobiliproteins (PBPs) which are unique and quite different, at least in the alpha chain, from red algal PBPs (Wilk et al. 1999): these have a beta polypeptide with distinct affinities to red algal PBPs but an alpha chain with no affinity to any other protein; and some of the chromophores of the phycobiliproteins are quite unique (Glazer and Wedemeyer 1995). Thus there is much evolutionary history in the production of these PBPs and it is not at all clear that there is any direct, linear descent from red algae.

As in dinoflagellates the water soluble PBPs are in the lumen of the thylakoids. Thus photons that are harvested must be passed onto the thylakoid membrane through interaction of these protein units. Recently there has been much excitement with the suggestion from several laboratories that coherent energy transfer may take place, i.e. that the migration of excitation energy is by exciton wave transfer. Exactly how this can take place is being taken up as a challenge by these laboratories.

4 The Need for Light Harvesting Antennae

Figure 1 shows the molecular layout of the reaction centres of photosystem I and photosystem II as currently configured from molecular X-ray diffraction studies. The two centres are built around a scaffold, which takes an absorbed photon of light and uses this to drive an electron across the reaction centre (inside to outside) forming a primary reductant and a primary oxidant molecule. From there the primary sites are stabilised by electron transfers to and from secondary sites. And from there an electron transport chain carries out the fixation of energy as ATP and NADPH (by which CO_2 is fixed into organic form).

The reaction centres have few pigment molecules and a large number of cofactors and are expensive in terms of light capture and the amount of protein per absorbed photons. It is therefore a second imperative of all photosynthetic systems to build light-harvesting centres with a much greater concentration of pigments per protein molecule. These pigment-protein complexes are arranged around the reaction centres and give a density of perhaps up to 1000 pigment molecules per RC. Under these conditions the absorption cross section of the RC increases by 1000 and for Chl molecules this results in a turnover of the RC of about once every 0.1 s under medium light intensities (Ruban 2013).

Clearly the absorption cross-section will be affected by the pigments that are linked into the light-harvesting pigment protein complexes and the quality of the incident light, e.g. whether this is in full sunlight, or the shade of a forest or the depths of the ocean.

Under low light conditions the need for more light harvesting molecules and a greater absorption cross-section is high. Thus it is not surprising that algal and plant systems adjust to lowered light by incorporating a higher density of pigments and thereby increase the absorption cross-section. Algae also have evolved the ability to place a large number of pigments in their LH antennae. However, surprisingly few have evolved pigments that can absorb in the green region: here phycobiliproteins are the example, *par excellence*; there are also the oxygenated carotenoids, fucoxanthin and peridinin, which are exceptional in terms of carotenoids by the extension of their absorption spectra out to 550 nm.

5 Light-Harvesting Antennae in Cyanobacteria and Eukaryotic Algae

In Cyanobacteria, the phycobiliproteins (PBPs) are the light-harvesting antenna *par excellence*. However, it seems likely that these are a secondary, although very successful, evolutionary development. PBPs are also water soluble and extrinsic to the thylakoid membrane. It is more likely that they were preceded by membrane intrinsic light-harvesting antennae; of which there are still some in existence. The isiA antenna, containing only Chl *a* (Fig. 6), the pcb antenna, containing Chl *a* + *b* and similar antennae which bind Chls *d* and *f* (see Sect. 2.1) are examples of such antennae, although whether these preceded PBPs is not known. It seems clear too that these pigments if not their antennae were passed on to plastids, although the LHC (CAB/CAC) proteins seem to have evolved from high-light protection proteins (see below) (Engelken et al. 2010; Niyogi and Truong 2013).

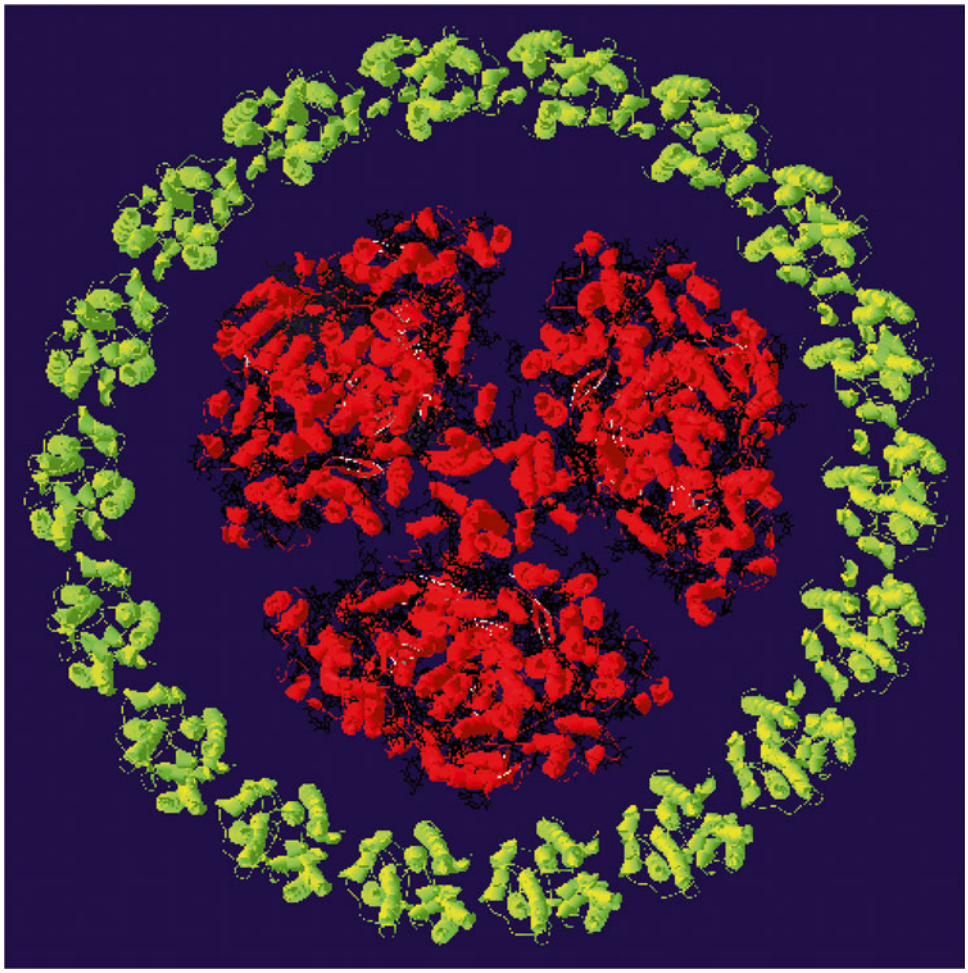


Fig. 6 Space-filling model of the structure of the isiA supercomplex of *Synechococcus* where 18 isiA units surround a trimeric Photosystem 1 reaction centre (See Zhang et al. 2010)

In all plastids, except the cyanelles of Glaucophyta, a related family of LHC membrane intrinsic proteins exists each with three membrane-spanning protein helices (Fig. 2). These bind eight Chl *a* and six Chl *b* or *c* and four xanthophyll molecules (Rochaix 2014). In nearly all plastids the LHCs are divided into those attached to PSI (LHCI), and those attached to PSII (LHCII). However it has long been known that LHCII is mobile and can reattach to PSI under conditions where this evens up the activity of the PS (Larkum 2003). Much is known on the sub-distribution of LHCI and LHCII in different plastids, particularly land plants. In eukaryotic algae most is known for *Chlamydomonas reinhardtii* (Rochaix 2014): 15 LHCII- and 6 LHCI-encoding genes have been identified. Clearly, there is much latitude here for adaptive responses in light harvesting strategies. In eukaryotic algae, LHCII trimers are connected to RCII (a core dimer plus CP43 and CP47) through two monomeric LHCII proteins, CP26 and CP29. PSI complex is monomeric, in contrast to Cyanobacteria (Fig. 6), where it is trimeric; the large number of Chls bound to extra sub-units act as antennae. In addition there are up to six LHCI proteins, bound asymmetrically in a crescent-shaped arc, and, during state transitions (see below), an additional number of LHCII molecules (see Fig. 8).

6 Control of Energy Supply to PSI and PSII: State Transitions, Absorption Cross-Section Changes and Spillover

Photosynthetic organisms must often be able to adapt to light intensities that may range over a thousand fold intensity. Such environmental changes may be slow, such as weather–based or seasonal changes. Or they may be fast, ranging from moderately fast, as between dawn and noon time, to very fast as between clouds and full sunlight, or light flecks that occur in forests and in the sea. To meet these circumstances photosynthetic organisms put in place two kinds of adaptive mechanisms: long-term changes brought about by protein formation to adapt to new levels of pigments and short term changes, which reassign the concentration of pigments already entrained. Here I will concentrate more on the short-term changes. Long–term changes, which are well known in Cyanobacteria, eukaryotic algae and land plants, have been

dealt with elsewhere (Larkum 2003; Falkowski and Raven 2007).

The ultimate "aim" of all these mechanisms is to match the energy received by PSI and PSII so that the optimum levels of ATP and NADPH are produced. Note that where cyclic electron transport (CET) is involved, as it often is, to balance the need of the Calvin-Benson Cycle for more ATP than NADPH (Lucker and Kramer 2013), there is a need to have PSI operating faster overall than PSII.

6.1 State Transitions

State transitions were one of the first light harvesting mechanisms to be documented and came from the work of Murata (1969, 1970) who showed in spinach chloroplasts and in red algae that the amount of variable fluorescence (assigned to PSII) was affected by the previous conditions of illumination. Bonaventura and Myers (1989) first defined the phenomenon, albeit in a spillover model, which may be restated, as follows:

State I in which there is an excess of light available to and absorbed by PSI (light I) and a decrease in the amount of excitation energy distributed from the light-harvesting pigments to PSI; and

State II in which there is an excess of light available to and absorbed by PSII (light II) and an increase in the amount of excitation energy distributed from the light-harvesting pigments and PSII to PSI (Fork and Satoh 1986). In darkness a State I condition is usually found.

Satoh and Fork (1983) proposed a putative third state (**State III**) from evidence using the red alga *Pyropia* (*Porphyra) perforata.* Illumination of this alga either in state I or state II with light II produced state III in which light energy reaching PSII was decreased with no attendant increase in the energy supply to PSI. Although there was no change in the distribution of energy between the two photosystems there was a decrease in the overall amount of excitation energy migrating to RCII from the light-harvesting pigments. This phenomenon is probably a photoinhibitory response whereby some phycobilisomes are decoupled under high light to protect the RCs from over-activity.

The first physical model of the fluorescence changes in State Transitions came from Butler and coworkers (see Butler 1978) again in terms of a spillover model. It now seems more likely that the changes are effected in terms of re-association of light harvesting complexes between the two PSs (rather than re-channelling of energy absorbed by one PS to the other, although there is still much debate over this). There is strong evidence that re-association occurs in higher plants and so one must expect a component of fluorescence change in situations which over-excite one photosystem against the other. However it is now known that down-regulation of PSII occurs, which is brought about, both by the xanthophyll cycle and by delta pH quenching (see below).

State Transitions have been observed in all oxygenic photosynthetic organisms. They are largest in Cyanobacteria and red algae and lowest in tracheophytes (land plants with tracheid vascular tissue). The mechanism of state transitions is likely to be similar to that for changes in optical cross-section of the PSs (see below). However, the two mechanisms may not be identical (Haldrup et al. 2001; Wollman 2001). State transitions have been a simple way to study short-term changes in energy distribution to PSI and PSII, and have been used extensively for this purpose (see e.g. Haldrup et al. 2001; Wollman 2001). Here, I concentrate on what is known from *Chlamydomonas reinhardtii* (see review e.g. by Rochaix 2014).

In *Chlamydomonas reinhardtii* state transitions involve 80 % mobile LHCII as compared with 15–20 % in *Arabidopsis thaliana* (Delosme et al. 1996). Work on mutants has revealed a mutant, Stt7, in which State 1 is blocked, is deficient in LHCII phosphorylation in State 2 and lacks Stt7 protein kinase (Depege et al. 2003); this has an equivalent in *Arabidopsis*, STN7. There is also an additional mutant Stt1 (STN8 in *Arabidopsis*) (Rochaix 2014). It is likely that in Stt7/STN7 a protein kinase is deficient and cannot phosphorylate LHCII. Thus under State II conditions the mutant is blocked in transferring mobile LHCII from PSII to PSI, i.e. the State Transition is blocked. The arrangement of PSII with LHCII antennae in *C. reinhardtii* was studied by Drop et al. (2014a) (see Fig. 8) and in a another study of structural rearrangements during State Transitions by Drop et al. (2014b) it was shown that, under State 2 conditions, PSI is able to bind two LHCII trimers that contain all four LHCII types, and one monomer, most likely CP29, in addition to its Lhca. This structure is the largest PSI complex ever observed, having an antenna size of 340 Chls/P700. Moreover, all PSI-bound Lhcs were efficient in transferring energy to PSI. Interestingly, only LHCII type I, II and IV were phosphorylated when associated with PSI, while LHCII type III and CP29 were not, but CP29 was phosphorylated when associated with PSII in state 2. That study underscores what has become clear from a number of recent studies, that the classical view of phosphorylation of LHCII under reduced PQH conditions is an oversimplification.

It should be pointed out that the suggested function of State Transitions in bringing about equal activity of the PSs was challenged long ago by Bulté et al. (1990), who suggested that the main function of State Transitions might be to balance the production of ATP and $NADPH_2$. They showed that inhibition of ATP production in intact cells in *Chlamydomonas reinhardtii* led to a transition to State II while an increase of ATP production caused a change to State I. This suggestion seems to have been upheld by later investigations (Delosme et al. 1996). Recent work on

Chlamydomonas has shown that CET is controlled by redox chemistry and not by State Transitions; for example State 2 was not required for inducing CET, since anoxic conditions enhanced CET, both in wild type and in an stt7 mutant blocked in State 1 and the PSI-Cytb_6f supercomplex involved in CET was formed independently of State Transitions (Takahashi et al. 2013). In the unicellular red alga *Rhodella violacea*, in contrast to *Chlamydomonas*, state transitions were not accompanied by phosphorylation of thylakoid proteins (Delphin et al. 1995). Also they occurred under conditions where the activity of PSI does not change and it was suggested that ΔpH changes across the thylakoid membrane triggered "state II" quenching possibly through a down-regulation process of RCII (Delphin et al. 1996).

State Transitions have been investigated in a number of other algae since the early work, which was mainly directed to cyanobacteria, green algae and higher plants: the groups investigated include brown algae (Fork et al. 1991); *Chromera velia* (Quigg et al. 2012); chlorophytes (*Dunaliella*) (Ihnken et al. 2014); cryptophytes (Bruce et al. 1986); chrysophytes (Gibbs and Biggins 1991); *Nannochloropsis* (Eustigmatophyceae) (Szabo et al. 2014); *Pleurochloris* (Xanthophyceae) (Büchel and Wilhelm 1990). In *Pleurochloris* the state transitions were wavelength-independent. In many cases the extent of State Transitions is much more pronounced in algae than in plastids of higher plants (Schreiber et al. 1995). Furthermore, apart from the streptophyte algae, it appears that there is little lateral heterogeneity in the thylakoids of algae (Larkum and Vesk 2003). Thus there is the possibility of energy transfer between the PSs; and therefore further scrutiny of light energy distribution to the PSs in algal plastids is more than justified.

6.2 Absorption Cross-Section Changes

State Transitions is a term that has been applied to changes in light quality, which bring about a change in the activity of PSI and PSII. As shown above these can be understood in general terms as brought about by changes in the oxidation-reduction potential of plastoquinone and the resultant phosphorylation/dephosphorylation of LHCII, although many fine details remain to be worked out. Such changes are largely artificial in that they do not occur naturally, and have been used only to examine the mechanisms behind these changes. A much more natural effect is the short-term change from high light to low light and vice versa, an occurrence which is very normal for an alga undergoing changes in shading due to clouds, diurnal events, sun flecks and wind effects, etc. Such changes have become a focus of several recent studies, the most insightful of which have been carried out on *Chlamydomonas reinhardtii*. Several genes have been identified which influence the placement of LHCII between PSI and PSII. Also, by analogy with *Arabidopsis*, it appears that the placement of LHCI and LHCII in the two PSs is regulated in different ways when the light intensity changes (Rochaix 2014). The recent demonstration of large changes in the antenna size and distribution of LHCI and LHCII in *C. reinhardtii* (Drop et al. 2014a, b; Unlu et al. 2014) indicate the potential of these techniques; the effects of light intensity changes will be awaited with interest.

Of course, changes in absorption cross-section in response to light intensity changes is only one mechanism of dealing with increases in light intensity. Another important mechanism is to down-regulate light energy uptake by the process of non-photochemical quenching (NPQ) and this phenomenon is dealt with next.

When the flux of photons to PSI and PSII is not equal, one way of effecting equal activity of PSI and PSII, is to reduce the excitation energy of one PS and to increase it to the other. Initially it was supposed that there was a mechanism ("**spill-over**"), which simply diverted energy from one photosystem to the other – predominantly from PSII to PSI (Larkum 1983). However, the mechanism now generally proposed is in terms of mobile light-harvesting units, which change the optical cross-section, and also maybe change the spectral properties, of one or both photosystems (Fosberg and Allen 2001; Minagawa 2013; Rochaix 2014).

A general mechanism for changes in absorption cross-section in higher plants has been available for over 30 years (Allen et al. 1981; Allen 1992). The mechanism is thought, in higher plants, to be as follows.

Preferential illumination of PSII (**Light 2**) leads to reduction of the plastoquinone (PQ) pool, between the two PSs (**State 2**). Under these conditions, and through the mediation of the Q_o site of the cyt b_6f complex, at least one, and possibly more than one, membrane-bound protein kinase becomes activated leading to the phosphorylation of mobile LHCII and other polypeptides. Phosphorylated LHCII then moves away from the appressed thylakoid regions, to unappressed thylakoids on the outside of grana or in the stroma, where it associates with PSI (Fig. 7). The membrane bound kinase is deactivated in the dark or in **Light 1** (light which preferentially activates PSI and when PQ is oxidised - **State 1**) and a latent phosphatase continually reverses the action of the kinase (Allen 1992). Evidence for a similar mechanism in the green alga *Dunaliella* has been presented (Escoubas 1996) and there is some evidence that it may also exist in dinoflagellates (ten Lohuis and Miller 1998). However, the greatest advance in the area of algae has come over the last two decades from studies of *Chlamydomonas reinhardtii* (Rochaix 2014), which can be transformed and has become the alga of choice, similarly to *Arabidopsis thaliana* in higher plants. This work is largely dealt with in the section on State Transitions (above), but it is obviously connected with changes in absorption cross-section brought about by reassignment of LHCs as a result of short-term rearrangements and long–term production (see Fig. 7). Particularly impor-

tant here is the realization that changes are brought about in going from low light to high light (and vice-versa) and that much can be learnt from such an approach. One recent example is for *C. reinhardtii* where changes in the LHCs have been physically mapped onto PSI and PSII (Drop et al. 2014a, b; Unlu et al. 2014); this is possible because through electron microscopy it is possible to carry out electron density scans of light-harvesting particles obtained on developed sucrose density gradients (Fig. 7). What these investigations show is that the standard model of state transitions needs changing. Phosphorylation does not necessarily induce a State II to State I transition.

While the general principles of this proposed mechanism have been supported, in the interval there has been much progress in many other areas – both in higher plant and in algal studies. In higher plants it has been shown that there is a specific LHCI, which acts to harvest light specifically for PSI (Green and Durnford 1996). Thus changes in cross-sectional area of PSI and PSII due to re-association of mobile LHCII can only contribute a small fraction of change in cross-sectional areas (usually <20 %). In higher plants too it has been shown that the PSI subunit H polypeptide is essential for docking of phosphorylated LHCII and in mutants lacking the H subunit phosphorylated LHCII stays associated with PSII – with no changes in cross-sectional area (Lunde et al. 2000; Haldrup et al. 2001). Furthermore it has been shown that phosphorylation of LHCII is not linearly dependent on the reduction of PQ (Haldrup et al. 2001): the degree of phosphorylation reaches a peak at rather low light intensities (of light which preferentially activates PSII).

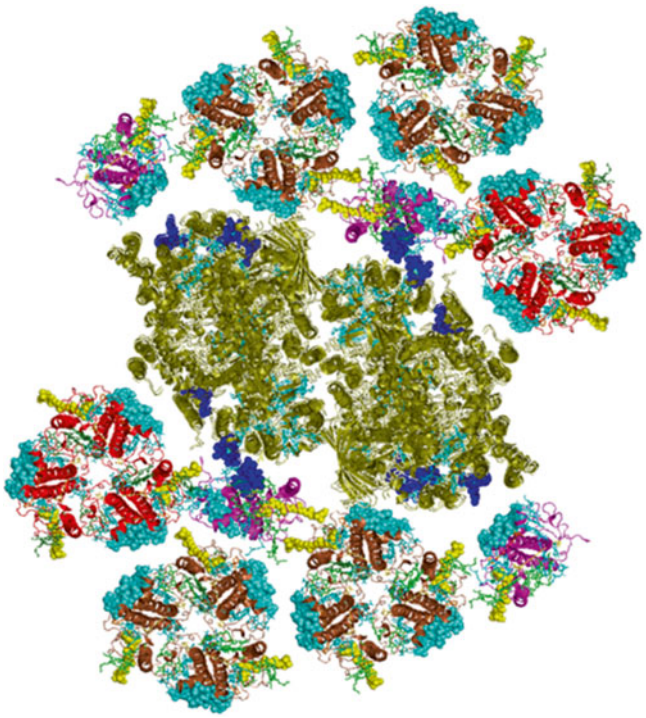


Fig. 7 The model has been assembled using the crystal structures of the cyanobacterial PSII core, LHCII trimer and CP29. For CP26, the structure of a monomeric LHCII has been used. Proteins of the PSII core (*lime green*), LHCII-S and -M (*brown*), novel LHCII-N (*red*), CP29 and CP26 (*magenta*), Chls *a* (*cyan*), Chls *b* (*green*), neoxanthin (*yellow spheres*), lutein L1 (*orange*), lutein L2 (*dark-yellow sticks*) (Taken from Drop et al. 2014a)

In algae (other than streptophyte green algae) the distribution of PSI and PSII appears to be much more homogeneous (Larkum 2003). Here there is less evidence for phosphorylation of LHCs driven by Light 1 and Light 2 (and the involvement of a PQ-driven mechanism). Gibbs and Biggins (1991) argued against such a phosphorylation mechanism in the Chl *c*-containing chrysophyte alga, *Ochromonas*. Allen (1992) gives a review of this early work on eukaryotic algae.

In Cyanobacteria and red algal plastids, the main light-harvesting system is the phycobilisome (PBS) and there has been much debate over whether the attachment of PBS to PSII or PSI is driven by the redox state of PQ involving phosphorylated proteins (for a detailed review see Allen 1992). Certainly there is good evidence that the absorption cross-sections of PSI and PSII change in response to Light 1 and Light 2. Many proteins are also phosphorylated in the light. Recent evidence seems to suggest that the PBS physically move between PSII and PSI sites (Sarcina et al. 2001; Mullineau and Sarcina 2002). However, there is certainly evidence for a protective mechanism in red algae whereby energy is directed from one PS to the other in what has been called **spillover** (Kowalczyk et al. 2013) (see below).

Almost certainly there are distinct differences in the mechanisms by which short-term accommodation of Light 1 and Light 2 effects changes in the cross-sectional areas of PSI and PSII in cyanobacteria, algae and land plants. Pursiheimo et al. (1998) proposed three categories: **Group 1**, Cyanobacteria and red algae, which did not show phosphorylation of any of the photosystem II (PSII) proteins; **Group 2** consisting of some of the remaining eukaryotic algae, mosses, liverworts and ferns, which phosphorylated both the light-harvesting chlorophyll a/b proteins (LHCII) and the PSII core proteins D2 and CP43, but not the D1 protein, and **Group 3** where reversible phosphorylation of the D1 protein of PSII was found only in seed plants and was seen as the most recent evolutionary event in the series. In terms of phosphorylation of LHCII they found that Groups 2 and 3 were similar with maximal phosphorylation of LHCII at low light and nearly complete de-phosphorylation at high light. Clearly this survey did not include any algae dependent on CAB light-harvesting systems. However, the large number of studies of *Chlamydomonas reinhardtii* over the last two decades has provided a solid base for understanding this system (Rochaix 2014), with some recent revaluation of the

finer details of the mechanisms (Tikkanen et al. 2012; Croce and van Amerongen 2014).

6.3 Non-photochemical Quenching – Sensu Lato

Non-photochemical quenching is a set of processes, whereby some light energy is deactivated as heat before the rest is channeled to the reaction centres; it is a protective mechanism that is activated under high light, protecting the reaction centres from damaging levels of excitation, which can give rise to photoinhibition, through the degradation of key peptides and the activities of Reactive Oxygen Species (ROS). The oldest known process is the Xanthophyll Cycle, which is activated by key carotenoids and triggered though the polypeptide PSBS, in land plants, or LHCSR (LHCX) in most algae (Niyogi and Truong 2013; Lepetit et al. 2013) (see Fig. 9). There is another process, which is simply triggered by low pH and involves a number of xanthophylls. Additionally, in some Cyanobacteria there is a specific mechanism brought about by the Orange Carotenoid Protein (OCP), which acts similarly to deactivate excitation energy as heat.

6.4 Spillover

Spillover is a term first used by Butler in the 1950s to denote a migration of excitation from one photosystem to the other (see above). In its earliest formulations it can probably be seen as a process that was confused with changes in absorption cross-section, brought about by reassignment of antenna unit or subunits. Nevertheless, a valid use of the term seems to have been that of Ley and Butler (1980) who studied the fluorescence changes in the unicellular red alga, *Porphyridium cruentum*. As recently reported by Kowalczyk et al. (2013) for an investigation of a similar situation in the high-light-stressed intertidal red alga, *Chondrus crispus*, a real migration of energy from PSII to PSI can be detected, rather than a deactivation through an NPQ pathway or a redistribution of light harvesting antennae. Another situation, where spillover has been shown is in the lichen *Parmelia sulcata*, which harbors a unicellular Trebouxian green alga (Slavov et al. 2013). It should be pointed out that few eukaryotic algae have thylakoids with grana and true appression (Larkum and Vesk 2003), i.e. where PSII is physically segregated from PSI (see e.g. Rochaix 2014). It is therefore possible in algae, and in Cyanobacteria, for PSI and PSII to be physically close to one another, and therefore, theoretically, for energy to migrate from one photosystem to the other. How widespread this phenomenon is has yet to be shown.

7 Non-photochemical Quenching

7.1 The Xanthophyll Cycle

The Xanthophyll Cycle in its generally recognised form occurs in most eukaryotic algae and in higher plants. The xanthophylls involved are violaxanthin, antheraxanthin and zeaxanthin or diatoxanthin and diadinoxanthin (Fig. 8). While much work has been carried out on higher plants and green algae (Demmig-Adams and Adams 1993) much less work has been carried out on algae, other than some green algae such as *Chlamydomonas* (Rochaix 2014). However, there is good evidence to believe that a similar cycle exists in many eukaryotic algae (Lichtlé et al. 1995; Goss and Bohme 1998; Brown et al. 1999; Lohr and Wilhelm 1999).

In the Xanthophyll Cycle, in the light, violaxanthin or diatoxanthin are converted by de-epoxidation to zeaxanthin or diadinoxanthin (Fig. 8). The details of this de-epoxidation and subsequent epoxidation in the light have been well documented, see e.g. Yamamoto (1979), Mohanty et al. (1995), and Demmig-Adams and Adams (1993). Thus one molecule of oxygen is liberated (de-epoxidated) or taken up (epoxidated) for a complete transition. In algae these changes were worked out in detail by Stransky and Hager (1970) and their general conclusions, shown in Fig. 8 were as follows:

Group 1 (Glaucophyta, Rhodophyceae, Cryptophyceae and Cyanobacteria), no epoxide cycle takes place although changes in levels of zeaxanthin occur;

Group 2 (Bacillariophyceae, Chrysophyceae, Xanthophyceae, Chloromonadophyceae, Dinophyceae and Euglenophyceae), diadinoxanthin is the oxygenated carotenoid and diatoxanthin is the de-epoxidated carotenoid;

Group 3 (Phaeophyceae and Chlorophyceae and odd species of some other Classes), the Xanthophyll Cycle is present. In micromonad algae only a part of the conventional Xanthophyll Cycle is present – that converting violaxanthin to antheraxanthin (Goss and Jakob 2010); furthermore, Lohr and Wilhelm (1999) have shown that some algae displaying the diadinoxanthin type of a Xanthophyll Cycle also display features of the violaxanthin-based cycle.

In general, as light levels increase, so the level of violaxanthin/diadinoxanthin decreases, reaching a steady level, and conversely, the level of zeaxanthin/diatoxanthin increases to an asymptote (Demmig-Adams and Adams 1993).

The Xanthophyll Cycle involves, generally, three factors in the stimulation of a non-photochemical quenching of energy in the PSII/LHCII assemblage:

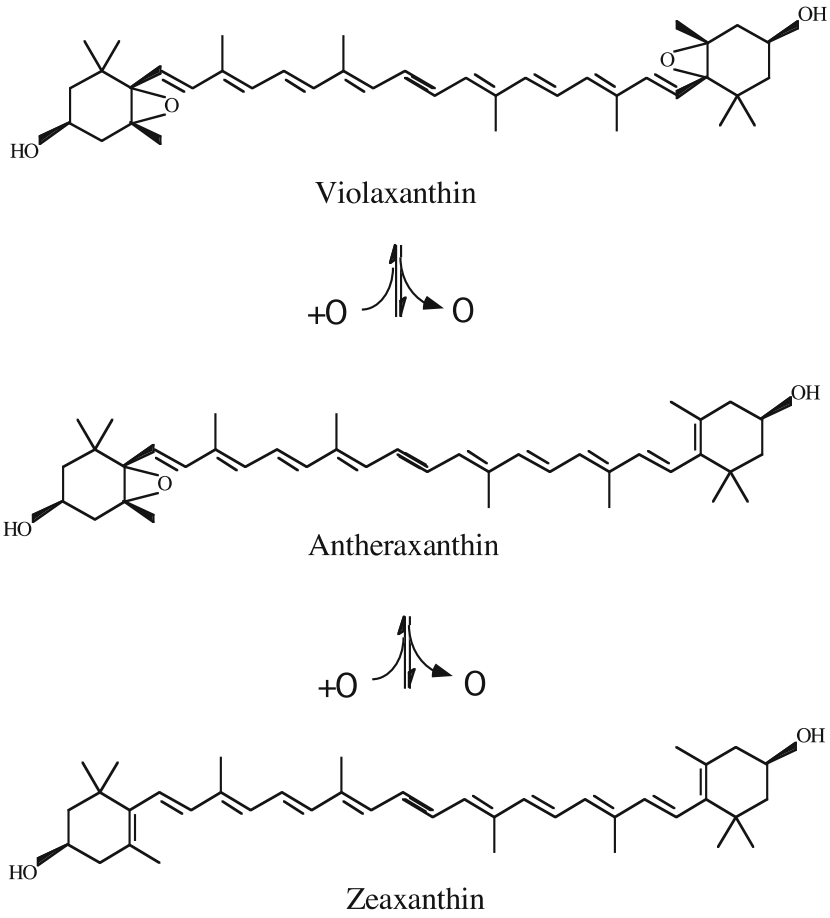


Fig. 8 The xanthophyll cycle: the interconversion of violaxanthin and zeaxanthin, with light triggering the conversion of violaxanthin to zeaxanthin (and diadinothanxin to diatoxanthin in those algae which possess these xanthophylls)

(i) an increased concentration of zeaxanthin (or diatoxanthin),
(ii) the presence of PSBS, in land plants, or LHCSR in eukaryotic algae (and to a lesser extent in liverworts, mosses and other non-vascular plants, and, streptophyte green algae (see Fig. 9),
(iii) a ΔpH across the thylakoid membrane.

With these three factors in operation, and in the presence of LHCII, excitation energy is transferred to the carotenoid and excitation energy is transduced to heat.

There is still much to be learnt in eukaryotic algae, however. In Rhodophyta and Glaucophyta, which rely on a phycobilisome system the operation of LHCSR is not well established, nor has it been found in dinoflagellates (see Fig. 9). In a number of algae from these groups it is not clear whether a delta pH is necessary. A number of workers had earlier implicated LHCII in this process (see e.g. Horton et al. 1996). However, more recently a specific role for the non-chlorophyll-binding, 22 kDa 4-α-helix membrane-spanning protein, PSBS, has been shown (Li et al. 2002; Niyogi and Truong 2013). This protein may lie in an intermediate position between LHCII and the inner antennae of RCII (Belgio et al. 2013). The evidence suggests that energy-dependent quenching, qE (which is defined as that component of the total non-photochemical quenching, qN, directly attributable to the energisation of the thylakoid membrane, and therefore the rapidly entrained component of qN) is directly dependent on PSBS.

A number of specific details are known concerning the reactions involved in quenching by zeaxanthin (and diatoxanthin). For instance, dibucaine stimulates the quenching and antimycin A, dithiothreitol (DTT) and the protein carboxyl-modifying agent dicyclohexylcarbodiimide (DCCD) inhibit the quenching. Horton et al. (1996) suggested that there is a pocket extending from the intrathylakoid lumen into the membrane by which low pH in the thylakoid lumen can influence a critical site in the thylakoid membrane. Since PSBS is essential for qE to occur it may be the protein, which senses the low pH and the binds zeaxanthin or it may play a crucial structural role in energy transfer/dissipation (Li et al. 2002). The mechanistic details of energy quenching have yet to be fully worked out. Clearly if the mechanism is to work zeaxanthin has able to change its molecular excitation states, which would then allow it to dissipate excitation energy as heat when triggered by low pH. Recent work of the group of Frank (see Josue and Frank 2002) suggest that the S1 state of carotenoids is important for this kind of down-regulation.

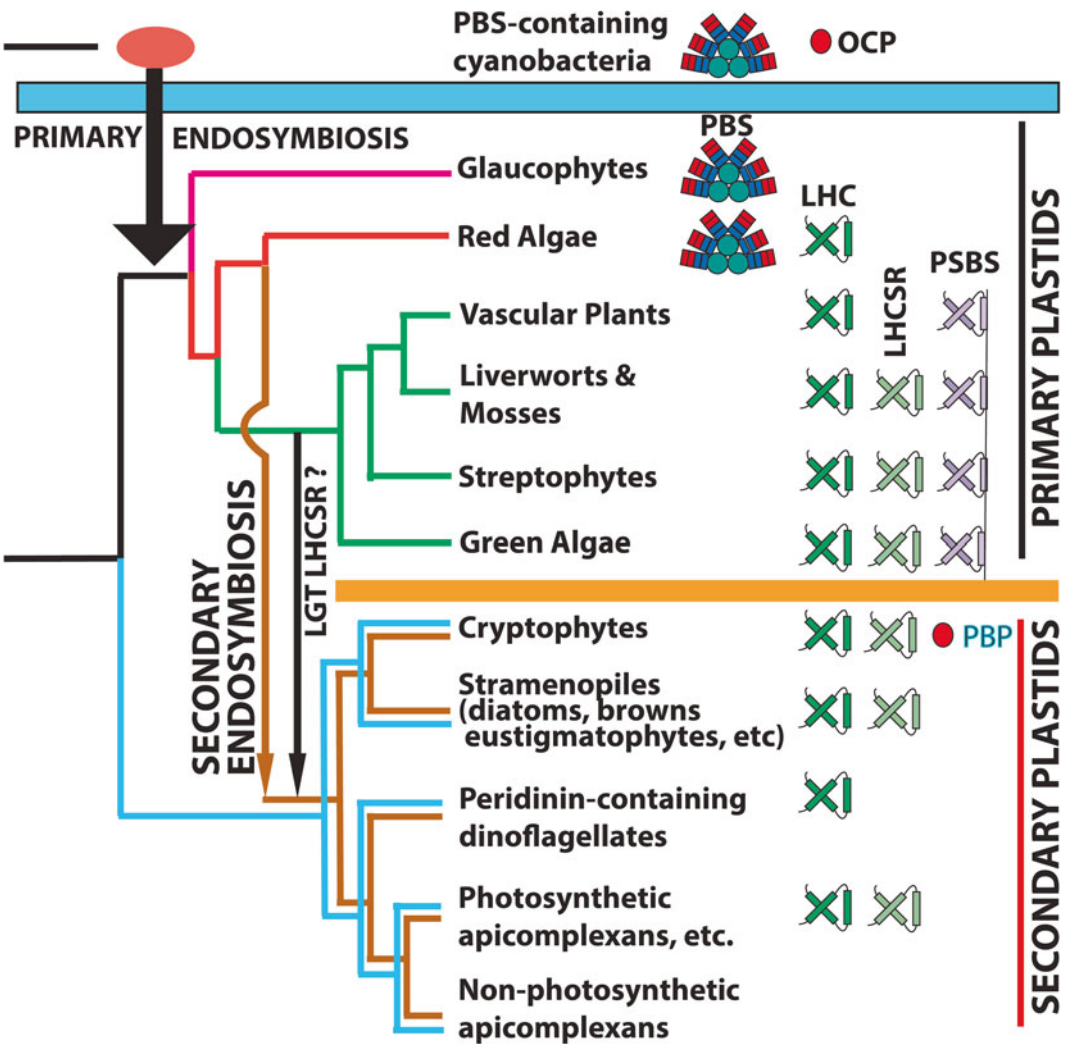


Fig. 9 Scheme showing the likely evolutionary events in the formation of photosynthetic proteins involved in light-harvesting and photoprotection (Copyright: AWD Larkum: based on the scheme of Niyogi and Truong 2013)(LGT = lateral gene transfer)

The situation in algae is more complex than in higher plants. Firstly chlororespiration may make up a larger component of electron flow than in higher plant plastids (Minagawa 2013; Rochaix 2014) and chlororespiration may induce a pH gradient even in the dark (e.g. in *Euglena*, Doege et al. 2000). Secondly, qE seems to be independent of the xanthophyll cycle in *Euglena* (Doege et al. 2000). Thirdly, Cyanobacteria, admittedly oxygenic photosynthetic bacteria and not algae, but nevertheless with similar photosynthetic mechanisms, and sharing a common origin, do not have the conventional xanthophyll cycle yet carry out down-regulation of photosynthesis, both by conventional carotenoids (Schreiber et al. 1995) and by the orange carotenoid protein.

7.2 pH Quenching

Many workers in the field have suggested a second mechanism by which eukaryotic algae and land plants can quench excitation energy. This is through the action of low pH on light-harvesting proteins in the vicinity of the reaction centres. However, much more work needs to be done before this is generally accepted (see, e.g. Allorent et al. 2013).

7.3 Orange Carotenoid Protein

The orange carotenoid protein was discovered relatively recently (Kirilovsky and Kerfeld 2012). It is, as its name suggests, a caroteno-protein, which is produced under high light in cyanobacteria; initially it was thought that it occurred in only certain cyanobacteria but it has been found to exist quite widely in cyanobacteria. The OCP is located on the stalk of the phycobilisome and under high light channels excitation energy into the carotenoid where the energy is released as heat. However, the OCP has a second role in quenching singlet oxygen (Sedoud et al. 2014).

When the light intensity is reduced to non-stressful levels another protein FRP decouples the OCP and excitation energy is once again passed on to the local reaction centre (Gwizdala et al. 2013).

8 Reactive Oxygen Species (ROS)

Reactive oxygen species are comprised of singlet oxygen ($^1O_2$), hydroxyl radical (OH*), hydrogen peroxide (H_2O_2) and a number of smaller constituents (Schmitt et al. 2014). All of these radicals have short half-lives and react with organic molecules to form oxidation products; singlet oxygen has a half-life of ~3.5 μs at normal temperatures. With lipids, lipid peroxides are formed which may be quite detrimental to normal membrane-based reactions. Thus the formation of ROS, which is unavoidable when electron transport processes are involved, is an activity, which is (a) curtailed as far as possible by special biochemical processes (see below) and (b) mitigated by repair processes. A prime example is in PSII where for reasons of proper functioning a carotenoid triplet valve cannot be put in place and D1 protein is actively degraded during photosynthesis and must be actively replaced (Larkum 2003).

A set of protective measures are set in place in eukaryotic alga systems to mitigate the formation of ROS. One of these is the alternate oxygenase (NOX). Another is the Mehler Ascorbate Peroxidase (MAP) pathway, whereby electrons are channeled out of the reducing side of PSI to oxygen, rather than feeding into NADP reduction.

These reactions have only attracted attention relatively recently and their true importance is only now being realized by an increasing number of sophisticated investigations.

9 Evolution of Photosynthetic Proteins Involved in Photoprotection and Light Harvesting

Balancing efficient capture of light against the damaging effects of high light is a problem faced by all photosynthetic organisms and results in the phenomenon of photoinhibition (Raven 2011). It is therefore clear why both oxygenic and anoxygenic photosynthetic organisms have evolved mechanism to deal with situations of low light and high light. As discussed at the beginning of this chapter, curiously there is no phylogenetic link between the light harvesting proteins of these two types of photosynthetic organisms. This is despite a clear phylogenetic link between the proteins of their Reaction Centres (Blankenship 2014). For the proteins involved in light harvesting and photoprotection in oxygenic photosynthetic organisms there are some clear links. Furthermore, it is likely that in Cyanobacteria, light-harvesting proteins involving Chl evolved before phycobiliproteins; however, these isiA proteins bear no relation to the light-harvesting proteins of eukaryotic algae and land plants, on the one hand, nor to the anoxygenic photosynthetic bacteria, on the other. Nevertheless there is a clear link back to Cyanobacteria both for the light harvesting proteins of eukaryotic algae (and land plants) and the photoprotective proteins (Fig. 9).

Some important recent advances have been made by the group of Adamska (Engelken et al. 2010; Sturm et al. 2013). It has long been known that Cyanobacteria have a group of proteins, which broadly fit into the category of High Light-Induced Proteins (HLIP). These have a single membrane spanning helix, which binds Chl *a* and probably acts as a means of supplying Chl under high light stress. These proteins are homologous with small Chlorophyll-Binding (CB) proteins (OHP1, OHP2); also with a single membrane spanning helix, which occur in plastids of eukaryotic algae and land plants (OHP1 occurs in green algae and land plants, OHP2 occurs in almost all algae and in land plants). Two-helix Stress Enhanced proteins (SEP) also occur in most eukaryotic algae and land plants and can be seen as gene duplications from HLIP/OHP. These two-helix proteins almost certainly gave rise by gene duplication to four-helix proteins, of which to PSBS and LHCSR are extant members, PSBS in green algae and LHCSR in eukaryotic algae and early land plants (liverworts and mosses). Early four helix proteins then likely evolved into the three-helix proteins, which include CAB/CAC proteins of many algae and land plants, the early light induced protein (ELIP) of green algae and land plants and a RedCAP (red lineage Chl a/b binding-like) protein of red algae, cryptophytes, haptophytes and heterokontophytes, including diatoms (Sturm et al. 2013). The evolutionary development of the RedCAP protein is not clear, and is probably lost in the events that led up to the formation of the primary plastids, over 1 Ga ago. During these events, glaucophytes did not inherit any of the new CAB/CAC proteins, red algae (but PSI only) and the red algal lineage inherited RedCAP proteins and green alga inherited CAB proteins (and associated, LHCSR, PSBS, ELIP and SEP proteins). During this time Chl *c* evolved and became the Chl that accompanied Chl *a* on the three-helix RedCAP (CAC) protein – not in red algae where only Chl *a* is bound, but in cryptophytes where Chl c_2 is bound, and in haptophytes and heterokontophytes, where Chl c_1 and c_2 are bound (Fig. 9). As pointed out by Sturm et al. (2013), this would have required considerable genetic readjustment in the lineages with secondary plastids. However, why Chl *c* was used and not Chl *b* is unclear (Green 2011).

In the green lineage, which includes Chlorophyceae, Euglenophyceae, Chlorarachniophyceae and land plants, CAB proteins with associated LHCSR, PSBS, ELIP and SEP proteins were inherited. However, during the evolution

Box 1: Glossary

Archaeplastida

A eukaryotic supergroup of organisms with primary plastids. Includes Chlorophyceae (including streptophytes) Rhodophyceae and Glaucophyceae.

Chromalveolates

A group of algae proposed by Cavalier Smith, which are in the lineage that inherited genes from red algae and have secondary (and tertiary) plastids. Depending on how it is defined it includes Cyptophyceae, Haptophyceae, Heterokontophyceae (including diatoms), Chromerids and apicomplexans. The group is largely consonant with the group Chromista used in this book (see AlgaeBase: http://www.algaebase.org/).

CAB/CAC proteins

As used here, this term refers specifically to the CAB/CAC superfamily that contain three transmembrane α-helices binding Chl *a* and Chl *b* or Chl *c*. Coded for by Lhc genes.

LHC superfamily

Proteins that contain a characteristic transmembrane domain called the LHC motif, but do not necessarily bind Chls.

LHCSR

Stress-induced LHC protein involved in flexible NPQ in algae, liverworts and mosses.

NPQ

Non-photochemical quenching of chlorophyll fluorescence. Used as a proxy for photoprotective thermal dissipation of excess light energy in photosynthesis.

OCP

Orange carotenoid protein involved in flexible NPQ in many phycobilisome-containing cyanobacteria.

PBS

Phycobilisome, the major soluble light-harvesting antenna in many cyanobacteria, red algae and glaucophytes. It is composed of water-soluble phycobiliproteins and is peripherally attached to thylakoids, where it transfers absorbed light energy to the reaction centers of PSII and PSI.

PSBS

A four-helix protein in the LHC superfamily that is involved in flexible NPQ in green algae and land plants. This protein does not appear to bind pigments.

Xanthophyll cycle

An interconversion of xanthophylls that involves one or two de-epoxidation reactions occurring in high light (with a reverse epoxidation reaction in limiting light). Three types of xanthophyll cycles are known: violaxanthin cycle, diadinoxanthin cycle, and lutein epoxide cycle.

of land plants LHCSR proteins were discarded in favor of PSBS in the evolution vascular plants (Niyogi and Truong 2013) (see Fig. 9).

10 Outlook

Currently it is apparent that photosynthesis and light-harvesting are continuing to undergo radical changes in our understanding. This is being brought about by new techniques such as molecular biology, electron diffraction, single molecule spectroscopy, fast fluorescence and new chromatographic techniques. As a result we can see that much of what we saw as unifying concepts in the past are opening up to detailed study, e.g. changes in absorption cross-section of PSI and PSII. Furthermore it is becoming clear that the rather crude understanding of the unity of primary plastids needs a radical overhaul and that this will pass valuable insights into our understanding of secondary (and tertiary) plastids. The discovery (or rediscovery) of Chl *d* and Chl *f* over the last 15 years has much to yield in our understanding of the role of Chl *a*, *b* and *c*, both in the modern and in the Archean world. And, most surprisingly of all, it appears that through the discovery of *Gloeobacter kilaueensis* (Saw et al. 2013) we may be able to glimpse some of the evolutionary events that gave rise to the mechanism by which water was split by a newly evolving PSII.

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Carbon Acquisition by Microalgae

John Beardall and John A. Raven

1 Introduction

Aquatic photoautotrophs contribute around 50 % of the approximately 111–117 Pg C assimilated into organic matter annually (Behrenfeld et al. 2001; Falkowski and Raven 2007). Of this, the majority of inorganic carbon is fixed by the microalgae that comprise the phytoplankton in the open ocean (mostly because there is a lot of ocean), though locally microalgal primary productivity on an areal basis can be high in a range of freshwater water bodies as well as in benthic populations and biofilms on submerged rocks or plants. Whereas terrestrial plants generally utilise CO_2 from the atmosphere, albeit after its solution in (extra)cellular water, aquatic plants are faced with acquiring inorganic carbon from a dense medium (water) in which the diffusion rate of CO_2 is much slower (by a factor of 10^4) than in air, and where much of the available dissolved inorganic carbon (DIC) is available (except in some inland waters) as the charged anion HCO_3^- rather than as CO_2. The latter is particularly exemplified in seawater where CO_2 concentrations at air equilibrium are in the order of 10–15 μM, but HCO_3^- is found at around 2 mM. The other component of DIC, carbonate ion, is only found in significant concentrations at high pH and is not used as a source of carbon in photosynthesis. CO_2 availability is frequently a major limiting factor in algal mass cultures (Beardall and Raven 2013a). With the burgeoning interest in growing microalgae for biotechnological purposes, the capacity for, and mechanisms behind, carbon acquisition in these organisms are currently of high priority.

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2 Rubisco Is the Central Feature of C Acquisition

All cyanobacteria and eukaryotic microalgae rely on the enzyme ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) and the Photosynthetic Carbon Reduction Cycle (PCRC; otherwise known as the Calvin Cycle) for net assimilation of inorganic carbon to organic matter. Rubisco is thus the core carboxylating enzyme and is involved in over 99 % of primary production on the planet (Raven 2009, Losh et al. 2013, Raven 2013b).

Rubisco catalyses the fixation of CO_2 to the acceptor molecule ribulose-1,5-bisphosphate (RuBP), giving rise to two molecules of 3-phosphoglycerate, as shown in Eq. (1).

$$\begin{aligned} &\mathrm{RuBP} + \mathrm{CO_2} \rightarrow \mathrm{6C\ carboxyketone\ intermediate} \\ &\rightarrow 2 \times \mathrm{3\text{-}phosphoglycerate} \end{aligned} \quad (1)$$

Rubisco also catalyses the oxygenation of RuBP (Eq. 2), giving rise to one molecule of phosphoglycerate and one of phosphoglycolate, instead of two molecules of phosphoglycerate.

$$\begin{aligned} &\mathrm{RuBP} + \mathrm{O_2} \rightarrow \mathrm{5C\ peroxyketone\ intermediate} \\ &\rightarrow \mathrm{3\text{-}phosphoglycerate} + \mathrm{2\text{-}phosphoglycolate} \end{aligned} \quad (2)$$

While one of the two carbons in phosphoglycolate can be recovered and converted to phosphoglycerate by the reactions of the photosynthetic carbon oxidation cycle (PCOC) in the process of photorespiration, one carbon is lost as CO_2 and represents a potentially significant inefficiency in the carbon assimilatory process. In some cases glycolate is excreted and this represents a major loss to the organisms of the C and energy involved in its biosynthesis (Raven et al. 2011; Raven and Beardall 2016), chapter "Dark Respiration and Organic Carbon Loss", in this volume).

The carboxylase (Eq. 1) and oxygenase (Eq. 2) activities of Rubisco are competitive and dependent on the ratio of oxygen and CO_2 at the active site of the enzyme, according

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to Eq. (3), where the selectivity factor S_{rel} defines the ratio of rates of carboxylase to oxygenase reactions.

$$S_{rel} = \frac{K_{1/2}(O_2)k_{cat}(CO_2)}{K_{1/2}(CO_2)k_{cat}(O_2)} \quad (3)$$

where $k_{cat}(CO_2)$ is the CO_2-saturated specific rate of carboxylase activity of Rubisco (mol CO_2 mol^{-1} active sites), $K_m(CO_2)$ the concentration of CO_2 at which the CO_2 fixation rate by Rubisco is half of $k_{cat}(CO_2)$, $k_{cat}(O_2)$ the O_2-saturated specific rate of oxygenase activity of Rubisco (mol O_2 mol^{-1} active sites) and $K_m(O_2)$ is the concentration of O_2 at which the O_2 fixation rate by Rubisco is half of $k_{cat}(O_2)$.

A number of different forms of Rubisco, with differing kinetic properties, have evolved in autotrophic organisms. These have been discussed in great detail elsewhere (Badger et al. 1998; Raven and Beardall 2003; Tcherkez et al. 2006; Whitney et al. 2011; Raven et al. 2011, 2012; Studer et al. 2014), so the following represents a general overview of what is known.

There are three known main forms of Rubisco, referred to as Forms I, II and III, plus a 4th group (Form IV) of Rubisco-like proteins which lack carboxylase activity and may instead function in S metabolism (Hanson and Tabita 2001). Most microalgae and cyanobacteria have forms of Rubisco with eight large and eight small sub-units (L_8S_8) and these fall into the category of Form I. Many marine *Synechococcus* and all *Prochlorococcus* species produce Form IA Rubisco – this was obtained by lateral gene transfer from an autotrophic proteobacterium (Raven et al. 2012). The majority of marine and freshwater cyanobacteria however have the ancestral Form IB Rubisco, which was transferred in primary endosymbiosis to glaucocystophyte and chlorophyte algae (and their evolutionary derivatives the embryophytes) and by secondary endosymbiosis to chlorarachniophyte and euglenophyte algae (Martin and Schnarrenberger 1997; Raven et al. 2012). Form 1D RUBISCO, obtained by lateral gene transfer from an autotrophic proteobacterium, is found in all red algae (displacing the Form IB Rubisco transferred in primary endosymbiosis), and by secondary endosymbiosis in those cryptophytes, haptophytes (e.g. cocolithophores) and heterokonts (= stramenoplies or ochristans, e.g. diatoms), that have been examined, and has the highest known selectivity for CO_2 over O_2 (Raven et al. 2012). On the other hand, the ancestral dinoflagellates, and *Chromera velia*,[1] both alveolates, obtained Form II Rubisco by lateral gene transfer from an autotrophic proteobacterium; Form II Rubisco comprises only two large subunits (L_2). The derived state in dinoflagellates is the replacement of their original peridinin-containing plastids by plastids from other eukaryotic algae in tertiary endosymbiosis (Raven et al. 2012). Form III Rubiscos also lack small subunits (but can have more complex structures based on the L_2 basal structure ($(L_2)_4$, $(L_2)_5$), but as these are found only in Archaea they will not be considered further.

These different forms of Rubisco have different kinetic properties. Form IA Rubiscos tend to higher S_{rel}, but lower $K_{0.5}(CO_2)$, values than do Form IB Rubiscos, though Scott et al. (2007) report a $K_{0.5}(CO_2)$ for Rubisco from the marine cyanobacterium *Prochlorococcus marinus* of 750 μM, which is the highest recorded value for a Form 1A Rubisco. Values reported for the Form 1D Rubiscos are variable, even for the same organism; for instance Boller et al. (2011) cite a $K_{0.5}(CO_2)$ of 72 μM, while Shiraiwa et al. (2004) report values for partially purified *E. huxleyi* Rubisco of 200 μM. However, given the $K_{0.5}(CO_2)$ values cited for Rubisco are many times higher than the whole cell $K_{0.5}(CO_2)$ in photosynthesis and that maximum rates of CO_2-saturated Rubisco activity in vitro are just sufficient to account for rates of light-saturated photosynthesis in vivo, then occurrence of a CCM in *E. huxleyi* would be essential, irrespective of which of these values is considered (Beardall and Raven 2013b). The Form II Rubiscos of dinoflagellates are poorly characterised because of their instability in vitro, but Form II Rubiscos from photosynthetic proteobacteria, from which it is believed the dinoflagellate Form II Rubiscos originated by lateral gene transfer (Badger et al. 1998), have very low S_{rel} values (Whitney and Andrews 1998; Leggat et al. 1999; see Table 1 of Raven and Beardall 2003). The general trend across all autotrophs is that a low $K_{½}(CO_2)$, and a high S_{rel} are correlated with a low $k_{cat}(CO_2)$, and vice versa (Tcherkez et al. 2006; Raven et al. 2012).

While there are six alternative metabolic pathways for autotrophic CO_2 assimilation in extant autotrophs (Raven et al. 2011, 2012), only the Photosynthetic Carbon Reduction Cycle (PCRC, =Calvin cycle) operates in cyanobacteria and the eukaryotic microalgae (though there is one possible example of C_3–C_4 intermediate C fixation in a single species of diatom – see below). The 3-phosphoglycerate formed by the carboxylase reaction of Rubisco (Eq. 1) is metabolised through the PCRC such that energy, as ATP, is expended to convert 3-phosphoglycerate to 1,3-bisphosphoglycerate which is then reduced, using NADPH, to glyceraldehyde-3-phosphate. For every three molecules of CO_2 assimilated via Rubisco, one molecule of glyceraldehyde-3-phosphate is produced for biosynthesis and three molecules of RuBP are regenerated, at the expense of further ATP expenditure, to allow the PCRC to continue. Overall, for every one CO_2 assimilated via the PCRC cycle at CO_2 saturation (assuming no oxygenase activity) the energy requirement is 2 ATP and 2 NADPH. However, oxygenase activity (Eq. 2) and photorespiration have the potential to increase the energy requirement for net CO_2 assimilation. This is further exacerbated by

[1]Wherever possible the currently accepted names for species are used. The name used in the paper cited is also indicated. For details of names see chapter "Systematics, Taxonomy and Species Names: Do They Matter?" of this book (Borowitzka 2016).

the energetic costs of CO_2 concentrating mechanisms (see below) employed by algae and cyanobacteria to enhance CO_2 supply to Rubisco and inhibit oxygenase activity (see Raven et al. 2000, 2012, 2013, 2014) for a full discussion of the energetics of photosynthesis in algae).

As pointed out by Raven and Beardall (2016) in their chapter on respiration in this volume, respiratory pathways in cyanobacteria and microalgae can be present in the same compartment as those of the PCRC. Thus in green algae for instance, activity of the oxidative pentose phosphate pathway means that different regulatory mechanisms need to be employed on key enzymes such as phosphoribulokinase, glyceraldehyde-3-phosphate dehydrogenase, fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase in order to avoid the operation of a futile cycle, expending ATP without net C fixation (Maberly et al. 2010). Thus these enzymes in plastids are activated in the light while the key enzyme of the oxidative pentose phosphate pathway, glucose 6-P dehydrogenase, is activated in the dark and inhibited by light (Maberly et al. 2010). Although the oxidative pentose phosphate pathway in marine diatoms appears to be incomplete (Kroth et al. 2008), there is still a need for independent regulation of plastid enzymes such as glyceraldehyde-3-phosphate dehydrogenase that can operate either, in opposing directions, in glycolysis or in the PCRC. Patterns of regulation of key enzymes in respiration and the PCRC, phosphoribulokinase and glyceraldehyde-3-phosphate dehydrogenase, showed a phylogenetic pattern, with three distinct clades, related to evolutionary origins of the 16 algae studied (Maberly et al. 2010; see also Robbens et al. 2007).

3 The Characteristics of Rubisco Necessitate Activity of CO_2 Concentrating Mechanisms

It is apparent from the above discussion that cyanobacteria and algae with different forms of Rubisco will perform differently at a given set of CO_2 and O_2 concentrations at the active site. Thus at present-day dissolved CO_2 levels of ~15 μmol^{-1} (the exact concentration depending on salinity and temperature), organisms with low affinity for CO_2 (high $K_{0.5}(CO_2)$) will have Rubiscos operating well below maximum capacity if internal CO_2 is in equilibrium (or lower) than external CO_2. Similarly, although organisms with Form IB Rubisco have a high selectivity, favouring carboxylation over oxygenase activity, species such as dinoflagellates with their low S_{rel} Form II Rubisco might struggle to perform net C assimilation under air equilibrium, as photorespiratory losses of C would be significant, or even exceed C gain via the carboxylation reaction. However, these "deficiencies" in Rubisco are generally countered by the operation of CO_2 concentrating mechanisms (CCMs), which act to increase the CO_2 concentration and hence the CO_2:O_2 ratio at the active site of Rubisco. This enhances the carboxylase activity of Rubisco and to a large extent suppresses photorespiration. The accumulation of CO_2 necessitates energy input, which is offset, at least in part, by the energy saved by the greatly decreased rate of photorespiration (Raven et al. 2012, 2014). There is a wide range of mechanisms that bring about this enhancement of CO_2 levels at the Rubisco active site and these have been the subject of detailed reviews recently (Giordano et al. 2005; Price et al. 2008; Reinfelder et al. 2011; Hopkinson et al. 2011; Raven et al. 2008, 2012, 2014). Intracellular accumulation of CO_2 also has implications for leakage of CO_2 out of cells. The latter has been recently considered by Raven and Beardall (2015).

3.1 CCMs Based on Active Transport

Microalgae and cyanobacteria possess biophysical CCMs based on active transport of inorganic carbon as HCO_3^- or CO_2 into cells. CCMs are found in almost all cyanobacteria and microalgae, the only major exceptions being the Chrysophyceae and Synurophyceae (Maberly et al. 2009; Raven et al. 2011) together with the lichen symbiont *Coccomyxa* (Palmqvist 2000, but perhaps not [based on genomic studies rather than the physiological methods used on the strain in Palmqvist 2000], in the Antarctic species *Coccomyxa subellipsoidea*: Blanc et al. 2012) and *Stichococcus minor* (Munoz and Merrett 1989). Earlier reports of a lack of CCM activity in the coccolithophore *Emiliania huxleyi* appear unfounded (Rost et al. 2007; Reinfelder 2011; Stojkovic et al. 2013).

There are five variants of inorganic carbon transport in cyanobacteria (Fig. 1a). The flux of CO_2 across the plasma membrane of cyanobacteria is, if present, by diffusion, but on either the cytosolic face of that membrane or on the thylakoid membrane there is an energized conversion of CO_2 to HCO_3^- via a NAD(P)H dehydrogenase. This then effectively acts like a Ci-pump, even though direct active transport of CO_2 does not occur. It is also possible that some CO_2 uptake may effected by aquaporins. There are two NAD(P)H dehydrogenase systems involved: an inducible, high-CO_2 affinity, system (NDH-I_3) at the thylakoid membrane and a constitutive, low affinity, one (NDH-I_4, located probably at the plasma membrane) (Price et al. 2008). In addition, cyanobacteria can actively take up HCO_3^- from the medium. A number of different HCO_3^- pumps have been identified: the genes for one low-CO_2 inducible, high affinity HCO_3^- transporter, BCT1, are encoded by the *cmpABCD* operon and belonging to the traffic ATPase family (Omata et al. 1999). This transported is found only in freshwater β-cyanobacteria (containing Form 1B Rubisco) and the genes are absent from genomes of all marine α- and β-cyanobacteria sequenced so

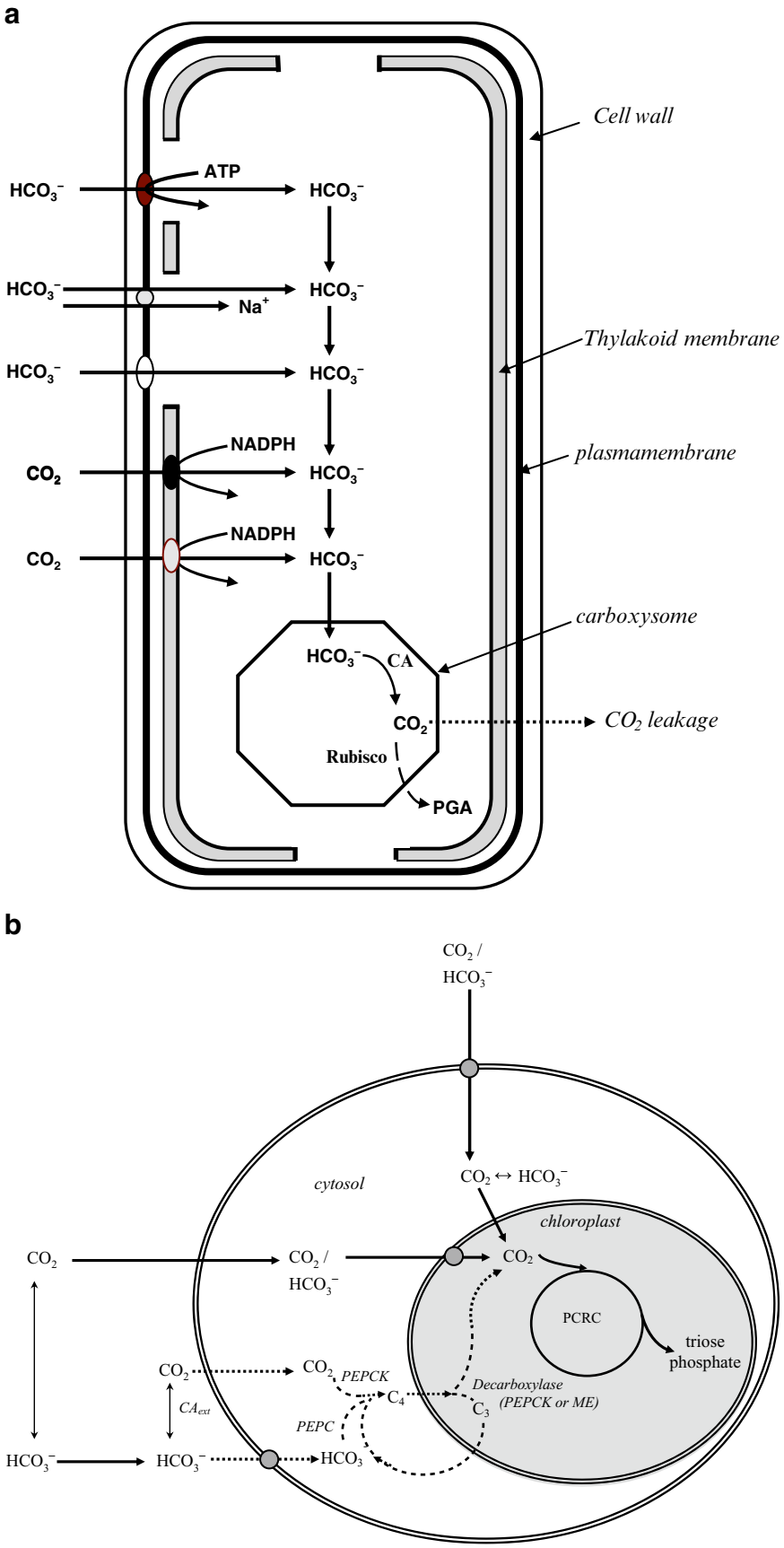

Fig. 1 A schematic model for CCMs in (**a**) cyanobacteria and (**b**) eukaryotic microalgae. Details are given in the text. In (**a**) the model incorporates low affinity transport systems (*grey*) and high affinity systems (*black*) at the plasmalemma and/or thylakoid membrane. Transporters whose characteristics are unknown are shown in *white*. Model (**b**) incorporates the possibilities for DIC transport at the

far. A high affinity, inducible, Na^+-dependent, HCO_3^- transporter (SbtA) is present in *Synechocystis* 6803 and various other β-cyanobacteria (Shibata et al. 2002). BicA is also a Na^+-dependent HCO_3^- transported aligned with the SulP family of transporters (Price et al. 2004). Unlike SbtA though, this is a low affinity system. BicA and SbtA may both be forms of Na^+/HCO_3^- symporters, but there is no conclusive evidence for this to date. Irrespective of the form of inorganic carbon transported, the HCO_3^- thus delivered directly or indirectly to the cytosol diffuses into the carboxysomes, which show the only carbonic anhydrase (CA) activity in the cytosol. The CO_2 generated within the carboxysomes by this CA leads to the build up to a higher steady state concentration than in the bulk medium, thus strongly favouring the carboxylase over the oxygenase activity of Rubisco (Smith and Ferry 2000; Price et al. 2002).

Inorganic carbon transport systems in the eukaryotic microalgae (Fig. 1b) are best characterised in the chlorophytes, though some studies have been carried out on other groups and especially on diatoms and coccolithophorids. Active transport mechanisms for inorganic carbon (as CO_2 or as HCO_3^-) could be based at the plasma membrane, at the inner plastid envelope membrane, or both (Fig. 1b). Equilibration between CO_2 and HCO_3^- in the various compartments (periplasmic space, chloroplast stroma, thylakoid lumen) of eukaryotic algal cells also involves a range of CAs. The evidence for a role of pyrenoids analogous to that of carboxysomes in the cyanobacteria is weak; though all pyrenoid-containing algae have CCMs, not all algae shown to be capable of expressing CCMs have pyrenoids.

Many algae have the capacity to use external HCO_3^- as the source of inorganic carbon acquired to support photosynthesis and this has been demonstrated by techniques such as mass spectrometry and isotope disequilibrium. In some cases active CO_2 transport has also been shown (Sültemeyer et al. 1991). Frequently HCO_3^- use is indirect, involving an external carbonic anhydrase (CA_{ext}) which converts HCO_3^- to CO_2 in the periplasmic space, thereby enhancing the potential for CO_2 uptake across the plasmalemma. In *Chlamydomonas reinhardtii*, in which both CO_2 and HCO_3^- are actively transported across the plasmalemma (although the former is the preferred species), there are two isozymes of CA_{ext} namely pCA1 (the gene product of *Cah1*) and pCA2 (the gene product of *Cah2*). The former is highly expressed and inducible by limiting CO_2 while the latter is of unknown function and is repressed under low CO_2 conditions and only weakly expressed under high CO_2. The activity of pCA1 is not though absolutely essential for growth in low CO_2 or for operation of the CCM (Spalding et al. 2002). Although a DIDS-sensitive anion exchange HCO_3^- transport system has been shown in the chlorophyte microalga, *Dunaliella tertiolecta*, this is much less significant than the CA_{ext} system as a means of HCO_3^- use (Young et al. 2001).

In chlorophyte algae there is good evidence for a role of the plastid envelope in CCMs based on active transport of DIC. Photosynthetically active chloroplasts from high- and low-CO_2 grown chlorophyte cells have been shown to possess low- and high-affinity DIC uptake systems respectively, as do the corresponding intact cells (Amoroso et al. 1998). Active uptake of both CO_2 and HCO_3^- , and CO_2 accumulation, have been demonstrated in isolated chloroplasts of *Chlamydomonas reinhardtii* and *Dunaliella tertiolecta* (Amoroso et al. 1998) and in *Tetraëdron minimum* and *Chlamydomonas noctigama* (van Hunnik et al. 2002). Active uptake by the plastid does not exclude additional active transport steps at the plasmalemma and there are some species where CCM activity appears to be based solely at the plasmalemma (Rotatore and Colman 1990, 1991).

There is also the possibility of a CCM based on acidification in the thylakoid. Here a α-CA (Cah3) based on the inner side of the thylakoid membrane in invoked in conversion of HCO_3^- to CO_2 following passive uniport of HCO_3^- to the thylakoid lumen. The CO_2 thus produced would leak out of the lumen to the site of Rubisco in the stroma or pyrenoid. Although compartmentation of various CA forms in *Chlamydomonas* is consistent with this model, direct evidence for operation of such a mechanism has yet to be obtained. An 'inside-out' variant on this kind of CCM involves localised acidification of the cell surface as a means of locally increasing the equilibrium $[CO_2]:[HCO_3^-]$ ratio, with equilibration of the inorganic carbon catalysed by the low pH and by external carbonic anhydrase(s), and subsequent diffusive (or active; see below) uptake of CO_2 (Raven and Hurd 2012; Raven 2013a; Raven et al. 2014). Such mechanisms are probably less effective in microalgae than in macroalgae and in aquatic embryophytic plants, since the acid (and the required alkaline) patches are smaller, so the lateral area over which leakage down the pH gradient occurs is greater relative to the surface area over which proton efflux (and, in the alkaline zones, proton influx) takes place (Raven and Hurd 2012; Raven 2013a; Raven et al. 2014). Smaller organisms are also prone to more proton leakage through the

Fig. 1 (continued) plasmalemma and/or chloroplast envelope of microalgae, as well as a putative C_4-like mechanism (*dashed lines*). CO_2 will cross membranes by diffusion, whereas active transport (*shaded circles*) can be of CO_2 or HCO_3^-. No attempt has been made to show the roles of the various internal CAs in the different compartments (Redrawn after (**a**) Price et al. (2002) as modified by Giordano et al (2005), and (**b**) Sültemeyer (1998) and Raven and Beardall (2003), as modified by Giordano et al. (2005))

thinner diffusive boundary layer that is a consequence of smaller size (Raven and Hurd 2012; Flynn et al. 2012; Raven 2013a).

For a number of years it was suggested that the process of calcification in organisms such as the coccolithophorid *Emiliania huxleyi* produced CO_2, which could act as a form of CCM by enhancing CO_2 supply to Rubisco (see Reinfelder 2011). While it is clear that HCO_3^- use in calcification will generate CO_2 within *E. huxleyi* cells, recent work has shown that coupling of calcification-derived CO_2 to photosynthesis is not obligatory and cannot drive the observed CCM activity (Raven and Crawfurd 2012; Bach et al. 2013; Beardall and Raven 2013b; Stojkovic et al. 2013).

3.2 CCMs Based on C_4 Metabolism

In some (C_4) higher plants photosynthetic carbon assimilation is based on an initial assimilation of HCO_3^- by the enzyme PEP carboxylase, which has a high affinity for its inorganic carbon substrate. The initial stable resulting products are the C_4 dicarboxylic acids malate or aspartate (depending on species) and these compounds are then transported from mesophyll cells to another type of cell, the bundle sheath cells, where decarboxylation leads to enhanced supply of CO_2 at the active site of Rubisco in these cells, i.e. this acts as a biochemical CO_2 pump improving CO_2 supply. This contrasts with the purely diffusing flux of CO_2 in the majority of (C_3) higher plants. Beardall and co-workers in the late 1970s first proposed a C_4-like photosynthetic metabolism in diatoms and to a lesser extent in other microalgae (Beardall et al. 1976), though their later work (Morris et al. 1978) ascribed their labelling patterns and other data to high rates of anaplerotic C fixation through PEPCase to top up the intermediates of the TCA cycle as these were used to supply biosynthetic processes such as protein synthesis. Subsequently, Reinfelder et al. (2000) and Morel et al. (2002) revisited the topic and proposed operation of single cell C_4 photosynthesis in the marine diatom *Thalassiosira weissflogii* (Fig. 1b). While the original paper of Reinfelder (2000) engendered a great deal of discussion (Johnston et al. 2001), subsequent work (Morel et al. 2002; Reinfelder et al. 2004; Roberts et al. 2007a, b) has provided better evidence for some form of C_4 (or more likely C_3–C_4 intermediate) mechanism in *Thalassiosira weissflogii*, though proposals for a similar mechanism in other diatoms (*T. pseudonana* and *Phaeodactylum tricornutum*) have not been substantiated (Haimovich-Dayan et al. 2013; Clement et al. 2015). Likewise, suggestions that high levels of enzymes involved in C_4 photosynthesis in the haptophyte *Emiliania huxleyi* were shown by pulse chase labeling experiments to be associated with anaplerotic β-carboxylation (Tsuji et al. 2009). Thus *T. weissflogii* remains the only microalga for which C_4 photosynthesis is a possibility, though it is more likely better aligned with C_3–C_4 intermediates (Roberts et al. 2007a).

The expression of CCMs is decreased as the concentration of CO_2 for growth is increased above the current value, to an extent that depends on the kinetic characteristics of the Rubisco used by the organism (Giordano et al. 2005; Raven et al. 2011, 2012). The extreme cases are those of cyanobacteria with Form IA or Form IB Rubisco with low CO_2 affinities and low CO_2:O_2 selectivity factors, and of those dinoflagellates with Form II Rubiscos (Raven et al. 2011, 2012; Raven and Beardall 2014). It is important to remember that down-regulation of CCM expression is often determined as a decreased affinity for external inorganic carbon in photosynthesis and growth, rather than the more rigorous measurement of the CO_2 accumulation ratio, and also that diffusive CO_2 entry cannot occur in parallel with the CCM until the external CO_2 concentration exceeds the internal CO_2 produced by the CCM (Raven et al. 2011, 2012; Raven and Beardall 2014).

CCM expression is also influenced by incident PAR (increased expression at higher PAR) and by the availability of the nutrients nitrogen, phosphorus and iron (with a general increase in expression with decreased concentration of nutrients) (Raven et al. 2011, 2012; Raven and Beardall 2014). We have previously argued that CCMs in organisms with Rubiscos with relatively high affinity for CO_2 and relatively high selectivity for CO_2 relative to O_2 could be retained in geological periods with high atmospheric CO_2 and warmer surface ocean; for instance in the case of phytoplankton where shoaling of the thermocline and shallower upper mixed layer lead to higher mean PAR and lower nutrient concentrations as a result of a smaller nutrient flux across the larger temperature difference across the thermocline (Raven et al. 2011, 2012). However, such effects could only last for a few thousand years. This is because the downwelling of warmer surface waters in the deep ocean thermohaline circulation eventually warms the deep ocean (Tyrrell 2013), approximately restoring the depth of the upper mixed layer and the previous mean PAR and nutrient concentrations, assuming other factors remain constant. A final aspect of the effect of the environmental temperature on CCMs in microalgae relates to the genetically determined (adaptive) effects of temperature. On land, the C_4 CCM in flowering plants follows, among other factors, temperature and PAR: C_4 plants are generally restricted to warmer, high-irradiance environments. However, CCMs in algae also occur in cooler and lower-irradiance habitats as well as in warmer, higher-irradiance habitats (Raven 2013b; Raven and Beardall 2014). Further investigation is needed to determine the basis for this difference between terrestrial C_4 plants and algae.

4 Dark Inorganic Carbon Fixation

Some aspects of dark inorganic carbon fixation have been dealt with in the respiration chapter in this volume (Raven and Beardall 2016). Although conventionally termed a 'dark' fixation process, it occurs more rapidly in the light than in the dark in most photolithotrophically growing cells since protein synthesis is almost always more rapid in the light and than in the dark phases of a diel cycle (Raven 1974, 1976a, b, c, 2013d). The essentiality of dark inorganic carbon fixation is that during periods of growth, intermediates are 'bled off' the TCAC to satisfy the needs of biosyntheses, especially of amino acids. This removal of intermediates means that continued energy transformation in respiration and TCAC operation requires replenishment of intermediates of the cycle and this is achieved through the activity of β-carboxylase reactions, catalysed by phosphoenolpyruvate carboxylase or pyruvate carboxylase. High levels of these enzymes have in the past been misinterpreted as evidence for C_4 photosynthesis (Beardall et al. 1976; Tsuji et al. 2009, 2012). Estimation of the rate of in vivo dark inorganic carbon fixation despite the much larger respiratory CO_2 production requires the use of tracers, and there is the likelihood of dilution of supplied ^{13}C- or ^{14}C-inorganic carbon by the internally produced $^{12}CO_2$, with consequent under-estimation of dark inorganic carbon fixation rate. Despite this, the measured rate of dark inorganic carbon fixation agrees very well with what is required to supply carbon skeletons for amino acid production to support the measured rate of protein synthesis (Granum and Myklestad 1999 and references therein). Another major process which requires dark inorganic carbon fixation is lipid synthesis. However, unlike the case of amino acid synthesis, the fixed inorganic carbon is not retained by the algal cell. This is because the inorganic carbon (as HCO_3^-) fixed by acetyl CoA carboxylase to produce malonyl CoA is released as CO_2 when the malonyl CoA adds its acetyl moiety to a growing fatty acid chain (Raven 1974; Raven and Farquhar 1990). These anaplerotic C fixation reactions and formation of downstream products of C fixation such as proteins and lipids are dealt with in more detail in the chapter on respiration by Raven and Beardall (2016) and elsewhere in this volume.

5 Algal Growth Using Organic Carbon Sources

Many photosynthetically competent algae can take up organic carbon and assimilate it into compounds used in growth, often with stimulation of the growth rate (mixotrophy, either involving organic carbon acquisition by osmotrophy or phagotrophy). Fewer such algae can grow in the dark using organic carbon as energy and carbon source, i.e. chemoorganotrophically. There are two extremes of the use of exogenous organic carbon. One involves photosynthetically competent algae which cannot use exogenous organic carbon to stimulate light- and inorganic carbon-saturated growth, even if the organic carbon can be taken up by the cells (obligate photolithotrophy). The other extreme involves organisms which are clearly derived from photosynthetically competent ancestors but which lack photosynthetic capacity and can only grow using exogenous organic carbon as the energy and carbon source (obligate chemoorganotrophs). The terminology related to these combinations of energy and carbon sources are defined in the Glossary.

Some cyanobacteria are obligate photolithotrophs; others are mixotrophic (osmochemoorganotrophy combined with photolithotrophy), while fewer are capable of osmochemoorganotrophy in the dark (Droop 1974; Raven 1976a, b, c; Zhang and Bryant 2011). *Gloeobacter violaceous*, the basal organism in the molecular phylogeny of extant cyanobacteria (Blank and Sanchez-Baracaldo 2010), cannot grow on the organic substrates most commonly able to support osmomixotrophy or osmochemoorganotrophy in cyanobacteria, so this organism is probably an obligate photolithotroph (Rippka et al. 1974). This is consistent with obligate photolithotrophy being ancestral in the cyanobacteria, although more data are needed. The reasons for obligate photolithotrophy in cyanobacteria are not clear; it is not universally a result of the absence of organic solute transporters in the plasmalemma (Zhang et al. 1998) or the result of an incomplete tricarboxylic acid cycle (Zhang and Bryant 2011).

As with cyanobacteria, some eukaryotic microalgae are obligate photolithotrophs, and the basis for this obligate photolithotrophy is unclear (Droop 1974; Raven 1976a, b, c). However, both osmotrophy and phagotrophy occur in some eukaryotic microalgae, giving mixoosmoorganotrophy and mixophagoosmotrophy, as well as osmoorganotrophy and phagoorganotrophy (Droop 1974; Raven 1976a, b, c; Raven et al. 2009; Flynn et al. 2013; Mitra et al. 2014). Since the origin of genetically integrated oxygenic photosynthesis in eukaryotic algae lies in endosymbiosis which apparently originated as arrested phagotrophy, it could be argued that phagotrophy is an ancestral trait in algae so that they were mixophagoorganotrophs or, in the dark, phagoorganotrophs. However, it is of interest that the algae arising by primary endosymbiosis (the Archaeoplastida, comprising glaucocystophytes, rhodophytes and chlorophytes and streptophytes) have few members, all in the Prasinophyceae, capable of phagotrophy (Raven et al. 2009; Flynn et al. 2013; Maruyama and Kim 2013; Raven 2013c; Schmidt et al. 2013; Mitra et al. 2014). Phagotrophy is much more common among the microalgae which arose by secondary (and tertiary) endosymbiosis (Raven et al. 2009; Flynn et al. 2013; Schmidt et al. 2013; Mitra et al. 2014).

Phagotrophy in algae not only gives an alternative (to photosynthesis) as a source of carbon and energy, but also an alternative (to osmotrophy) source of combined nitrogen, phosphorus, iron and (for vitamin auxotrophs) vitamins (Raven et al. 2009; Flynn et al. 2013; Schmidt et al. 2013). For mixophagoorganotrophs, photosynthesis could be regarded as a way of making more effective use of the nitrogen, phosphorus and iron than is the case for phagochemoorganotrophy without photosynthesis. With a similar C:N:P:Fe in the prey as in a phagochemoorganotroph, the necessary respiration used in producing more biomass from the prey means an excess of nitrogen, phosphorus and iron. Photosynthesis in mixophagochemoorganotrophs could partly, completely, or more than, compensate for the loss of carbon in respiration, and limit the excretion of nitrogen, phosphorus and iron (Raven et al. 2009; Flynn et al. 2013). These considerations could also apply to kleptoplastidic algae which continue phagotrophy (Raven et al. 2009; Flynn et al. 2013).

Mixoosmochemoorganotrophy and osmochemoorganotrophy have been relatively less well explored recently than their phagotrophic counterparts, but is it clear that mixoosmochemoorganotrophy is more common than is osmochemoorganotrophy (Droop et al. 1974; Raven 1976a, b, c). In the diatom *Phaeodactylum tricornutum* the expression of a human or *Chlorella* glucose symporter in the plasmalemma permits dark osmochemoorganotrophic growth of the alga (Apt et al. 2011). However, this cannot be taken as showing that all cases of microalgal obligate photolithotrophy is a result of the lack of a transporter for an organic substrate that could otherwise support osmochemoorganotrophy. Recalling the role of phagotrophy in supplying photosynthetic algae with resources other than organic carbon (nitrogen, phosphorus, iron, vitamins), uptake of organic carbon by microalgae often seems to be related to the acquisition of organic forms of nitrogen (Schmidt et al. 2013). The extent to which osmochemoorganotrophic uptake of organic carbon contributes to microalgal growth in the natural environment is constrained by the concentration of available organic carbon compounds in the environment, and also by the efflux of dissolved organic carbon across the algal plasmalemma (see Respiration Chapter, Raven and Beardall 2016).

6 Conclusions and Prospects

The processes of inorganic carbon fixation in cyanobacteria and eukaryotic microalgae are essentially as found in C_3 plants and based on assimilation via the Photosynthetic Carbon Reduction Cycle (PCRC) elucidated by Calvin, Benson and Bassham, though some details of its regulation may differ. However, due to the characteristics of cyanobacteria and microalgal Rubiscos, efficient net carbon assimilation under present-day CO_2 levels requires operation of a CO_2 concentrating mechanism (CCM). These CCMs take a variety of forms, based on biophysical active transport processes leading to accumulation of CO_2 at the active site of Rubisco. There is only very limited evidence for a biochemical CCM (analogous to intermediate C_3–C_4 photosynthesis in higher plants) in one marine diatom species. CCMs have a significant energetic cost and are regulated *inter alia* by light availability as well as CO_2 concentration. Further work on modulation of CCMs by environmental factors is required, especially in regard to long-term adaptation under global change scenarios. It would also be useful to have information on CCMs of more species of cyanobacteria and eukaryotic algae, as most of the work to date has been carried out on only a handful of species.

Some eukaryotic microalgae have a capacity to obtain organic carbon by phagotrophy. Others can utilise external organic carbon sources through mixoosmochemoorganotrophy or osmochemoorganotrophy, though the former is more common. Clearly more work is needed in this area to determine the constraints to utilization of organic matter by algae.

Glossary (Modified from Raven et al. 2013; See Also Flynn et al. 2013)

Autotroph An organism which uses exergonic inorganic chemical reactions, or photons, as the energy source for growth and inorganic chemicals taken up on a molecule by molecule basis across the plasmalemma to supply nutrient elements

Auxotroph An organism whose growth requires external provision of specific organic molecules, e.g. vitamins.

Chemolithotroph An organism which uses the catalysis of exergonic inorganic chemical reactions as the energy source for growth and inorganic chemicals taken up on a molecule by molecule basis across the plasmalemma to supply nutrient elements

Chemoorganotroph An organism which uses the catabolism of organic compounds as their energy source for growth and organic compounds as the source of carbon and, in many cases, the source of nitrogen.

Genetically Integrated Used of endosymbionts: an endosymbiont which has transferred some genes needed for independent growth to the host nuclear genome, with loss of these genes from the endosymbiont genome. Vertically transmitted.

Heterotroph = Chemoorganotroph

Kleptoplasty Retention by a phagochemoorganotroph of plastids obtained from photosynthetic eukaryotic food items in a photosynthetically competent state for various lengths of time. The limitation on the time for which kleptoplastids are functional is presumably a result of damage

to plastid protein that are encoded in the. Photosynthetic competence can be prolonged (in the ciliate *Myrionecta*) by retention of nuclei from the photosynthetic food item, thus allowing replacement of damaged examples of the majority of those many plastid proteins that are encoded in the nucleus. Not vertically transmitted.

Mixotroph An organism which combines autotrophic and chemoorganotrophic sources of energy and carbon for growth; usually applied to phototrophic autotrophs.

Obligate Chemoorganotroph An organism which can only grow osmochemoorganotrophically and/or phagomixotrophically

Obligate Photolithotroph A photolithotroph whose growth is not increased by the presence or external organic matter, even if organic solutes can be taken up on a molecule-by-molecule basis.

Osmochemoorganotroph A chemoorganotrophic organism taking up organic and inorganic nutrients on a molecule-by-molecule basis across the plasmalemma as the means of acquiring carbon and other elements.

Osmotrophy Uptake of molecules by cells on a molecule-by-molecule basis.

Phagotrophy Uptake of particles by cells.

Phagochemoorganotroph A chemoorganotrophic organism taking up particles of live or dead organic matter as the means of acquiring carbon and other elements.

Photolithotroph An organism which uses photons as the energy source for growth and inorganic chemicals taken up on a molecule by molecule basis across the plasmalemma to supply nutrient elements

Saprochemoorganotroph = Osmochemoorganotroph

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Fundamentals and Recent Advances in Hydrogen Production and Nitrogen Fixation in Cyanobacteria

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1 Introduction

Oil, natural gas and coal are not only our primary energy sources but also serve as the feedstock of several derived synthetic materials such as diesel, plastics and pharmaceuticals. Rapid consumption of these fuels has not only led to their near exhaustion but has also been attributed as one of the primary drivers of global warming. Recent research findings have shown that microorganisms may hold a solution to the twin crises of global warming and rapidly declining rate of fossil fuels. Photosynthetic microorganisms have the potential to mitigate carbon dioxide at the source and produce high-end biofuels, including zero carbon fuels such as hydrogen and high-carbon fuels such as biodiesel. Cyanobacteria in particular have gained a lot of attention in recent years because of their potential applications in the sector of bioenergy (Mata et al. 2010). Two reasons have made them especially powerful: they have minimal nutrient requirements and have the potential to be genetically modified. This is in sharp contrast to bacteria that require carbohydrates as feedstock, which significantly adds to the budget of an entire project. On the other hand, cyanobacteria are known to harvest light more efficiently than terrestrial plants. In fact, cyanobacteria are responsible for 20–30 % of Earth's photosynthetic productivity and are known to convert solar energy into biomass-stored chemical energy at the rate of ~450 TW (Pisciotta et al. 2010). Additionally, bulk cultivation of cyanobacteria for commercial application is much more feasible as compared to plants as they have a faster growth rate and can be grown on non-arable, non-productive land. Cultivation of cyanobacteria for biotechnological applications thus poses no threat to food crops, in contrast to the cultivation of plants for the same end. Furthermore, cyanobacteria are also capable of utilizing a wide variety of water sources including wastewater (Tamagnini et al. 2007) to produce both biofuels and valuable co-products (Parmar et al. 2011). In view of this, research groups are now considering growing marine cyanobacteria in bags on the surface of oceans to generate biomass for extraction of cyanobacterial fuel and value-added co-products.

The current use of photosynthesis to produce biofuels follows a biphasic, indirect process. In the first step, biomass is generated using the energy provided by the sun, which in a second step is converted into biofuels. The presence of cellulose in the biomass is a major bottleneck in the fermentation process and requires energy-intensive pretreatment steps using heat or chemical reagents. Further downstream processing of biofuels such as ethanol requires yet another energy-intensive step of distillation after fermentation (Tanksale et al. 2010). Thus, in such cases yields are limited by the amount of biomass that can be fermented. However, the problem can be circumvented if cyanobacterial hydrogen is considered as the final goal, as its production does not rely on biomass digestion but instead employs a direct approach. Therefore, considering the different array of biofuels, biohydrogen production using photosynthetic organisms appears promising.

The journey of hydrogen production from cyanobacteria to be used as a fuel has come a long way since Benemann introduced the concept in the 1970s (Benemann and Weare 1974). Today the availability of techniques such as large scale sequencing, global transcriptomics and in depth proteomic analysis promises to take this research to greater heights. In fact, with the advent of synthetic biology the possibility to create novel pathways has already rekindled new hopes (Heidorn et al. 2011). In this chapter, the fundamentals and recent advances made in the field of cyanobacterial hydrogen production will be analyzed in detail.

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2 Hydrogen-Producing Cyanobacteria

Several species and strains of cyanobacteria have shown the ability to produce hydrogen. To date, hydrogen production has been observed in 14 cyanobacterial genera, under diverse culture conditions (Lopes et al. 2002). These cyanobacteria include unicellular and filamentous freshwater or marine organisms. Work on hydrogen production from cyanobacteria was initiated in the 1970s during the first oil crisis. At that time, *Anabaena cylindrica* B-629[1] was considered a suitable organism for hydrogen production (Benemann and Weare 1974; Weissman and Benemann 1977; Daday et al. 1977, 1979; Lambert and Smith 1977; Jeffries et al. 1978; Lambert et al. 1979). Hydrogen production from the organism was studied based on different physico-chemical parameters. Following the success with *A. cylindrica*, several other strains from the genus *Anabaena*, including *A. cylindrica* IAM M-1, *A. variabilis* IAM M-4, *Anabaena* PCC 7120, *A. flos-aquae* UTEX amongst others, became popular models for studying hydrogen production from cyanobacteria (Masukawa et al. 2001). Additionally, various research groups isolated cyanobacteria from different locations and hence started the search for hydrogen production from a diverse group of cyanobacteria. The different genera include *Oscillatoria*, *Calothrix*, *Nostoc*, *Synechococcus*, *Synechocystis*, *Gloeobacter*, *Microcystis*, and *Cyanothece*. A complete description of all species and their taxonomic details is beyond the scope of this review; therefore, the authors of this chapter have selected model organisms to showcase hydrogen producing systems.

3 Physiology of Hydrogen Production from Cyanobacteria

The complexity of the molecular machinery that has evolved to produce hydrogen defies the deceptive simplicity of the reaction that it catalyzes (Eq. 1).

$$2\,H^+ + 2e^- \rightarrow H_2 \quad (1)$$

The protons and electrons represented in the above equation are not "free", as it is implied. They are derived from various sources depending upon the organism and the metabolic pathway involved.

Photosynthetic cyanobacteria share similarities to organisms from the eukaryotic world, such as green algae and plants: they possess two photosystems, photosystem I (PSI) and photosystem II (PSII), similar to the eukaryotes (Björn and Govindjee 2008). The presence of PSII enables the organism to evolve oxygen by splitting of water molecules using light energy. Oxygen serves as the terminal electron acceptor in the respiratory chain. Only under anaerobiosis do the organisms favour hydrogen production. Since production of hydrogen implies loss of reductants to the cell, the organism carries out this activity only under the conditions of anaerobic stress. With the exception of the NAD-reducing hydrogenase of *Ralstonia eutropha* (now referred to as *Cupriavidus metallidurans*) that can tolerate microaerobic conditions (Schneider and Schlegel 1976; Burgdorf et al. 2005), nature crafted most of the enzymes involved in the hydrogen production process as oxygen-labile. This ensured limited or no functionality of the hydrogen-producing enzymes during aerobic conditions, thus ensuring conservation of energy.

In fact, most of the [NiFe] hydrogenases inactivated by oxygen may take hours to recover. Cyanobacterial hydrogenase [NiFe] homologs are however atypical in this aspect: they are inactivated in the presence of oxygen but are reactivated in less than a minute when the cells regain an anaerobic environment. In vitro the enzyme can be activated within minutes by NADH and NADPH (Vignais et al. 2002; Cournac et al. 2004).

Depending upon the choice of microorganism, the pathway and enzymes involved in the hydrogen production process may differ considerably. Hydrogen production in cyanobacteria may involve one or a combination of the enzymes nitrogenase, uptake Hup-hydrogenase (encoded by *hupSL*), and bidirectional Hox-hydrogenase (encoded by *hoxEFUYH*) (Tamagnini et al. 2002, 2007; Bothe et al. 2010). Various organisms have evolved different mechanisms for protection of the oxygen-labile enzymes. This includes spatial separation where the oxygen-sensitive enzymes are separated in space from the oxygen-evolving photosystem II. This occurs mostly in heterocystous cyanobacteria like *Nostoc* and *Anabaena* where oxygen-evolving photosynthesis occurs in vegetative cells, whereas oxygen-sensitive nitrogen fixation/hydrogen production is restricted to the PSII-free heterocysts (Ramasubramanian et al. 1994; Serebriakova et al. 1994; Lindberg et al. 2002; Tamagnini et al. 2002, 2007; Khetkorn et al. 2013). Non-heterocystous filamentous cyanobacteria adopt different strategies to create anaerobiosis. Some filamentous cyanobacteria have a tendency to form clumps or groups. Notably, only certain filaments within the group acquire the potential to fix nitrogen while the remaining filaments act as a shield to protect the nitrogen-fixing filaments (Ohki and Taniuchi 2009). This behavior has been observed only in the filamentous strains of cyanobacteria. On the other hand, in some unicellular cyanobacteria functionality of hydrogenase is achieved by temporal separation where the photosynthetic oxygen evolution

[1]Wherever possible the currently accepted names for species are used. The name used in the paper cited is also indicated. For details of names see chapter "Systematics, Taxonomy and Species Names: Do They Matter?" of this book (Borowitzka 2016).

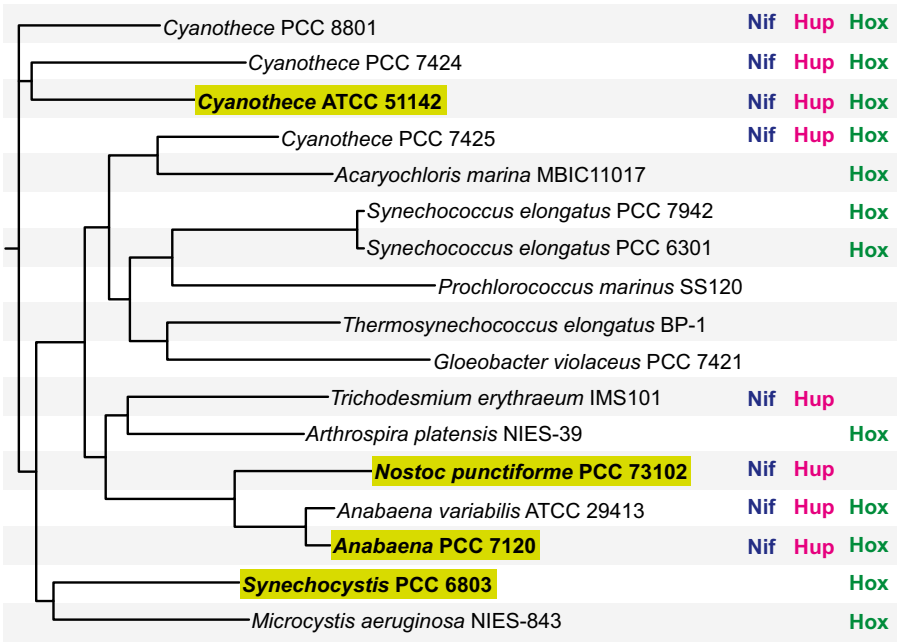


Fig. 1 Phylogenetic tree of representative cyanobacterial strains based on 16S rRNA sequences. The relative positions of *Synechocystis* PCC 6803, *Nostoc punctiforme* PCC 73102, *Cyanothece* ATCC 51142 and *Anabaena* PCC 7120 (strains discussed in the text) are highlighted. The presence of full sets of genes encoding nitrogenase, uptake hydrogenase or bidirectional hydrogenase is noted next to each strain with Nif, Hup and Hox, respectively

is separated in time from hydrogen evolution (Hallenbeck and Benemann 2002). This is common in unicellular non-nitrogen fixing organisms like *Synechocystis* (Appel et al. 2000; Tamagnini et al. 2007), while in some other unicellular nitrogen-fixing organisms such as *Cyanothece*, there appears to be a circadian control over photosynthetic oxygen evolution and nitrogen reduction/hydrogen production (Min and Sherman 2010).

Thus, considering the diversity of cyanobacterial hydrogen production, in this review four different model strains have been selected as representatives to holistically discuss the different strategies and mechanisms of the physiology of hydrogen production. These model biological systems are *Synechocystis* PCC 6803, *Nostoc punctiforme* PCC 73102, *Anabaena* PCC 7120 and *Cyanothece* ATCC 51142. This choice was based on the following criteria:

(i) Their position in the cyanobacterial phylogenetic tree.
(ii) Presence of enzymes involved in hydrogen production/uptake.
(iii) Morphology: unicellular/filamentous/ability to develop heterocysts.
(iv) Ability to fix nitrogen.
(v) Ease of genetic manipulation for future metabolic engineering.

While freshwater *Synechocystis* and marine *Cyanothece* are unicellular, *Nostoc* and *Anabaena* are filamentous in morphology. With the exception of *Synechocystis*, all are diazotrophs. 16S rRNA phylogenetic analysis of the four strains shows their evolutionary divergence from a common ancestor, with *Cyanothece* having evolved parallel to *Synechocystis*, *Anabaena* and *Nostoc* (Fig. 1). This correlates well to the difference in physiology and metabolism observed in *Cyanothece* in comparison with the other organisms described in the text below. The relative proximity of *Anabaena* PCC 7120 and *Nostoc* PCC 73102 reflects their similar pattern of evolution and physiology.

Based on the presence of different enzymes within each genus, their physiology of hydrogen production is described in greater details below. Table 1 summarizes the hydrogen production properties and characteristics of all the four organisms.

3.1 Hydrogen Production by Unicellular *Synechocystis* PCC 6803

Synechocystis PCC 6803 is a freshwater, unicellular, non-nitrogen-fixing cyanobacterium, isolated in 1968. It was the first cyanobacterium to be sequenced and characterized (Kaneko and Tabata 1997). Additionally, tools for its genetic engineering have been well developed and characterized (Huang et al. 2010; Heidorn et al. 2011; Yu et al. 2013; Camsund and Lindblad 2014). It is therefore an ideal candidate to use as a model system to study genetic engineering for enhancement of hydrogen production.

The genomic sequence of the strain reveals the presence of a single *hox* gene cluster, which offers the simplest native configuration for hydrogen production in cyanobacteria. Hox is a bidirectional hydrogenase that is able to catalyze both uptake and evolution of hydrogen. The direction of the reaction is largely governed by the redox potential of the substrates that are interacting with the enzyme and by the concentration

Table 1 Comparison of the four selected model cyanobacterial strains in terms of morphology, function, enzymes involved in hydrogen production, adapted mechanisms for hydrogen production and maximum rate of hydrogen production

Organism	Morphology	N_2-fixing/ non-N_2-fixing	Enzymes involved in H_2 production	Mechanism to facilitate nitrogenase/hydrogenase activity	Maximum H_2 production rate (μmol mg^{-1} chl *a* h^{-1})	Growth conditions; photon fluence rate	References
Synechocystis PCC 6803	Unicellular	Non-N_2-fixing	Hox-hydrogenase	Temporal separation	0.13	Air; 20 μmol photons $m^{-2}s^{-1}$	Howarth and Codd (1985)
Cyanothece ATCC 51142	Unicellular	N_2-fixing	Nitrogenase	Circadian rhythm	465	Air; 100 μmol photons $m^{-2}s^{-1}$	Bandhopadhyay et al. (2010)
			Uptake hydrogenase				
Nostoc punctiforme PCC 73102	Filamentous/ heterocystous	N_2-fixing	Nitrogenase	Spatial separation	9.7	Air; 200 μmol photons $m^{-2}s^{-1}$	Yeager et al. (2011)
			Uptake hydrogenase				
Anabaena PCC 7120	Filamentous/ heterocystous	N_2-fixing	Nitrogenase	Spatial separation	2.6	Air; 20 μmol photons $m^{-2}s^{-1}$	Masukawa et al. (2001)
			Uptake hydrogenase				
			Hox-hydrogenase				

of those substrates (Dickson 2011). When an electron acceptor is present, the Hox-hydrogenase will act as a hydrogen uptake enzyme, while, in the presence of an electron donor, it will produce hydrogen (Melis et al. 2000; Vignais and Colbeau 2004). *Synechocystis* is characterized by the absence of nitrogenase. Not surprisingly, the uptake hydrogenase Hup, which is usually found along with nitrogenase enzyme, is also absent (Fig. 1). Hydrogen production in *Synechocystis* is considered to serve as an electron valve for the cell (Cournac et al. 2004). The Hox-hydrogenase is capable of utilizing the low potential electrons generated during photosynthesis, thus preventing the slowdown of the electron transport chain (Appel et al. 2000).

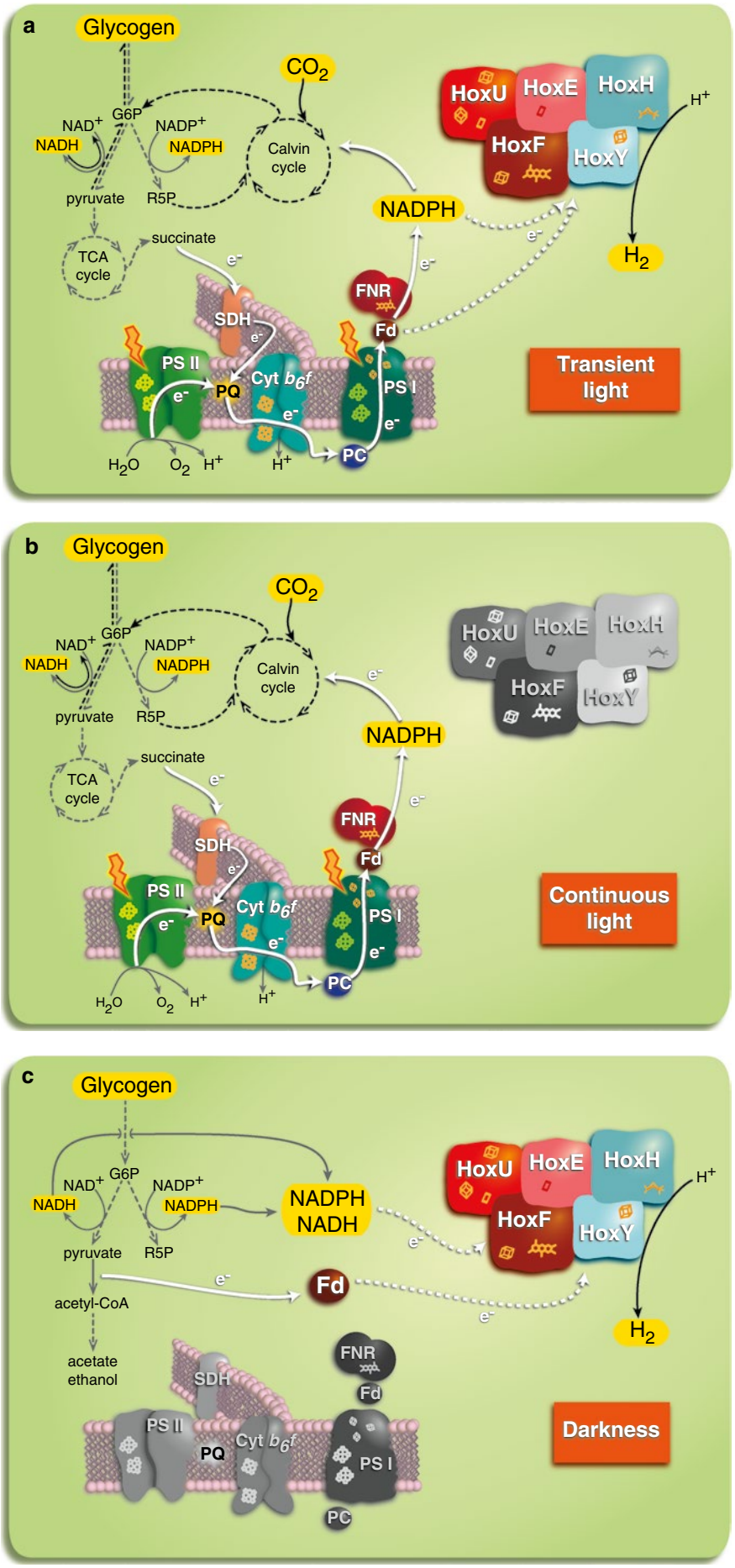
Hydrogen production in *Synechocystis* occurs by both direct biophotolysis as well as indirect biophotolysis. Direct biophotolysis utilizes the reductants directly generated from the photolysis of water at PSII. This production occurs at the onset of photosynthesis with light exposure. However, the production is transient in nature and ceases upon inactivation of the enzyme by the oxygen evolved concomitantly with photosynthesis. In contrast, indirect biophotolysis is of steady nature and occurs in the dark as the cells anaerobically feed on accumulated glycogen. Both the processes are described in greater detail below.

3.1.1 Direct Biophotolysis in *Synechocystis* PCC 6803

In *Synechocystis* PCC 6803 there is no temporal or spatial separation of hydrogen production and oxygen evolution (Tamagnini et al. 2002). However, the hydrogen produced under light, when photosynthesis is active, is intermittent in nature. Hydrogen production is initiated at the beginning of photosynthesis on exposure to light and is soon inhibited by the oxygen evolved during photosynthesis. The rate of oxygen evolution is higher than the rate at which aerobic respiration can consume oxygen, hence the damage to hydrogenase. This adaptation highlights nature's efficiency in preventing the loss of reductants that can instead be redirected towards growth (Dickson 2011).

The physiology of hydrogen production by direct biophotolysis is illustrated in Fig. 2a. In this process, the reductant ferredoxin for hydrogen production comes directly from photosynthesis rather than through the catabolism of the stored carbohydrates. On splitting of water, the protons are used to create a potential gradient across the thylakoid membranes to enable the production of ATP from ADP. Electrons from water splitting enter the photosynthetic electron transport chain through a series of transport molecules, including plastoquinone, cytochrome *b6f* and plastocyanin, and move through PSI to reduce ferredoxin. In *Synechocystis*, as in green algae like *Chlamydomonas reinhardtii*, the reduced ferredoxin can directly act as an electron donor to [FeFe] hydrogenase. This was recently established in a detailed study to determine the redox partner of the bidirectional Hox hydrogenase in *Synechocystis* (Gutekunst et al. 2014). The authors did not observe a strict requirement for NADPH/NADH to stimulate the electron transfer by ferredoxin. The results were further authenticated by the development of merodiploid FNR (ferredoxin:NADP(H) oxidoreductase) mutants that were limited in their capacity to transfer electrons from ferredoxin to $NADP^+$. These mutants demonstrated prolonged hydrogen production capacity in keeping with the observation that ferredoxin was the direct electron donor to the hydrogenase. Also the thermodynamics of such a system is highly favorable as the mid-point reduction potential of ferredoxin is −440 mV as compared to −420 mV of the H_2/H^+ redox couple. However, this study was in contradiction to earlier reports that suggested NADPH/NADH as the redox coupler to the bidirectional Hox hydrogenase in *Synechocystis* (Appel et al. 2000; Cournac et al. 2004). The thermodynamic feasibility of such a system was always widely debated. Ferredoxin via NADPH serves as a reductant for various pathways, like the Calvin cycle for fixation of carbon dioxide. Under favourable conditions, the excess reducing equivalents are disposed of by the Hox-hydrogenase as molecular H_2. Thus, in direct biophotolysis, ferredoxin derived from photosynthesis can be directly oxidized to reduce protons and yield hydrogen. This process occurs only as a stress buster within the cells to release the excess electron load away from the electron transfer chain. The phenomenon can be observed at a laboratory scale when a culture kept in the dark is suddenly exposed to light. Transient hydrogen production is detected, and then fades away. This phenomenon of an initial burst of hydrogen on transient light exposure has been attributed to a delayed onset of PSII activity (Cournac et al. 2004). Direct biophotolysis represents the most ideal way of linear transfer of energy from sunlight and splitting of water into molecular hydrogen. However, the process is fraught with several limitations, such as oxygen sensitivity of the hydrogenase, and competition for the reducing equivalents with different metabolic pathways.

One way to combat oxygen sensitivity would be to use oxygen-tolerant hydrogenases. However in nature, oxygen-tolerant hydrogenases do not occur in cyanobacteria and modelling one in the laboratory has not been successful to date. To overcome this challenge in green algae, Melis and coworkers (2000) developed the mechanism of sulfur deprivation by inhibiting the D2 subunit of PSII. This is known to cause a reversible decline in the photosynthetic activity, without affecting the rate of mitochondrial respiration (Melis et al. 2000). Experiments conducted in sealed flasks using sulfur-deprived medium showed that the net imbalance developed due to the skewed photosynthetic-respiration relationship led to the onset of anaerobiosis within the flask that triggered hydrogen production by the algae (Melis et al. 2000). However, recent RNA sequencing studies on the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Glycogen
CO2
NAD+
NADH
G6P
NADP+
NADPH
Calvin cycle
pyruvate
R5P
TCA cycle
succinate
HoxU
HoxE
HoxH
HoxF
HoxY
H+
H2
NADPH
e-
FNR
Fd
SDH
PS II
Cyt b6f
PQ
PS I
PC
H2O
O2
Transient light
b
Glycogen
CO2
Continuous light
c
Glycogen
NADPH NADH
acetyl-CoA
acetate ethanol
Darkness

effect of sulfur deprivation on hydrogen production by *Synechocystis* PCC 6803 revealed no change in the transcripts of the hydrogenase gene (Zhang et al. 2008).

3.1.2 Indirect Biophotolysis in *Synechocystis* PCC 6803

In indirect biophotolysis, hydrogen production occurs in a two-step process (Fig. 2b). In the first step, similar to the direct biophotolytic process, photolysis of water occurs in the presence of light. The reducing equivalents derived from it are mainly channeled towards the Calvin cycle for CO_2 fixation into the polymeric carbohydrate glycogen, as well as other pathways, such as respiration and nitrate assimilation. In the dark and under anaerobic conditions fermentation is induced (Fig. 2c). In this second step, the polymeric carbohydrates break down and glucose is oxidized by the glycolytic pathway and/or the oxidative pentose phosphate pathway (OPP) to generate energy. In the absence of oxygen as an electron acceptor, catabolism of sugars occurs to feed the energy requirement of the cell by substrate-level phosphorylation of ADP via the Embden-Meyerhof-Parnas pathway (glycolysis). The generated reducing equivalents are released by the cell as volatile fatty acids. Concomitantly, hydrogenase is also induced to partly release the electron stress as molecular hydrogen. Therefore, in indirect biophotolysis oxygen-evolving photosynthesis is largely used in one stage to fix and store carbon that can later be used in a second anaerobic, hydrogen-producing stage. In this way, the oxygen-sensitive hydrogenase reaction is separated in time and/or space from the oxygenic photosynthesis (Hallenbeck et al. 2012).

Reductants are generated either from fermentation (ferredoxin from glycolysis) or from catabolism of the stored glycogen (NADPH, derived from the pentose phosphate pathway). With the recent publication by Gutekunst et al. (2014), although it is now largely accepted that ferredoxin is the likely endogenous redox partner of hydrogenase, the feasibility of NADPH/NADH serving the same function under favorable conditions cannot be completely overruled. NADH and NADPH are interconvertible by a transdehydrogenase. The Hox-hydrogenase can then utilize either NADPH or NADH and protons as substrates to produce molecular hydrogen. Under anaerobic conditions, a larger pool of reduced NADPH is available that can be directly oxidized by the diaphorase moiety of Hox; it may alternatively be first converted into NADH through transdehydrogenases and subsequently reoxidized by the diaphorase moiety (Ananyev et al. 2008). It has been hypothesized that the photosynthetically-generated reductant is consumed first, followed by the reductant generated by catabolism of sugars (Ananyev et al. 2008).

Drawing reductants away from other pathways into hydrogen production has been viewed as one of the primary ways of enhancing hydrogen production efficiency. For instance, enhanced hydrogen production from *Synechocystis* was carried out under nitrogen starvation conditions (Skizim et al. 2012). This is because the nitrate assimilation pathway functions as an electron sink and competes for the same reductant as the hydrogen pathway. Therefore, enhanced H_2 production has been reported in *Synechocystis* under environmental stress conditions (Oxelfelt et al. 1995; Axelsson and Lindblad 2002; Baebprasert et al. 2010). Nitrate is actively transported into the cells and reduced first into nitrite by nitrate reductase (encoded by *nar*B) and further to ammonium by nitrite reductase (encoded by *nir*A). The two reactions require two and six electrons respectively. Baebprasert et al. (2011) have shown that mutants created by either disruption of nitrate reductase or nitrite reductase, or both, indeed showed higher hydrogen production activity as compared to the wild type.

3.2 Hydrogen Production by *Anabaena* PCC 7120

Anabaena PCC 7120 is a N_2-fixing filamentous and heterocyst-forming cyanobacterium. The 7.21 Mb genome includes all three enzymes known to be involved in cyanobacterial hydrogen production (Kaneko et al. 2001). The FeMo-containing nitrogenase is found both in the heterocyst and in the vegetative

Fig. 2 Biochemistry of hydrogen production in *Synechocystis* PCC 6803. (**a**) Direct biophotolysis pathway. In this pathway the reducing equivalents are obtained directly from the splitting of water at photosystem II (*PSII*). The electrons are transferred into the photosynthetic electron transport chain through a series of transport molecules including plastoquinone (*PQ*), cytochrome b_6f (*Cyt* b_6f) and plastocyanin (*PC*), and move through photosystem I (*PSI*) to reduce ferredoxin (*Fd*). Ferredoxin then reduces $NADP^+$ to NADPH via the enzyme ferredoxin NADP(H) reductase (*FNR*). The Calvin cycle and the hydrogen production pathway (via the bidirectional Hox hydrogenase) compete briefly for reducing equivalents thus generated. Ferredoxin is also able to deliver electrons directly to Hox. There is H_2 production until Hox is inactivated due to the presence of O_2. These pathways are therefore possible only under short illumination periods. (**b**) Indirect biophotolysis pathway, continuous light conditions. Under continuous light, the reducing equivalents are generated in a manner similar to the direct biophotolytic pathway. All the generated reductants are however directed towards the Calvin cycle to fix CO_2 into sugars, stored as glycogen. (**c**) Indirect biophotolysis pathway, dark conditions. In darkness, under anaerobiosis, glycogen is broken down to glucose-6-phosphate (*G6P*), which is then further oxidized either by the oxidative pentose pathway (to ribulose-5-phosphate, R5P) or by glycolysis (to pyruvate) to generate the reductants NADPH and NADH. Oxidation of pyruvate (via pyruvate:ferredoxin oxidoreductase) delivers further reducing equivalents to a ferredoxin. Both pathways are able to subsequently deliver electrons to the Hox hydrogenase

cells (Masukawa et al. 2010). Significantly, the non-heterocyst nitrogenase is functionally expressed in the vegetative cells only under anaerobiosis and in the presence of an exogenous carbohydrate supply (Masukawa et al. 2010). Under aerobic conditions the nitrogenase-related *nif* genes are expressed in the PSII-lacking heterocysts. The *nif* operon comprises the *nifHDK* genes that encode the molybdenum-containing nitrogenase enzyme complex. Upstream of the *nifHDK* operon another *nif* operon has been identified, *nifB-fdxN-nifS-nifU* (Mulligan and Haselkorn 1989). The vegetative cells carry out the oxygenic photosynthesis and fix CO_2 in the form of glycogen. The polymeric glycogen is broken down into simpler units that are transported by a putative ABC transporter, which exports polysaccharides beyond the cell wall (Maldener et al. 1994). The bidirectional Hox-hydrogenase is also present in *Anabaena* PCC 7120 and is found active in both vegetative cells and heterocysts in anaerobically grown filaments.

The basic physiology of hydrogen production in *Anabaena* PCC 7120 is outlined in Fig. 3a, b. The differentiation of the heterocysts occurs between 18 and 24 h after nitrogen deprivation (Golden et al. 1985). Heterocyst development is also accompanied by changes in photosynthetic and carbon metabolism, which help to provide low potential reductants and ATP for the energy-intensive nitrogenase reaction (Meeks et al. 2002). To further ensure anaerobiosis, high-rate respiration sets in by heterocyst-specific *cox2* and *cox3* cytochrome *c* oxidase that also helps in generating ATP (Jones and Haselkorn 2002). This activates the nitrogenase complex for nitrogen fixation and hydrogen production. To prevent waste of energy, the heterocyst-specific uptake hydrogenase recovers electrons from the H_2 produced by nitrogenase (Tamagnini et al. 2007).

Additionally, the vegetative cells are also capable of producing hydrogen via the Hox-hydrogenase through indirect biophotolysis (Fig. 3a). Here, the stored glycogen is broken down and the reducing equivalents are produced via OPP, and then delivered to ferredoxin. Reduced ferredoxin transfers the electrons to reduce $NADP^+$ to NADPH via FNR. NADPH has the potential to deliver the electrons to bidirectional Hox-hydrogenase, so as to evolve molecular hydrogen. Besides being respectively generated by the OPP and glycolysis, NADP(H) and NADH can be generated by several other pathways, including the photosynthetic electron transport chain. With the recent findings that ferredoxin is the likely redox coupler to the bidirectional Hox hydrogenase in *Synechocystis* (Gutekunst et al. 2014), the possibility of ferredoxin as a direct donor of reductants to the bidirectional Hox hydrogenase of other cyanobacteria including *Anabaena* is now open to research.

Unlike the vegetative cells, in the heterocysts, FNR catalyzes the ferredoxin-dependent oxidation of the NADPH derived from the pentose phosphate pathway (Fig. 3b). In the closely-related organism *Anabaena variabilis*, the heterocyst-specific ferredoxin FdxH, a variant of the PetF found in the vegetative cells, is optimized for reverse electron flow between NADPH and FNR, as compared to the vegetative cells (Schrautemeier and Böhme 1985; Razquin et al. 1994). The physiological implication of this is immense. Since photoreduction of $NADP^+$ does not occur, under light, reduced FNR can transfer the electrons to PSI via the Cyt b_6/f complex and ultimately to FdxH, which serves as the immediate electron donor to nitrogenase (Böhme and Schrautemeier 1987). This occurs when the ratio of NADPH to $NADP^+$ is high, as measured in isolated heterocysts under nitrogen-fixing conditions (Böhme 1987). Notably, deletion mutants of FdxH could still carry out high-rate nitrogen fixation and hydrogen production (Masepohl et al. 1997). This indicated that although presence of FdxH was required for maximal nitrogenase activity, it was not absolutely essential to carry out nitrogenase-derived hydrogen production. It could perhaps be substituted by other ferredoxins.

3.3 Hydrogen Production by *Nostoc punctiforme* PCC 73102/ATCC 29133

Nostoc punctiforme PCC 73102 was isolated from a symbiotic association with the cycad *Macrozamia* and brought into the Pasteur Culture Collection (PCC) in 1973. Later a subculture was incorporated into the American Type Culture Collection (ATCC). It is a nitrogen-fixing heterocystous cyanobacterium. Whole genome sequence analysis of this organism revealed the presence of a single uptake hydrogenase along with a single nitrogenase but no Hox-hydrogenase (Meeks et al. 2001). However, this is not true for all strains of *Nostoc*: some contain a bidirectional Hox-hydrogenase in addition to nitrogenase and uptake hydrogenase.

Hydrogen production in *Nostoc punctiforme* is mediated by nitrogenase (Lindberg et al. 2002). The physiology of hydrogen production by this strain of *Nostoc* is similar to that of *Anabaena* PCC 7120 even though this organism does not possess a Hox-hydrogenase (Fig. 4). *Nostoc punctiforme* contains only the molybdenum nitrogenase (Haselkorn and Buikema 1992), in contrast to organisms such as *Anabaena variabilis*, which possesses two molybdenum-containing nitrogenases and also an alternative, vanadium-containing nitrogenase (Thiel 1993; Thiel et al. 1997). The nitrogenase is located in the heterocysts and is encoded by the structural genes *nifHDK* (Meeks et al. 2001; Ramasubramanian et al. 1994). The accessory *nif* genes are organized in a cluster as *nifB-fdxN-nifS-nifU-nifH-nifD-nifK-orf-nifE-nifN-nifX-orf-orf-nifW-hesA-hesB-fdxH* (Meeks et al. 2001).

Absence of water-splitting PSII activity and the existence of a high rate of respiration ensure an anaerobic environment in the heterocyst. In addition, the structure is surrounded by specialized

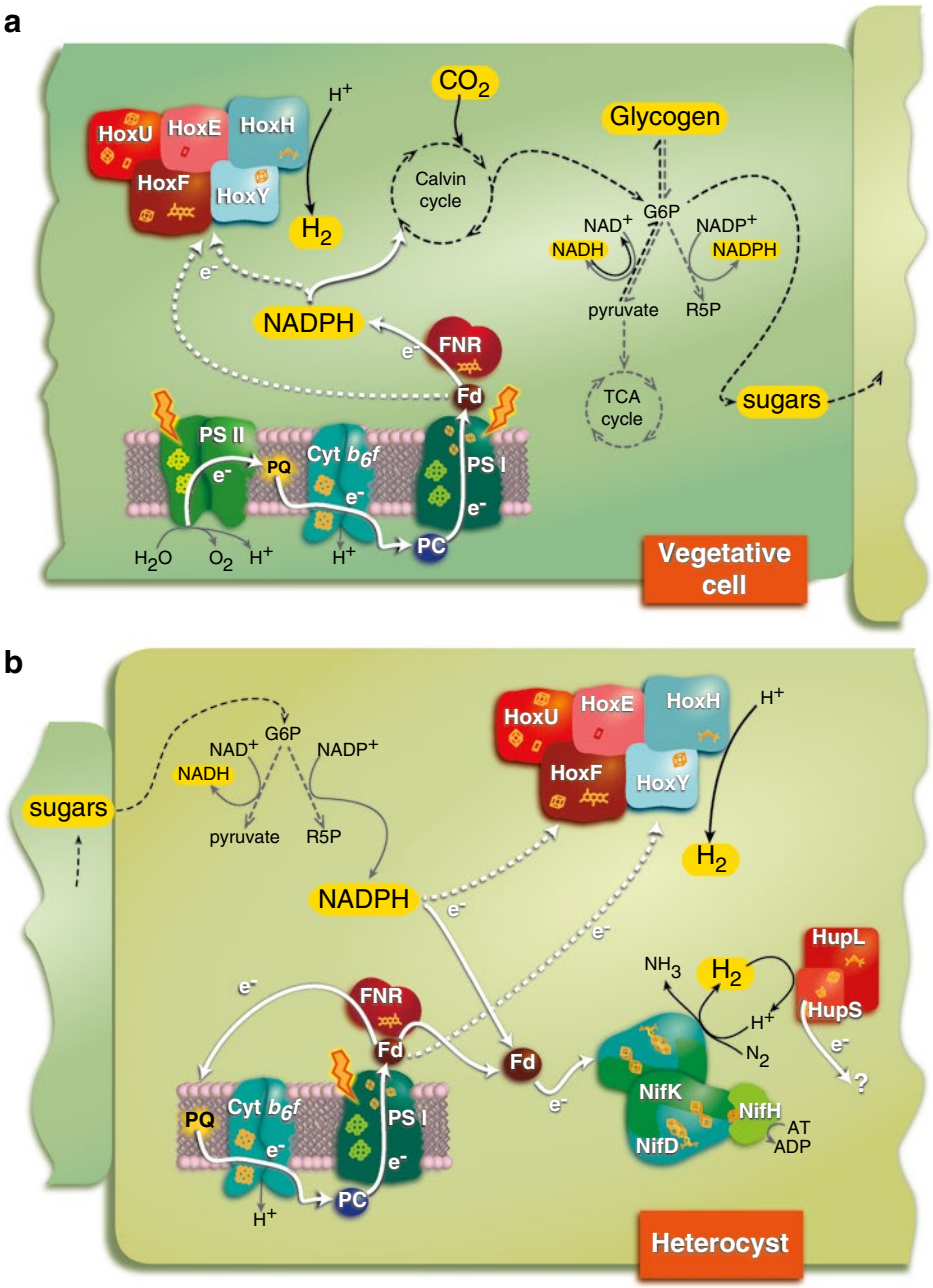

Fig. 3 Biochemistry of hydrogen production in *Anabaena* PCC 7120. Hydrogen production in *Anabaena* is mainly mediated by the nitrogenase and the bidirectional Hox hydrogenase. The bidirectional hydrogenase is present in both the vegetative cells and the heterocysts. (**a**) In the vegetative cell, the captured solar energy (photons) is used to split water, liberating oxygen, protons and electrons. The electrons are transferred through PSII to PSI and ultimately reduce ferredoxin. Reduced ferredoxin can reduce $NADP^+$ to NADPH via FNR. NADPH can drive hydrogen production by the bidirectional Hox hydrogenase, or it can drive CO_2 fixation by the Calvin cycle. Hypothetically, reduced ferredoxin may be able to deliver electrons directly to Hox, as it happens in *Synechocystis* PCC 6803. (**b**) In the heterocysts, reduced ferredoxin can reduce plastoquinone, driving cyclic photophosphorylation through PSI, thus generating additional ATP. Ferredoxin can also indirectly donate electrons to the nitrogenase system (NifHDK), which catalyzes the ATP-dependent reduction of nitrogen and production of hydrogen. As above, the direct donation of electrons by ferredoxin to Hox cannot at this point be excluded. Hydrogen produced by nitrogenase is recaptured by the uptake Hup hydrogenase

membrane structures made up of layers of polysaccharides and glycolipids, which make the heterocyst less permeable to the environmental gases (Kumar et al. 2010). The primary source of electrons for the nitrogenase in the heterocysts is the reserve carbohydrates, imported from the vegetative cells. Electrons are transferred to nitrogenase via NADPH and a heterocyst-specific ferredoxin. Using an alternative path, the electrons can pass to nitrogenase via ferredoxin reduced by PSI (Lindberg 2003).

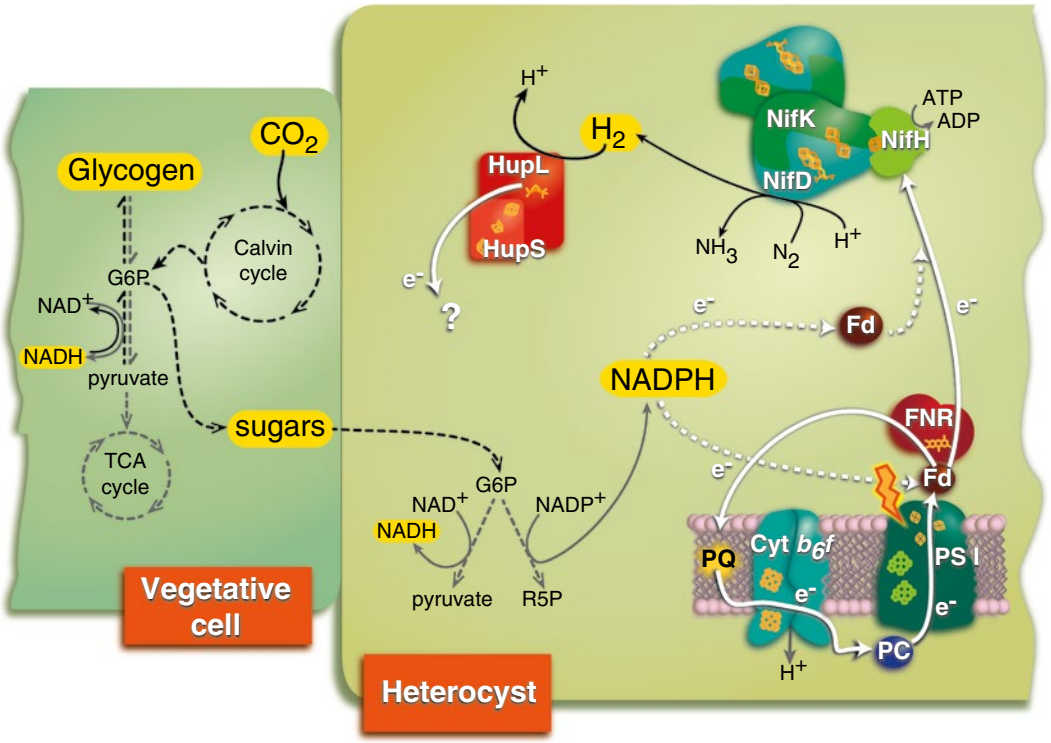

Fig. 4 Biochemistry of hydrogen production in *Nostoc punctiforme* PCC 73102. Hydrogen production in *Nostoc* is primarily mediated by the nitrogenase present in the heterocysts, where PSII activity is absent. The reducing equivalents for nitrogen fixation/hydrogen production are primarily obtained from the degradation of carbohydrates (synthesized from fixed CO_2 via the Calvin cycle) in the vegetative cells. Electrons are transferred to nitrogenase via NADPH and a heterocyst-specific ferredoxin, which reduces the nitrogenase Fe-protein (NifH). Hydrogen produced by nitrogenase is recaptured by the uptake Hup hydrogenase

3.4 Hydrogen Production by *Cyanothece* ATCC 51142

Cyanothece ATCC 51142 is a marine, unicellular, diazotrophic cyanobacterium, a benthic strain isolated off the U.S. Gulf Coast (Reddy et al. 1993). The strain possesses a bidirectional Hox-hydrogenase, an uptake hydrogenase, and the MoFe nitrogenase (Min and Sherman 2010). Hydrogen production by both the Hox-hydrogenase and the nitrogenase occur under appropriate metabolic conditions and are regulated differentially (Fig. 5a, b). The *hox* genes encoding Hox-hydrogenase are diurnally regulated, that is, they are under the light/dark control and are preferentially turned on in the dark (Toepel et al. 2009). Min and Sherman (2010) demonstrated that in *Cyanothece* ATCC 51142 hydrogen production was found both under continuous light and under light/dark cycles. Addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which blocks the plastoquinone binding site of photosystem II, completely inhibited hydrogen production. Similarly, incubation of the cells in the dark under an atmosphere of argon gas also inhibited hydrogen production. This indicated that the organism could not utilize the reductant generated by catabolism of starch alone. This is an unusual adaptation as compared to the other cyanobacteria.

In *Cyanothece* ATCC 51142, hydrogen production by nitrogenase is under circadian control, where nitrogenase is transcribed every fixed number of hours. During a diurnal cycle of 12 h light and 12 h darkness, photosynthesis takes place only in the light phase. Photosynthesis leads to the accumulation of stored carbohydrates as starch (unlike other cyanobacteria where it is glycogen) in between the photosynthetic membranes (thylakoids). When the organism is cultured in the dark, photosynthesis ceases and the accumulated carbohydrates are consumed, yielding energy and reducing equivalents required for nitrogen fixation (Fig. 5b). High-rate respiration creates a microanaerobic environment for the induction of nitrogenase activity. However, when the organism is cultured in alternate periods of continuous light or continuous dark, hydrogen production by nitrogenase is still observed, reflecting its circadian control. It was found that during culture under continuous period of light, the organism still carries out nitrogen fixation in cycles of approximately 16 h, corresponding to the light/dark periods. It was observed that at this time, even though light was available, the capacity

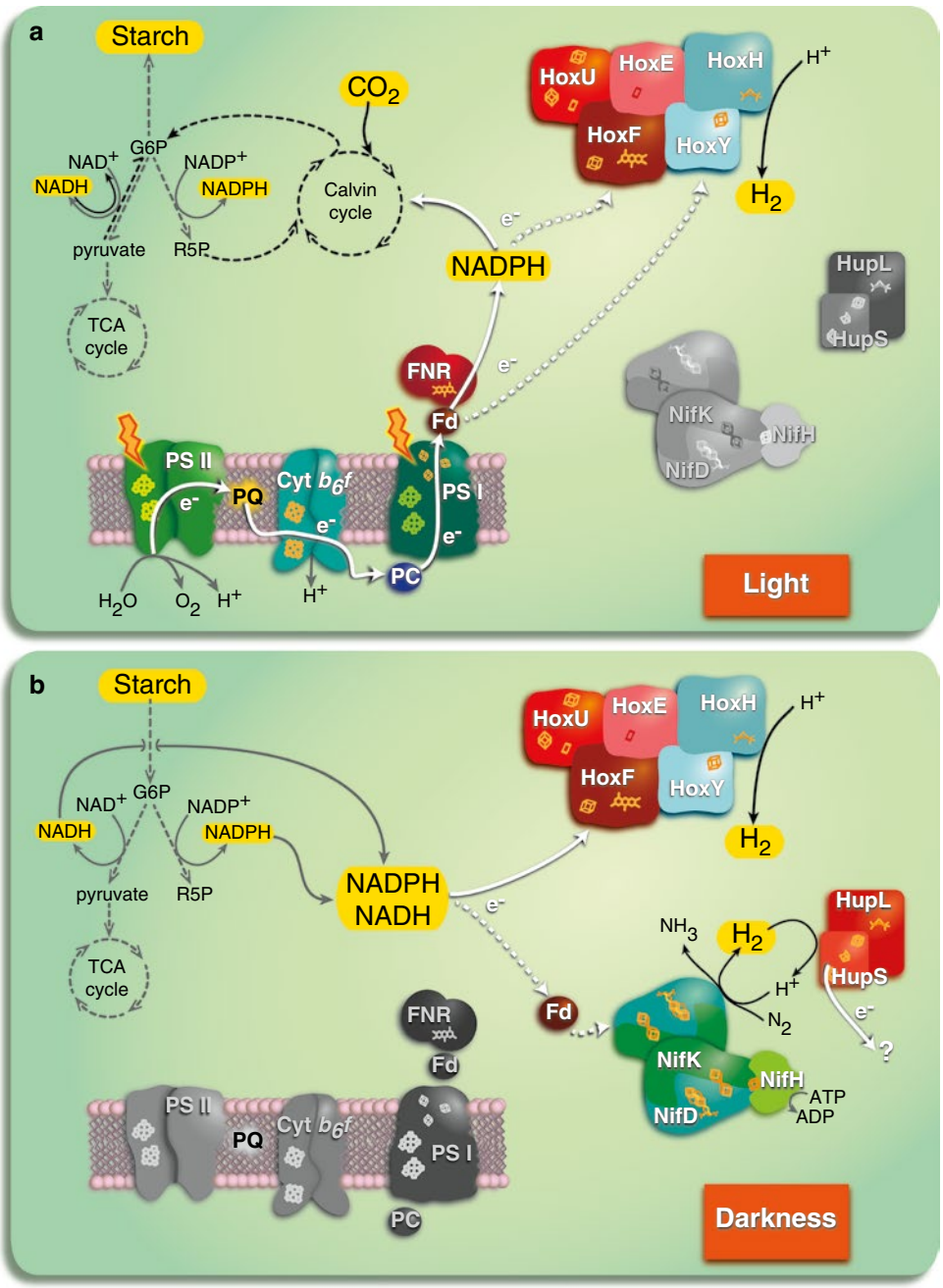

Fig. 5 Scheme of the predicted photobiological hydrogen production pathway in *Cyanothece* ATCC 51142. *Cyanothece* is able to produce hydrogen aerobically because it controls its metabolic processes with a circadian clock. (**a**) The organism photosynthesizes and fixes CO_2 via the Calvin cycle during daytime, storing it as starch. Hydrogen evolution has been observed during light periods, due to Hox hydrogenase activity. (**b**) In darkness, *Cyanothece* begins fixing nitrogen using starch as an energy source and nitrogenase to convert N_2 to NH_3 with H_2 as a byproduct. Although oxygen is present, high rates of respiration create an anaerobic environment in the cells, which allows nitrogenase to function. Hydrogen produced by nitrogenase is recaptured by the uptake Hup hydrogenase

of photosynthesis is extremely reduced and the stored carbohydrates are utilized in intensive respiration. On the other hand, when the organism was cultured in periods of continuous darkness, it was able to survive under heterotrophic conditions by supplying the growth medium with a carbon source such as glycerol (Min and Sherman 2010).

4 Enzymes Related to Hydrogen Production in Cyanobacteria

There are two types of enzymes that are directly related to cyanobacterial hydrogen production: hydrogenases and

nitrogenase. Structurally and functionally these enzymes differ. The primary role of the enzymes within the biological system is unique. While nitrogenase is primarily involved in nitrogen fixation, the role of uptake hydrogenase is in conservation of energy by catalyzing the oxidation (uptake) of the hydrogen released by nitrogenase during nitrogen fixation. In addition, bidirectional Hox-hydrogenase acts as an electron valve within the cell to release excess reducing equivalents as molecular hydrogen. The Hox-hydrogenase may also be involved in uptake activity, essential to preserve the redox balance of the cell. Another major difference between the catalyzed reactions is in terms of energy requirements. Nitrogenase catalyzes hydrogen production in an energy-intensive reaction requiring 16 ATP molecules per molecule of hydrogen evolved in addition to reductants, while hydrogenase does not have any energy requirements (Watt et al. 1975). Moreover, it has been estimated that the light required for hydrogen evolution by nitrogenase is approximately 9 ± 1 quanta/H_2. In principle, hydrogenase requires less than 1 quantum/H_2 and should therefore exhibit three times the efficiency of any nitrogenase-mediated system (Benemann 1994).

4.1 Cyanobacterial Nitrogenase

Nitrogen is needed by the organism for synthesis of amino acids and nucleotides. Nitrogenases are complex enzymes and catalyze the reaction shown in Eq. (2):

$$8H^+ + 8e^- + N_2 + 16MgATP \rightarrow 2NH_3 + 16MgADP + 16P_i + H_2 \quad (2)$$

Nitrogenase appears to be a highly conserved enzyme complex. It is found in a diverse group of prokaryotes from the Bacteria and Archaea, but is not encoded in any eukaryotic genome (Berman-Frank et al. 2003). The phylogenetic ancestry of nitrogenase reveals a single common ancestor to all the nitrogenases sequenced so far, and also indicates its origin at a time prior to Earth's oxygenation (Broda and Pesheck 1983). Since the energy-intensive reaction requires 16 mol ATP for every mole of nitrogen fixed, the enzyme is activated only when the organism does not have an easy access to any other inorganic nitrogen sources present in the surrounding media.

Nitrogenases are classified depending upon the presence of metals present in the active site. The active site contains iron as one of the metal cofactors, the other being either molybdenum, vanadium or an additional iron ion (Zehr et al. 2003; Burgess and Lowe 1996; Eady 1996). Amongst all types of nitrogenase, molybdenum nitrogenase occurs most commonly in nature (Zehr et al. 2003), and is therefore described in some detail here. Nitrogenase is a complex enzyme and comprises two protein units. The smaller protein is called dinitrogen reductase (Fe protein or protein II), while the larger unit is called dinitrogenase (MoFe protein, or protein I) and contains the active site. The dinitrogenase is a heterotetramer ($\alpha_2\beta_2$) of about 220–240 kDa; the α and β subunits are encoded by *nifD* and *nifK*, respectively. The smaller dinitrogenase reductase is a homodimer (γ_2) of about 60–70 kDa and is encoded by *nifH* (Howard and Rees 1996). The smaller unit comprises redox active Fe_4S_4 clusters similar to those present in smaller molecular weight electron carriers. It is thus responsible for mediating the electrons from the external donor (ferredoxin or flavodoxin) to the active site (Burgess and Lowe 1996). The larger subunit comprises two types of clusters, a P cluster, and an M cluster. The P cluster is a Fe_8S_7 center and is thought to function as a relay that accepts electrons from the redox active Fe_4S_4 center of the smaller subunit and further transfers them to the M cluster. The M cluster is the active site and comprises an inorganic Fe_7MoS_9C component (the FeMo cofactor) and an organic component, homocitrate (Howard and Rees 1996; Spatzal et al. 2011). The FeMo cofactor is the postulated site of substrate binding and reduction, while the specific role of homocitrate has not yet been elucidated (Bothe et al. 2010). It is to be noted that both the Fe_4S_4 center of the small subunit and the Fe_8S_7 P center are highly oxygen labile. In fact, the Fe_4S_4 center is much more oxygen-sensitive and gets irreversibly damaged on exposure to oxygen (Postgate 1998). The problem of oxygen sensitivity is enhanced in photosynthetic cyanobacteria owing to the oxygen-evolving PSII. To circumvent this issue, cyanobacteria have evolved different mechanisms to separate nitrogenase activity and oxygen evolution. This is ensured either by the localization of the nitrogenase in the microanaerobic environment of the heterocyst, or by temporal separation of the photosynthetic oxygen evolution and nitrogen reduction activities. Both mechanisms are further discussed below. Nitrogenase is known to be inhibited by carbon monoxide (Christiansen et al. 2000). However, the chemistry is only partially understood.

Besides nitrogen reduction, nitrogenase can catalyze other reactions that involve a reactant with a triple bond. These include nitriles (RCN), isonitriles (RNC), hydrogen cyanide (HCN), nitrous oxide (N_2O), and acetylene (C_2H_2), as well as the double bond compound azide (N_3^-). Of particular interest for nitrogen research is the reduction of acetylene to ethylene. Ten to twelve percent acetylene in the headspace is routinely used to estimate the nitrogenase activity of a culture using gas chromatography (Carpenter 1983; Capone 1993).

4.1.1 Nitrogenase in Non-heterocystous Cyanobacteria

Some unicellular (such as *Synechococcus* and *Gloeothece*) and certain filamentous cyanobacteria (such as *Trichodesmium*) are known to possess nitrogenase. They have all developed unique strategies to safeguard their nitrogenase from oxygen. In certain organisms like *Synechococcus*, nitrogen fixation is temporally separated in the diurnal cycle. Oxygenic photosynthesis is known to take place in the daytime (under light), while nitrogenase is known to function in dark when PSII is inactive and the low amounts of oxygen present in the environment are consumed by high-rate respiration (Bothe et al. 2010). This occurs mostly when the organism forms dense mats or biofilms. However, this is not the case with all unicellular cyanobacteria. In *Cyanothece*, nitrogenase is known to be under the circadian control (Min and Sherman 2010). It can thus reduce nitrogen in air even under continuous light. It does so by temporarily inactivating PSII and increasing the rate of respiration (Reddy et al. 1993). The filamentous cyanobacterium *Trichodesmium* shows division of labor, where some cells carry out nitrogen fixation while others carry out oxygenic photosynthesis. Ohki and Taniuchi (2009) showed that 77 % of all vegetative cells in *Trichodesmium* stained positive for nitrogenase, indicating the absence of any heterocyst-like structure for nitrogenase protection.

Some uncultured unicellular marine cyanobacteria that lack oxygen evolution and CO_2 fixation capacity have been recently identified. Whole genome sequencing of such organisms has revealed the presence of nitrogenase in their DNA (Zehr et al. 2008).

4.1.2 Development and Differentiation of the Heterocyst, Site of Nitrogenase Localization

Under nitrogen-fixing conditions in the filamentous organisms, some vegetative cells differentiate irreversibly into highly specialized cells called heterocysts. *Anabaena* and *Nostoc* are two popular examples where this differentiation takes place. Their differentiation relies on complex intercellular communication and is highly regulated.

Heterocyst differentiation is irreversible (Adams and Carr 1989). About 10 % of the vegetative cells differentiate into heterocysts (Flores and Herrero 2010). Under common laboratory conditions, heterocysts are distinguishable after adapting the organism in nitrogen source-free media, forcing the organism to convert the atmospheric nitrogen to ammonia. The heterocysts become morphologically distinguishable 8–9 h after adaptation. An external reduced nitrogen source added during this time may repress the formation of heterocysts, but any later addition has no effect on the morphologically distinct cells, indicating their irreversible character (Ehira 2013). Proheterocysts differentiate from the walls of the thylakoid membrane, which reorganize to form a honeycomb-like structure (Pastor and Hess 2012). The specialized honeycomb membrane structure and the "necklike" interconnections with the vegetative cells effectively reduce the gas exchange area (Merino-Puerto et al. 2011). Moreover, the respiratory enzymes are embedded in the membrane located at the cell junctions. This architecture effectively reduces the amount of oxygen diffusing into the heterocyst cells (Kumar et al. 2010). To study the regulatory machinery of the cell in response to heterocyst differentiation, several mutants have been created over the years. More recently, RNA sequencing and proteomic studies have further confirmed these findings (Flaherty et al. 2011; Ow et al. 2008).

It is still unknown which vegetative cells in a non-nitrogen fixing filament will differentiate into heterocysts (Fan et al. 2005). However, it is known that the differentiation is under the regulation of several factors. In the absence of easily accessible nitrogen sources in the medium, transcriptional factor NtcA, a DNA-binding dimer, is triggered (Herrero et al. 2004). Another associated protein, DevH, is expressed and has been characterized in *Anabaena* PCC 7120 (Hebbar and Curtis 2000). NtcA belongs to the CRP (cyclic AMP receptor protein) family of proteins, and is the main 2-oxoglutarate sensor for the initiation of heterocyst differentiation. 2-oxoglutarate provides the carbon skeleton for the incorporation of inorganic nitrogen (as ammonium), and serves as a signal molecule of the relative organic carbon/nitrogen (C/N) content in the cells (Kumar et al. 2010). NtcA is crucial for the early steps of heterocyst differentiation. Other molecules and ions, including calcium and cyclic-di-GMP, have been identified as signals for the C/N imbalance (Zhao et al. 2005; Shi et al. 2006; Kumar et al. 2010). In *Anabaena* PCC 7120, the calcium-binding luminescent protein aequorin was used to detect the enhanced intracellular calcium levels following nitrogen deprivation (Torrecilla et al. 2004). Zhao et al. (2005) showed that the increased calcium ion concentrations are related to decreased CcbP, a calcium-sequestering protein. More than 500 proteins are differentially expressed in heterocysts during cellular transformation from vegetative cells, showing that this complex process is under the control of many genes (Kumar et al. 2010). The deletion of the *all2874* gene, which encodes a diguanylate cyclase, caused a significant reduction in heterocyst frequency and reduced vegetative cell size (Neunuebel and Golden 2008). This indicated cyclic GMP as a signal for heterocyst differentiation.

Another factor that has been identified in heterocyst differentiation is the protein HetR, a serine protease that is known to bind to the promoters of several heterocyst-related genes, such as *hetR*, *hetP*, *hetA*, *hetZ*, and *patS*. It is however not known how the DNA-binding activity of HetR relates to its regulatory effects (Zhou et al. 1998; Zhou and Wolk 2003; Zhao et al. 2005). Knockout strains of *hetR* gene were found

to have lost their ability to differentiate into heterocysts, while overexpression of HetR increased the frequency of occurrence of heterocysts (Buikema and Haselkorn 1991, 2001). In the absence of any NtcA-binding site on its promoter, HetR is found to be autoregulated (Buikema and Haselkorn 2001). However, NtcA and HetR are also found to regulate each other in an interdependent manner such that in the deletion mutants of *ntcA*, *hetR* is not induced, while in the deletion mutant of *hetR*, *ntcA* is only transiently expressed (Frias et al. 1994; Muro-Pastor et al. 2002).

HetR is localized in the heterocyst with the help of HetF. This was identified in *hetF* deletion mutants (Wong and Meeks 2001), which failed to develop differentiated heterocysts. On the other hand, in the absence of nitrogen, overexpression of HetF produced a multiple contiguous heterocyst phenotype (Wong and Meeks 2001). It is also suggested that HetF acts as a protease (Risser and Callahan 2008) and thus plays a role in regulating the accumulation of HetR within the heterocysts (Wong and Meeks 2001).

The proteins PatS and HetN are also found to regulate heterocyst differentiation; their overexpression downregulates heterocyst frequency (Wu et al. 2004). PatS is a small polypeptide whose C-terminal pentapeptide RGSGR (PatS-5) inhibits heterocyst differentiation when added exogenously or when expressed from a heterocyst-specific promoter (Yoon and Golden 1975). HetN contains an identical RGSGR motif that has been shown to be a functional moiety (Higa et al. 2012).

The products of *hetN* and *patA* genes are known to regulate heterocyst differentiation by positive regulation of *hetR* (Liang et al. 1992). It is possible that PatA influences heterocyst development by attenuating the negative effects of the main inhibitory signals of heterocyst pattern formation, PatS and HetN (Orozco et al. 2006). Mutants of *patA* were found to develop heterocysts at the end of the filaments (Liang et al. 1992).

The *hetC* gene encodes a member of the family of ATP-binding cassette type exporters. It is required for an early step in the differentiation of heterocysts as observed by heterologous expression of a PhetC-GFP reporter construct, which showed an increase in expression in proheterocysts and heterocysts (Khudyakov and Wolk 1997; Muro-Pastor et al. 1999). Two other novel genes, *hetL* and *asr1734*, have been shown to be involved in regulating heterocyst development, but their exact roles and biochemical functions remain unclear. Also, overexpression of another protein, HetP, promotes differentiation even in *hetR* mutants. Its role remains unclear (Higa and Callahan 2010).

After the early heterocyst precursors have been developed, the cell undergoes further morphological and metabolic changes. These include the deposition of a thickened cell wall as described in the early part of this section, in addition to the expression of cytochrome *c* oxidase, nitrogenase and hydrogenases. In *Anabaena* PCC 7120, the final steps of differentiation encode three rearrangement processes in *nifD* of nitrogenase, *hupL* of uptake hydrogenase and *fdxN* of ferredoxin (Golden et al. 1985, 1988, Golden 1997). Golden and Weist (1988) studied these processes in detail and found that a 11 kb DNA element is excised by XisA in *nifD*, a 55 kb element is excised from *fdxN* by XisF (Golden et al. 1988; Carrasco et al. 1994) and finally a 10.5 kb element is excised from *hupL* by XisC (Carrasco et al. 2005). It is only after these programmed rearrangements that the heterocyst is fully functional and is able to fix nitrogen. This rearrangement is not found in the *hupSL* of *N. punctiforme* PCC 73102 (Oxelfelt et al. 1995). The physiological consequence of this difference has not yet been ascertained.

4.2 Cyanobacterial Hydrogenases

Hydrogenase was first discovered by Stephenson and Stickland (1931). It catalyzes the simple reaction depicted in Eq. (1). Hydrogenases are essentially considered as electron valves that help in maintaining the redox balance within the cell. The release of fermentative products balances the redox build up inside the cells; release of hydrogen from the cell also achieves this purpose. Hydrogenases are classified depending on the nature of metal clusters present in the active center. Accordingly, they are broadly classified as [Fe]-only, [NiFe] or [FeFe] hydrogenases. These hydrogenases are functionally different and do not share a common ancestor (Adams 1990). Among these, [NiFe] hydrogenases are the most common in nature (Constant et al. 2011), but are limited to prokaryotes. In contrast, [FeFe] hydrogenases may be present both in bacteria and in the eukaryotic green algae (but not in plants). In cyanobacteria, presence of [FeFe] hydrogenases has not been reported to date. Cyanobacteria contain homologs of [NiFe] hydrogenases, most of which are the bidirectional Hox-hydrogenase and the uptake Hup-hydrogenase. Both of these hydrogenases serve different roles in the cell as explained in the case studies cited above. Organization of the loci containing the genes encoding the uptake hydrogenase (*hup*) and the bidirectional hydrogenase (*hox*) in the selected cyanobacterial strains is shown in Fig. 6. The cyanobacterial hydrogenases and their transcription and regulation are discussed in some detail below.

4.2.1 Cyanobacterial Uptake Hydrogenase

The uptake hydrogenase is a membrane-associated enzyme and is ubiquitously found in nitrogen-fixing cyanobacteria. The enzyme is completely absent in non-nitrogen-fixing cyanobacteria, such as *Synechocystis*. This hydrogenase is expressed under conditions of nitrogen starvation and is closely associated with nitrogenase activity (Tamagnini et al. 2002). The enzyme, HupSL, consists of two subunits, the

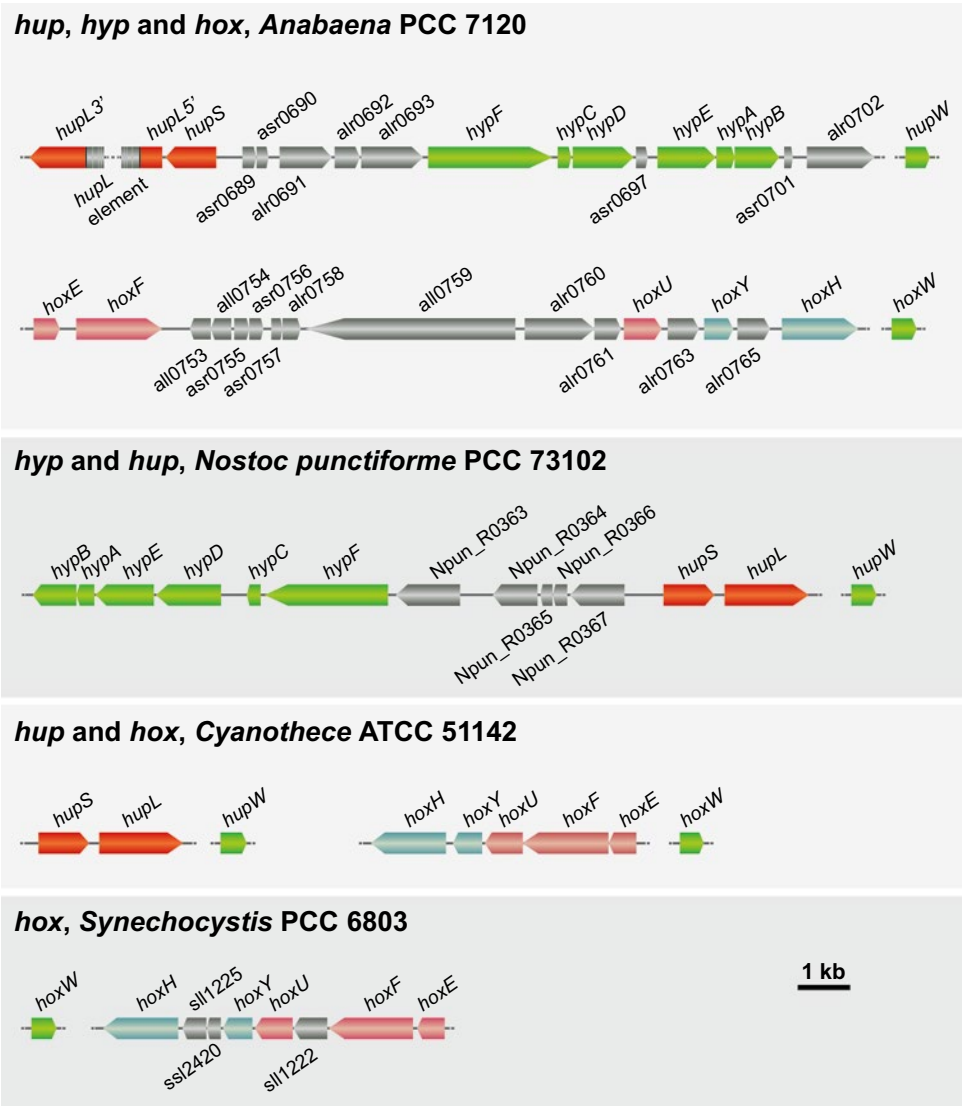

Fig. 6 Organization of the loci containing the genes encoding the uptake hydrogenase (*hup*), the bidirectional hydrogenase (*hox*) and the hydrogenase maturation genes (*hyp*) in selected cyanobacterial strains. In *Anabaena* PCC 7120 and *Nostoc punctiforme* PCC 73102, the maturation *hyp* genes are clustered near the structural *hup* genes; in *Cyanothece* ATCC 51142 and *Synechocystis* PCC 6803, the *hyp* genes are spread throughout the genome (not depicted). In *Anabaena*, the large subunit-encoding gene *hupL* is only formed after rearrangement upon heterocyst development, under nitrogen-fixing conditions. The *hoxUFE* and *hoxYH* structural genes are found in *Anabaena*, *Cyanothece* and *Synechocystis* in close clusters. The *hupW*/*hoxW* maturation proteases, specific for HupL/HoxH maturation respectively, are present in strains containing the corresponding *hup*/*hox* operons

large subunit HupL and the small subunit HupS (approximately 60 kDa and 30 kDa respectively) (Houchins and Burris 1981a, b; Lindberg et al. 2000). The large subunit contains the active site and is characterized by a dimetallic NiFe center, while the small subunit comprises iron-sulfur clusters that mediate the electron transport from the active site to the electron acceptor. Studies on the transcription of *hupSL* revealed that the enzyme is co-transcribed as a single operon with the gene encoding the smaller subunit located upstream from the gene encoding the larger one (Oxelfelt et al. 1995; Happe and Schühme 2000; Lindberg et al. 2000; Oliveira et al. 2004; Carrasco et al. 2005). However, in several heterocystous strains including *Anabaena* PCC 7120, the chromosomal arrangement of the *hupL* gene is intervened by a 10.5 kb element (Golden et al. 1985), as mentioned above. Further studies have shown that under nitrogen-fixing conditions, the mRNA comprises the *hupSL* transcripts alone. This indicated that under specific conditions *Anabaena* PCC 7120 undergoes a programmed DNA rearrangement by a site-specific recombinase that is encoded at one end of the *hupL* element (Carrasco et al. 2005).

The NtcA transcription factor that regulates nitrogenase is also found to regulate *hupSL* transcripts (Weyman et al. 2008). The binding site ($GTAN_8TAC$) of NtcA is highly conserved and is found approximately 40 bp from the transcription start site. *Anabaena variabilis* is an exception where the binding site has been identified 427 bp upstream of the transcription start site (Weyman et al. 2008). Additionally, several other factors may control the expression of HupSL, including the availability of nickel, anaerobiosis, the presence of H_2, and the absence of combined nitrogen. The expression of the enzyme may proceed in parallel with heterocyst formation (Happe and Schühme 2000). The enzyme is also known to be regulated at the post-transcriptional level, i.e. activated by photosynthetically-reduced thioredoxin (Papen et al. 1986). This may be attributed to the fact that

more hydrogen is released during light due to the high demands of ATP and reduced ferredoxin by the enzyme (Bothe et al. 2010).

In an effort to increase hydrogen production from filamentous nitrogen-fixing cyanobacteria, several uptake hydrogenase deletion mutants have been studied in strains such as *Anabaena variabilis* ATCC 29413 (Happe et al. 2000), *Anabaena* PCC 7120 (Carrasco et al. 1998), *Nostoc punctiforme* PCC 73102 (Lindberg et al. 2002), and *Nostoc* PCC 7422 (Yoshino et al. 2007). Studies showed almost three times increase in hydrogen production on deletion of the hydrogen uptake genes. These results suggest that the role of uptake hydrogenases is primarily associated with the oxidation of hydrogen released from nitrogenase. Breakdown of hydrogen into protons may serve essentially two functions within the organism:

(i) It provides the organism with ATP via the oxyhydrogen (Knallgas) reaction. In this reaction, the hydrogen evolved by nitrogenase is consumed in an oxygen-dependent reaction within the heterocysts. The reaction works therefore as an additional protection mechanism to maintain the activity of the oxygen-labile nitrogenase.
(ii) It supplies reducing equivalents (electrons) to nitrogenase or towards other cell functions.

Absence of uptake hydrogenase activity signifies loss of electrons/energy for the cell. Thus it may be hypothesized that the cell may downregulate the nitrogenase activity as a compensatory measure. However, comparison of the nitrogenase activity of the *hup* deletion mutants with the corresponding wild type strains showed either comparable or slightly enhanced nitrogenase activity. This indicated that some alternate measures were activated to compensate for the loss of energy. This was further investigated by Ekman et al. (2011) by large scale proteomic analysis of the *Nostoc punctiforme* PCC 73102 uptake mutant NHM5 developed by Lindberg et al. (2002). Their studies indicated that the inactivation of uptake hydrogenase in *N. punctiforme* changed the overall metabolism, in particular around processes involved in the scavenging of oxygen and reactive oxygen species, as well as in pathways related to nitrogen fixation. This allowed the identification of suitable targets for optimization of nitrogenase-based hydrogen photogeneration.

Cellular Localization of the Uptake Hydrogenase

The cellular localization of the uptake hydrogenase is a debatable question. In *E. coli*, the uptake hydrogenase is characterized by a TAT (Twin Arginine Translocation) signal at the N-terminus, which causes the transport of the matured protein to the periplasm. In cyanobacteria, however, this signal is characteristically absent (Penfold et al. 2006). Thus, uptake hydrogenase in cyanobacteria is localized on the cytoplasmic side of the cytoplasmic or thylakoid membrane. Available molecular data indicate that the enzyme is a membrane-associated enzyme, but the available hydropathy profiles fail to indicate a transmembrane domain (Tamagnini et al. 2002). This has led to speculations that the enzyme may be attached to the membrane by another polypeptide that serves as an anchor. In *E. coli*, a third subunit HupC is found to anchor the enzyme to the membrane (Allakhverdiev et al. 2010). However, to date no molecular evidence has been found for a HupC-homolog in cyanobacteria. Analysis of the sequenced genomes revealed the presence of ORFs whose products could potentially fulfill this anchoring role (Lindberg 2003). These anchor units in cyanobacteria are believed to be *b*-type cytochromes that participate in electron transfer from the small subunit to the respiratory electron transport chain (Tamagnini et al. 2002).

The subcellular localization of uptake hydrogenases along the filaments is also a debatable question. Though the activity of uptake hydrogenases is generally believed to be localized in the heterocysts in close association with nitrogenases, immunolocalization studies carried out in *Nostoc punctiforme* have shown expression of the *hupSL* genes in both vegetative cells and heterocysts (Martinho 2009). Recent studies conducted by Camsund et al. (2011) give some new perspectives. They constructed a reporter construct consisting of the green fluorescent protein (GFP) translationally fused to HupS, within the complete *hupSL* operon. The construct was electroporated into *N. punctiforme* PCC 73102. Laser scanning confocal microscopy and fluorescence imaging showed the presence of GFP exclusively in the heterocysts. This indicated that the presence of uptake hydrogenases occurs in close association to nitrogenases in *Nostoc punctiforme*. Additionally, in *Anabaena* PCC 7120, programmed DNA rearrangement of the *hupL* under nitrogen fixation indicated the strict confinement of HupL to the heterocyst (Carrasco et al. 1998, 2005).

4.2.2 Cyanobacterial Bidirectional Hox-Hydrogenases

The bidirectional Hox-hydrogenase is not unique to cyanobacteria nor is it universal. Hox-hydrogenases have been identified in other bacteria, including the Gram-positive bacterium *Rhodococcus opacus*, the Gram-negative bacterium *Ralstonia eutropha*, and the purple sulfur photosynthetic bacteria *Thiocapsa roseopersicina* and *Allochromatium vinosum*. In fact, phylogenetic analysis has shown that the origin of HoxH and HupL in cyanobacteria can be traced to the non-sulfur bacterium *Chloroflexus aurantiacus* (Carrieri et al. 2010). The Hox-hydrogenase can be found both in nitrogenase and non-nitrogenase-containing cyanobacteria; however, its distribution appears to be random (Kentemich

et al. 1989; Serebriakova et al. 1994). Though it is found in most of the cyanobacteria, Hox-hydrogenase is characteristically absent in most of the marine strains isolated from the ocean surface, suggesting its requirement for microaerobic/anaerobic conditions, a highly unlikely environment in the open surface of oceans. Also, its presence does not depend on the morphology of the organism. For instance, both unicellular *Synechocystis* and filamentous *Anabaena* are known to possess the enzyme.

Cyanobacterial Hox-hydrogenases are characterized by their oxygen labile nature, thermotolerance, and high affinity for hydrogen (Houchins 1984; Houchins and Burris 1981a, b). Methyl viologen assays, with methyl viologen acting as a promiscuous electron donor, support the hydrogen evolving nature of the hydrogenase. Further, it has been observed that the enzyme activity remains unaffected by the presence or absence of nitrogen within their micro-environment. This supports the fact that their activity, unlike that of uptake hydrogenase, is not associated with nitrogenase activity (Howarth and Codd 1985).

The Hox-hydrogenase is the only enzyme in cyanobacteria that is capable of reaching 10–13 % solar-to-hydrogen conversion efficiency (Carrieri et al. 2011). The enzyme is loosely attached to the thylakoid membranes and catalyzes a physiologically reversible reaction that converts protons and electrons to hydrogen gas. The redox partners of this enzyme are ferredoxin and/or NADPH/NADH, as shown in Eq. (3).

$$\mathrm{Fd}_{\mathrm{reduced}} \text{ and / or } \mathrm{NAD(P)H} + \mathrm{H}^{+} \leftrightarrow \mathrm{H}_{2} + \mathrm{Fd}_{\mathrm{oxidised}} \text{ and / or } \mathrm{NAD(P)}^{+} \tag{3}$$

As a potential hydrogen catalysis enzyme, the bidirectional Hox-hydrogenase has been very well studied. It consists of a complex comprising the HoxE, HoxF, HoxU, HoxY and HoxH proteins. The bidirectional hydrogenase was earlier thought to be composed of only four subunits, in which HoxFU constitute the diaphorase (NAD(P)H-oxidizing) part, and HoxYH constitute the [NiFe] hydrogenase part (Schmitz et al. 1995; Appel and Schulz 1996; Boison et al. 1999, 2000; Sheremetieva et al. 2002), as was the case with the most extensively studied Hox enzyme in *Ralstonia eutropha*. Later, HoxE was shown to co-purify with the active bidirectional enzyme, the cyanobacterial bidirectional hydrogenase has since been considered to be a heteropentameric enzyme encoded by *hoxEFUYH*, with HoxE belonging to the diaphorase part (Schmitz et al. 2002).

In the HoxYH complex, HoxH contains the catalytic core that catalyzes the oxidation of hydrogen/reduction of protons. The HoxY comprises a Fe_4S_4 cluster that facilitates the transfer of electrons to and from the catalytic site. The electrons consumed/released by the active site in HoxH flow through the small subunit, HoxY, and through the HoxEFU iron-sulfur clusters (Schimtz et al. 1995).

The Hox subunits that constitute the diaphorase moiety bear similarity to the respiratory complex I. It has been hypothesized that they may represent a part of the missing subunits of the respiratory complex (Schmitz and Bothe 1996; Appel et al. 2000; Appel and Schulz 1996). Cyanobacteria possess an incomplete version of respiratory complex I (NADH:Q oxidoreductase, or NDH I). Out of the minimal 14 subunits required to form the complex in *E. coli*, only 11 are found in cyanobacteria. The other three units, namely *nuoE*, *nuoF* and *nuoG*, encode the NADH dehydrogenase module, which in *E. coli* functions as the energy input device; consequently, the mechanism by which cyanobacteria use NAD(P)H via this enzyme is still unknown. Some of the conserved sequence motifs of the bidirectional hydrogenase are similar in the two corresponding complex I subunits, but apart from this there are only low sequence similarities. Mutant strains and wild type strains deficient in Hox subunits homologous to NDH I show similar rates of respiration. These include wild type *Nostoc punctiforme* (which does not possess a bidirectional hydrogenase) (Boison et al. 1999), and bidirectional hydrogenase deletion mutants of *Synechocystis* that show respiratory activity comparable to the wild type (Howitt and Vermaas 1999). More in-depth analysis is necessary to establish the role of the NDH I subunits as part of the respiratory mechanism. Mutants lacking the bidirectional *hox* structural genes have properties comparable to the wild type, reflecting the plasticity and metabolic adaptability of cyanobacteria.

Regarding the physical organization of the *hox* genes, the arrangement is not uniform (Fig. 6). In some cyanobacterial strains, all the *hox* genes are clustered in one operon but with multiple transcription start points, as in *Synechocystis* PCC 6803, while in others they are divided in two operons, as in *Anabaena* PCC 7120 (Sjöholm et al. 2007). The operon may be interrupted by one or several other unrelated ORFs, as in *Anabaena variabilis*, *Anabaena* PCC 7120, *Synechococcus* PCC 6301, and *Synechocystis* PCC 6803 (Tamagnini et al. 2002). Transcriptional studies indicate that the structural genes form a single transcriptional unit in *A. variabilis* ATCC 29413; in *Synechococcus* PCC 6301 two transcripts were detected (Boison et al. 2000).

Evolutionary Significance of Bidirectional Hox-Hydrogenase

The physiological significance of bidirectional hydrogenase is debatable. In most organisms it does not appear to perform any necessary function for sustenance. There are several hypotheses about the presence of Hox-hydrogenase. In cyanobacteria, the Hox-hydrogenase may function as a stress valve to remove the excess low potential electrons generated during photosynthesis, or the excess reductants from anaerobic metabolism, in the form of molecular hydrogen (Troshina et al. 2002; Tamagnini et al. 2007). Under other conditions,

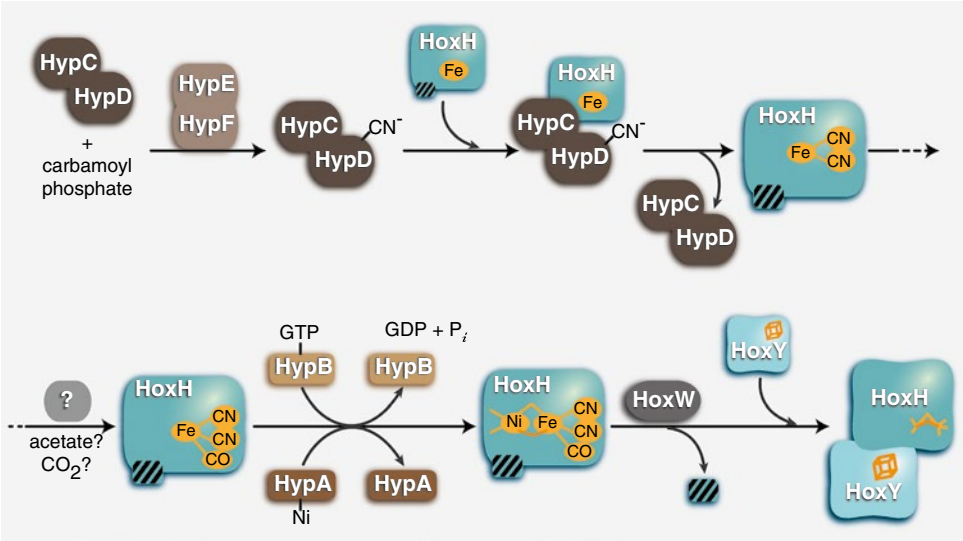

Fig. 7 Maturation of hydrogenases in cyanobacteria, exemplified by HoxYH assembly. A similar mechanism occurs with HupSL. HypEF are involved in the cyanide (CN^-) ligand synthesis from carbamoyl phosphate. CN^- is transferred by HypCD to the iron atom on HoxH (HupL). HypC interacts with HoxH, working as a chaperone to stabilize the protein. The HypCD complex is released and a CO ligand is added by an unknown mechanism. The nickel ion is inserted into the active site by HypA, with HypB providing energy from GTP hydrolysis. Correctly inserted nickel is a checkpoint for the protease HoxW (HupW) to cleave off the C-terminal end, which enables a conformal change of the large subunit making it possible for the small subunit to attach

it can also oxidize molecular hydrogen to deliver electrons to the respiratory chain (Schmitz et al. 1995). In the purple sulfur bacterium *Thiocapsa roseopersicina*, the Hox-hydrogenase catalyzes H_2 production both under dark, fermentative conditions, and in light when thiosulfate is present. Alternatively, it functions as uptake hydrogenase to oxidize H_2 in light under anaerobiosis (Rakhely et al. 2004). In *R. eutropha*, the Hox-hydrogenase functions in H_2 oxidation linked to the regeneration of NADH in support of carbon fixation (Burgdorf et al. 2005).

Respiration (NDH I) and nitrate assimilation (Nir and Nar) mutants exhibit increased H_2 photo-evolution rates by Hox-hydrogenase (Howitt and Vermaas 1999; Gutthann et al. 2007). Therefore, it has been hypothesized that the Hox-hydrogenase functions as an electron valve for cells using H_2 production/oxidation in response to changes in redox states. On the other hand, Hox deletion mutants do not appear to have any difference in growth profiles or photosynthetic activity (Pinto et al. 2012). In addition, as explained in the previous section, of all the Hox subunits, only HoxEFU are homologs for NuoEFG of respiratory complex I in cyanobacteria, but there is no evidence to date that these diaphorase subunits play any role in respiration (Boison et al. 1999; Howitt and Vermaas 1999).

4.2.3 Cyanobacterial Hydrogenase Maturation Proteins

Production of functional cyanobacterial hydrogenases, Hox and Hup enzymes, employs the activity of both the structural genes that form the chassis of the enzyme and the accessory maturation proteins that encode the chaperons and co-factors necessary for assembly of the active site and for correct folding. The maturation factors are encoded by the *hyp* (hydrogenase pleiotrophic) genes. It should be noted that the maturation factors known to date are only those encoding for proteins needed for the maturation of the hydrogenase part in the bidirectional enzyme (HoxYH) and the large subunit of the uptake hydrogenase (HupL). It is unknown if there are any other maturation proteins required for the assembly of the Hox diaphorase units, or for the small subunit HupS. For the synthesis and the insertion of the metallocenter, the gene products of *hypABCDEF* are required, in addition to ATP, GTP, and carbamoyl phosphate. Additionally, a specific protease, HoxW for the Hox-hydrogenase and HupW for the Hup enzyme, is needed to cleave off the last amino acids of the C-terminus of the large subunit of the respective hydrogenase (Fig. 7) (Böck et al. 2006; Vignais and Billoud 2007).

The *hyp* genes are conserved and can either be clustered, as in *Anabaena* PCC 7120 and *N. punctiforme*, or spread in the genome, as in *Synechocystis* PCC 6803 (Kaneko et al. 2001). Independently of type and number of hydrogenases, there is only one set of *hyp* genes per genome, indicating a co-regulation, or at least similar regulation, of the *hyp* genes on the assembly of both the Hox and the uptake hydrogenases. The only difference is with the protease, which is specific for each type of hydrogenase. The function of the various *hyp* genes has been well characterized only in *E. coli* and has not yet been separately verified in cyanobacteria. Their role is nevertheless considered to be homologous in both types of microorganisms.

Synthesis of the [NiFe] active site requires the synthesis and incorporation of the unusual ligands CO and CN^-. Interaction of the products of the two genes *hypE* and *hypF* is used to synthesize CN^-, using carbamoyl phosphate as precursor to form a thiocarbamate (Brazzolotto et al. 2006). However, the mechanism of incorporation of CO into the active site is still a debatable question. Various different metabolic origins have been suggested for its derivation (Forzi et al. 2007). According to Roseboom et al. (2006), generally acetate or CO_2 may serve as substrates. The current model describes the biosynthesis of the diatomic ligands and the coordination of Fe by CN^- and CO as representing independent steps in the assembly of the enzyme (Böck et al. 2006). Once the $Fe(CN)_2(CO)$ complex is established, Ni is introduced into the active site and is assembled without the help of accessory proteins. However, under physiological limitations, Ni is provided by a complex of maturation factors HypA and HypB. The *hypA* and *hypB* genes are usually clustered together in the genome and are involved in the Ni delivery and incorporation. While HypA is a Ni-binding metallo-chaperone, HypB provides GTPase activity, and therefore energy for the insertion of Ni and subsequent release of the maturation factors. The *hypC*, *hypD* and *hypE* genes are grouped together. The C-terminal region of HypD binds a Fe_4S_4 cluster and is able to form a complex with HypC. The HypC/HypD complex is further proposed to mobilize the CN^- for incorporation into the active site. This complex readily interacts with the large subunit of the enzyme. The final step in the maturation requires the cleavage of a C-terminal peptide of the large subunit by an endopeptidase. It has been suggested that the C-terminal peptide keeps the protein and active site in a particular conformation accessible to metal insertion and may even serve as the site of interaction between the large and the small subunits (Sawers et al. 2004).

4.2.4 Hydrogenase Transcriptional Regulation

Two factors control the transcription regulation of Hox-hydrogenase: the transcriptional regulators LexA and AbrB. LexA was identified as a protein interacting with the promoter region of the *hox* operon in *Synechocystis* PCC 6803 (Oliveira and Lindblad 2005), suggested to work as a transcription activator of the *hox* genes (Gutekunst et al. 2005). Moreover, two distinct DNA-binding regions, −198 and −338 bp in relation to the *hoxE* start site, have been identified in the promoter region of *hox* in *Synechocystis* PCC 6803. This suggests that the LexA mechanism of regulation may be through the formation of a DNA loop.

In addition to LexA, other transcription factors have been found to be involved in the regulation of *hox* operon. Oliveira and Lindblad (2008) showed that the gene product of *sll0359* (AbrB1) interacts with the *hox* promoter in *Synechocystis* PCC 6803. Experiments using partially segregated deletion mutants of the *abrB*-like gene as well as mutants overexpressing AbrB1 protein suggested that they may function as a regulator of the Hox-hydrogenase (Oliveira and Lindblad 2008). In addition, AbrB2 was recently shown to be a repressor of hydrogen production in *Synechocystis* PCC 6803 (Sakr et al. 2013). A cysteine residue of the AbrB2 protein has been identified as the target for glutathionylation, which affects the binding of AbrB2 on the *hox* operon promoter, as well as the stability of the AbrB2 protein.

5 Recent Advances and Goals to be Achieved in Cyanobacterial Hydrogen Production Research

The prospective production of hydrogen directly from water and sunlight has stimulated a strong interest of the scientific community in using photosynthetic microorganisms. However, hydrogen production from these organisms is fraught with several bottlenecks, including oxygen sensitivity of hydrogenase and inefficient utilization of solar light energy. Several of these bottlenecks have been discussed in detail by Khetkorn et al. (2013); selected issues are detailed below.

5.1 Genetic Engineering of Nitrogenase and Hydrogenase

The theoretical maximal hydrogen production from cyanobacteria can be determined as explained by Bothe et al. (2010). The stoichiometry of the reaction can be determined independently from the equations of nitrogen fixation and hydrogen production. According to the calculations of Bothe et al. (2010), only 20 μmol NH_4^+ can be stoichiometrically produced per hour per milligram of chlorophyll. Consequently, only 40 μmol H_2 can theoretically be produced per hour per milligram of chlorophyll. This is based on the assumption that four electrons are required to produce 1 mol of NH_4^+ and only two electrons are required to produce 1 mol of hydrogen. Considering this, the model organisms amenable to genetic engineering have already demonstrated the theoretical maximum hydrogen production. Therefore, it cannot be hoped that changes in process conditions alone will initiate any significant differences in the amount of hydrogen produced. However, with the advent of synthetic biology and systems biology, it is hoped that sustained research efforts in this direction may bring about a significant difference (Huang et al. 2010; Heidorn et al. 2011).

Enzyme engineering has been shown to hold the key in improvement of hydrogen production from organisms. As discussed before, in cyanobacteria nitrogenase, bidirectional Hox-hydrogenase, and uptake Hup-hydrogenase are the key enzymes related to hydrogen production. Different groups

around the world have tried to engineer the enzymes to switch their bias towards hydrogen production. Among the first such proofs-of-concept was the successful demonstration by Masukawa et al. (2010) of engineering of *Anabaena* PCC 7120 nitrogenase to tilt its bias away from nitrogen reduction towards hydrogen production. Masukawa and coworkers developed 49 variants on the basis of changes in 6 amino acid positions of the 19 amino acids found within 5 Å of the FeMo active site of nitrogenase. The choice of the amino acid substitution was based on the highly conserved structure of *Azotobacter vinelandii* nitrogenase. Interestingly, in *A. vinelandii* there are a number of natural mutants whose nitrogenase enzyme can reduce protons but not nitrogen; however, there are no natural mutants that reduce nitrogen but not protons. This occurrence suggests that proton reduction is essential for enzyme activity (Kim et al. 1995; Dilworth et al. 1998). Masukawa et al. (2010) found that several of the mutants that they had developed synthetically demonstrated significantly increased in vivo rates of hydrogen production. Amongst these, one mutant in particular, where the arginine at position 284 had been replaced by histidine, was found to be very promising. The cumulative hydrogen production by this strain over 1 week was found to be enhanced by 87 % over the reference strains.

Another radical approach may be to express foreign hydrogenases within the cyanobacterial systems that could possibly be coupled and exploited to degrade the photosynthetically-produced organic carbon for maximal hydrogen production. Over the years, limited success has been achieved in this field. The topic has been explained in detail by Khetkorn et al. (2013) and includes the recent study by Ducat et al. (2011) that successfully demonstrated the expression of non-native hydrogenases in *Synechococcus*. The economic feasibility of scale-up of such a process is yet to be ascertained.

In the future, it may be more rewarding to focus on development of oxygen-tolerant versions of these high hydrogen-evolving enzymes. Cournac et al. (2009) demonstrated genetic alterations of amino acids in the gas-substrate channel of [NiFe] hydrogenases that changed their intramolecular gas transport kinetics. Similarly, Leroux et al. (2008) showed that substitutions of two amino acids at the end of the channel (valine and leucine, both replaced by methionine) made the [NiFe] hydrogenase from the sulfate reducing bacterium *Desulfovibrio fructosovorans* more oxygen-tolerant. [NiFe] hydrogenases have lower potential to generate high rate hydrogen as compared to the [FeFe] hydrogenases, therefore similar genetic engineering of a [FeFe] hydrogenase could be promising. Some naturally occurring oxygen-tolerant [NiFe] hydrogenases have been described, in particular the membrane-bound hydrogenases from *Escherichia coli*, *Ralstonia eutropha*, and *Aquifex aeolicus* (Lukey et al. 2010; Goris et al. 2011; Pandelia et al. 2011). These particular enzymes owe their tolerance to O_2 to the presence of a unique six-cysteine-coordinated Fe_4S_3 proximal iron-sulfur cluster in the small subunit. This unusual iron-sulfur cluster is able to harbor high oxidation states that allow reduction of O_2 at the active site, thus protecting it from oxidative inactivation (Wulff et al. 2014). Heterologous expression of modified hydrogenases in cyanobacteria could allow their coupling directly with ferredoxin and the photosynthetically generated reducing power, while being insensitive to the photosynthetically produced O_2.

5.2 Increasing Reductant Supplies for Enhanced Hydrogen Production

Some scientists are of the opinion that hydrogen production from photosynthetic organisms is not limited by hydrogenase, but rather by the availability of the reductants (Ananyev et al. 2008). During indirect biophotolysis, the intermediate compounds may enter either the oxidative pentose phosphate pathway or the glycolysis pathway, generating NADPH or NADH as reductant respectively. In the OPP pathway, the C3 compound is stripped of hydrogen to produce NADPH and carbon dioxide as by-products, whereas in the glycolysis pathway the compound is only partially stripped of hydrogen to generate NADH and organic acids. The reductant yield differs threefold for OPP versus glycolysis; however, entry into each of these pathways is highly regulated. The reductant thus generated is used as substrate by hydrogenase to produce molecular hydrogen. Therefore, it has been proposed that enhancing the flux through the OPP pathway may help enhance hydrogen production (Skizim et al. 2012). A proof-of-concept was demonstrated by Woodward et al. (2000), who showed that a synthetic in vitro OPP circuit linked to hydrogenase was capable of producing 11.6 mol H_2 mol^{-1} glucose, close to the theoretical maximum of 12 mol H_2 mol^{-1} glucose.

Similarly, it has been suggested that increasing the carbon reserve as glycogen may also accelerate hydrogen production by enhanced catabolism. This was observed in salt tolerant strains where environmental stress increases the carbohydrate accumulation, which then increases the rate of catabolism eightfold (Carrieri et al. 2010). Earlier, McNeely et al. (2010) showed that the rate of NAD(P)H generation under dark auto-fermentative conditions is limited by the rate of catabolism of sugar and is feedback-inhibited by the accumulation of glycolytic. They showed that, by developing a lactate dehydrogenase deletion mutant, hydrogen production could be increased in *Synechococcus* PCC 7002. This increase was caused by lactate competing for the same reductant as hydrogenase. Elimination of competitors to reductants has thus been suggested as a successful strategy to enhance hydrogen production. Additionally, modifying

metabolic bottlenecks that limit the rate of carbohydrate catabolism may enhance the rate of hydrogen production. Overexpression of enzymes related to the glycolytic pathway is also considered as one of the strategies to enhance hydrogen production by enhancing auto-fermentation. In view of this, Kumaraswamy et al. (2013) recently developed overexpression and knockout mutants of GAPDH1 enzyme (NAD^+-dependent glyceraldehyde-3-phosphate dehydrogenase) in *Synechococcus* PCC 7002. The choice of this enzyme was crucial, as it is situated at the major point of regulation between the glycolysis and the OPP pathway. Overexpression of the enzyme resulted in increased accumulation of glycogen reserves and a twofold increase in the NADH pool. Subsequent dark autofermentation led to obviously enhanced hydrogen yields. The knockout strain showed enhanced flux towards the OPP and upper glycolysis pathway, which resulted in a 5.7-fold increase in the NADPH pool and a subsequent 2.3-fold increase in hydrogen production.

5.3 Fine-Tuning the Cyanobacterial Photosynthetic Machinery for Maximal Hydrogen Production

Although cyanobacteria appear as ideal candidates for hydrogen production, they need to be further optimized for maximization of their hydrogen production potential. This includes optimization of the PSII/PSI ratio, the linear/cyclic electron transport ratio, and the antenna size of the light harvesting complex in relation to the cell mass (Bernát et al. 2009). The evolutionary adaptation of cyanobacteria is such that they have a lower PSII/PSI ratio as compared to eukaryotic algae and higher plants (Fujita and Murakami 1987). Studies have shown that due to the reduced PSII/PSI ratio, most of the absorbed photons are used in cyclic electron transport as compared to the net flow through the linear electron transport chain. Over the years, different groups have tried to decrease the antenna size by various knockout mutants. The first such mutant was developed by Rögner et al. (1990), where a mutant (Olive) was created that lacked phycocyanin. This was followed by Shen et al. (1993), who developed an *apcE*-less mutant lacking a 99 kDa core membrane protein linker, which resulted in an impaired binding between phycobilisomes (PBS) and photosystems. Later, Ajlani and Vernotte (1998) developed the PAL mutant, which lacked the complete PBS proteins. Recently, Bernát et al. (2009) carried out a comparative hydrogen production analysis from the mutants. They concluded that the Olive and PAL mutants were strong candidates for future genetic manipulation studies, since both of them displayed an enhanced PSII/PSI ratio accompanied by an increased pool of photosynthetically derived reductants. The authors further showed that while the PAL mutant increased the level of enzymes related to oxidative and osmotic stress to compensate the over-reduction of the cellular metabolism, the Olive mutant compensated this effect by an up-regulation of carbon fixation. The group thus suggested a phycocyanin-less Olive mutant being the most promising candidate as a design cell for future hydrogen production studies.

5.4 Use of RNA Sequencing and Proteomics Analysis to Improve Hydrogen Production

To further improve hydrogen production from cyanobacteria, specific knowledge of the bottlenecks in all the pathways and processes is required. Detailed analysis of the transcriptome and proteome under different physiological conditions could help identify these limitations and also identify potential target areas for improvement. Recent whole genome sequencing analysis has helped to make this possible. Several studies have been published on RNA sequencing and proteomic analysis of *Chlamydomonas reinhardtii* under different physiological conditions for hydrogen production (Mus et al. 2007; Nguyen et al. 2008). There are however very few published reports in cyanobacteria to date. These include the studies by Pinto et al. (2012), where the authors compared the proteomics of wild-type *Synechocystis* PCC 6803 and the *hoxYH* deletion mutant. The mutant was characterized at different levels: physiological, proteomic and transcriptional. Proteomic analysis revealed that, under conditions favoring hydrogenase activity, only a few proteins, mostly related to the redox and energy state of *Synechocystis*, presented significant changes in the mutant, as compared to the wild-type. These results, in conjunction with the absence of significant differences in growth patterns, suggested that *Synechocystis* is able to easily adapt to different metabolic settings, and can therefore be used as a robust chassis for expression of synthetic constructs. The study also shows that *hox* may serve as an additional neutral site where other genes of interest related to hydrogen production can be expressed. Finally, the authors demonstrated that hydrogen production forces the cell to deal with redox and intracellular energy balances.

For future studies in non-model organisms, it may be ideal to try hybrid approaches of RNA sequencing with proteomics analysis to get the holistic picture, since effective proteomic profiling is generally dependent on high quality DNA reference databases. The integrated "omics" analysis may provide a comprehensive view of the complicated molecular mechanisms employed by cyanobacteria for hydrogen production, and may allow identification of a series of potential gene candidates for knockouts to channel the electron flow towards hydrogen production.

5.5 International Efforts in Promoting Biohydrogen for Future Energy Use

In spite of sustained research efforts, there is still a wide gap between the demand and supply to produce and store hydrogen. Concerted research efforts are required by governments around the world to ensure the realization of a hydrogen economy. To be economically competitive with today's fossil fuel economy, the cost of hydrogen production must be reduced by a factor of at least four (Hydrogen and fuel cell program, DOE 2009 report, USA). By sponsoring long-term innovative basic research, the US DOE Hydrogen and Fuel Cells Program is working to bridge this gap. Additionally, different energy programs have been drafted by various energy agencies to envision a hydrogen road map for the successful implementation of this technology. In its 2013 energy report, the International Energy Agency has considered it important to improve and develop processes for hydrogen production from renewable sources. The agency has considered this as one of the prerogatives in its track of clean energy. Further, the EU strategic energy technology plan (2008) has considered hydrogen as an important source of alternate fuel to meet the demands of the future energy. Advancing ahead, Japan recently announced the possible commercialization of an innovative technology that produces hydrogen from biomass, specifically from sewage sludge (News article, Staff reporter 2012). It is still a long way before the ideal way of producing hydrogen from sunlight and water can be scaled up and commercialized.

6 Conclusions

If harnessed with greater care, cyanobacteria have the potential to power the future hydrogen economy. In this direction, this review article gives a holistic picture of the physiology, enzymology and recent advances in cyanobacterial hydrogen production. Despite sharing similar ancestral origin and close evolutionary proximity, different genera and even different strains within the same genus have evolved different physiological strategies to release their excess reductant stress in the form of molecular hydrogen. Understanding the varied mechanisms is central to enhancing the hydrogen production prowess of these mini-cell factories. Significant research efforts have been made in understanding the key enzymes involved in hydrogen production. Several research milestones have been achieved in enhancing the hydrogen production from cyanobacteria, including demonstration of expression of foreign hydrogenases, alterations in the stored glycogen capacity and engineering of related enzymes to trend their bias towards hydrogen production. Though several technical achievements have been made, successful implementation of the hydrogen economy requires much more commitments and sustained efforts.

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Dark Respiration and Organic Carbon Loss

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1 Introduction

Algae are major contributors to global primary productivity, assimilating approximately 50 % of the CO_2 converted annually into organic matter (see Beardall and Raven (2016), chapter "Carbon Acquisition by Microalgae", in this volume). Microalgae, especially the phytoplankton in the oceans, are responsible for a significant proportion of this. Running counter to the reductive assimilation of inorganic into organic carbon are a number of processes that see organic carbon oxidised to yield energy and provide C skeletons for growth processes. These are generally lumped together under the overall heading of "respiration" but include a range of different metabolic pathways. Matters are further complicated by the complexity of trophic modes found in algae, which include not just photoautotrophy, but also mixotrophy, osmochemoorganotrophy and phagochemoorganotrophy, processes that involve cells taking up organic carbon compounds or particles from the external environment.

Some respiratory processes such as photorespiration and chlororespiration involving the plastid terminal oxidase (PTOX) are closely aligned with photosynthesis. This is also the case of the Mehler-Peroxidase reaction involving both photosystems and ascorbate peroxidase, as well as PTOX consuming electrons from Photosystem II, although these involve the consumption of oxygen without a concomitant breakdown of organic C to CO_2 (Beardall et al. 2003; Cardol et al. 2008). Others though are not directly associated with photosynthesis or the photosynthetic apparatus and these are usually termed "dark respiration", though as we shall see these processes also occur in the light and their rates may be influenced indirectly by irradiance.

Broadly speaking "dark" respiratory rates average ~10–15 % of gross photosynthetic rates (Raven 1976a, b; Geider and Osborne 1989; Beardall and Raven 1990), though variation between species can be quite large and some taxa such as dinoflagellates appear to have higher ratios of respiration to photosynthetic C assimilation than other groups (Geider and Osborne 1989).

In this chapter we examine the evidence for different "dark" respiratory metabolic pathways and consider their energetics, their roles in maintenance and growth processes and how, in photoautotrophic cells, respiration rates are affected by light. We also consider the role of respiration in mixotrophy and chemoorganotrophy.

2 Definition of the Trophic States of Algae with Relevance to Respiration

Modified from Flynn et al. (2013) and Raven et al. (2013).

Autotroph An organism using exergonic inorganic chemical reactions (chemolithotroph), or photons (photolithotroph) as the energy source for growth, and inorganic chemicals taken up on a molecule by molecule basis across the plasmalemma to supply nutrient elements. In the case of microalgae autotrophy almost invariably involves photolithotrophy.

Chemolithotroph An organism using the catalysis of exergonic inorganic chemical reactions as the energy source for growth and inorganic chemicals taken up on a molecule-by-molecule basis across the plasmalemma to supply nutrient elements. Widespread in Archaea and Bacteria, generally only occurs in Eukarya by means of symbiosis with Archaea or Bacteria. While CO_2 can be reduced to carbohydrate in hydrogenase-expressing algae in the dark when supplied

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with O_2, CO_2 and H_2, it is not likely that this can support growth (Gaffron 1944).

Chemoorganotroph An organism using the catabolism of exogenously derived organic compounds as their energy source for growth and organic compounds as the source of carbon and, in many cases, the source of nitrogen. The carbon skeletons needed for biosynthesis in growth are, if not supplied in the mixture of organic carbon compounds supplied, produced in respiration by the catabolism of some organic compound that is supplied.

Heterotroph = Chemoorganotroph.

Osmochemoorganotroph A chemoorganotrophic organism taking up organic and inorganic nutrients on a molecule-by-molecule basis across the plasmalemma as the means of acquiring carbon and other elements.

Mixotroph An organism combining autotrophy (in this case photolithotrophy) and chemoorganotrophy.

Phagochemoorganotroph A chemoorganotrophic organism taking up particles of live or dead organic matter as the means of acquiring carbon and other elements.

Photolithotroph An organism using photons as the energy source for growth and inorganic chemicals taken up on a molecule by molecule basis across the plasmalemma to supply nutrient elements.

Saprochemoorganotroph = Osmochemoorganotroph

3 Respiratory Pathways and Their Distribution among Algae

3.1 Embden-Meyerhoff Parnas (EMP), Entner-Doudoroff (ED) and Oxidative Pentose Phosphate (OPP) Pathways: Cytosol-Plastid-Mitochondrion

Most microalgae use the EMP pathway to convert hexose to pyruvate, with some partial duplication in the cytosol and the plastid stroma (acknowledging that some of the enzymes are common to the gluconeogenetic pathway converting triose phosphate from the Calvin-Benson cycle into compounds such as storage poly-hexoses, linear or cyclic polyols, and disaccharides). Triose phosphate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase enzymes occur not just in the plastid and cytosol of diatoms, but also in the mitochondria (Liaud et al. 2000). A further novelty of diatoms is the occurrence of the ED pathway, which is rare in eukaryotes, that, as well as the EMP pathway, also converts hexose to pyruvate (Fabris et al. 2012). The analysis of the *Phaeodactylum tricornutum*[1] genome by Fabris et al. (2012) shows that the EMP pathway occurs in the cytosol and also in the stroma, while the ED pathway and the glyceraldehyde-3-phosphate to pyruvate part of the EMP pathway, occurs in the mitochondrial matrix. The OPP pathway occurs in the diatom cytosol (Fabris et al. 2012). Flamholz et al. (2013) discuss the inefficiency, as net ATP produced in the conversion of hexose to pyruvate, of the ED pathway relative to the EMP pathway in terms of the trade-off between rate and efficiency and the cost of producing the metabolic machinery necessary to produce one mol ATP in a given time; see also Raven and Ralph (2015).

3.2 Anaerobic Metabolism

Energy transformation by microalgae under anaerobic conditions has been known for several decades to be quite complex (Raven 1984). Genomics has significantly broadened our understanding of the biochemical and compartmental diversity of anaerobic energy metabolism in unicellular photosynthetic eukaryotes (Atteia et al. 2013), with implications for the production of algal biofuels.

3.3 Mitochondria: The Tricarboxylic Acid Cycle (TCAC)

Danne et al. (2012) used genomic and transcriptomic data on the alveolates to show that dinoflagellates, like apicomplexans but unlike the ciliates, lacked pyruvate dehydrogenase, the enzyme which normally takes pyruvate produced by the EMP pathway and transported into mitochondria, or produced within mitochondria from malate entering from the cytosol, to produce acetyl CoA and NADH in the mitochondrial matrix (Fig. 1). Experiments in which ^{13}C-labelled glucose was fed to intact dinoflagellates showed the typical labelling pattern found in aerobic organisms containing pyruvate dehydrogenase, so there must be some uncharacterised enzyme which carries out the conversion of pyruvate to acetyl CoA (Danne et al. 2012). The dinoflagellates and apicomplexans also lack the NADH-dependent isocitrate dehydrogenase in the mitochondrial matrix, coincident with the loss of complex I of the mitochondrial electron transport chain which is specific for NADH, and its replacement with a NAD(P)H-UQ oxidoreductase (see below) (Fig. 2).

[1]Wherever possible the currently accepted names for species are used. The name used in the paper cited is also indicated. For details of names see chapter "Systematics, Taxonomy and Species Names: Do They Matter?" of this book (Borowitzka 2016).

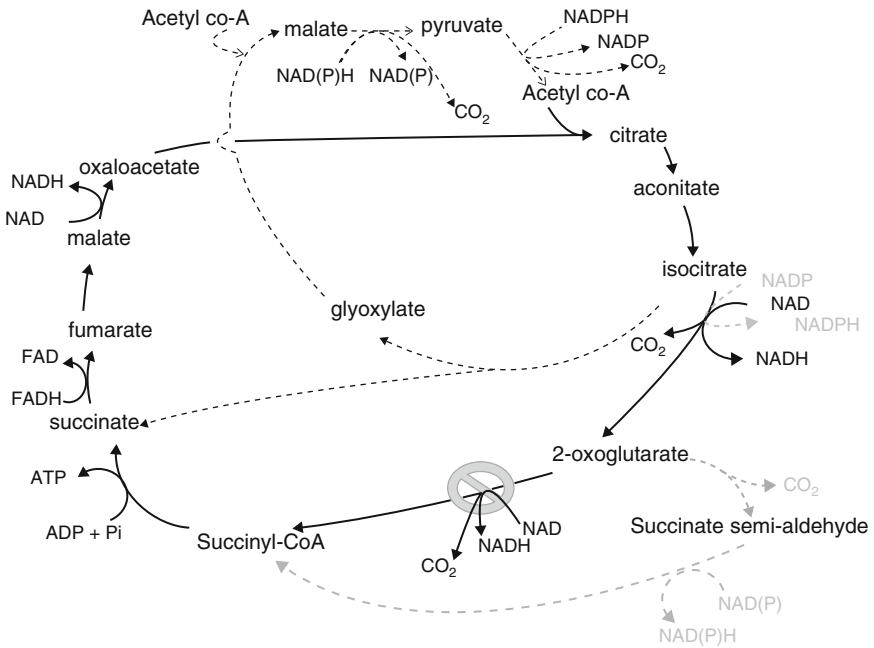

Fig. 1 The TCAC and its variations in cyanobacteria and some eukaryotic microalgae. The 'normal' reactions of the TCAC are shown in *black* and indicate the complete oxidation of acetyl CoA to CO_2 and the generation of reductant as NADH and FADH, as well as substrate level phosphorylation in the conversion of succinyl CoA to succinate. As detailed in the text, dinoflagellates and apicomplexans lack NADH-dependent isocitrate dehydrogenase, using a NADPH-dependent form of the enzyme instead (*grey*). In *Euglena gracilis* and many cyanobacteria, the step from 2-oxoglutarate to succinyl CoA and then to succinate is replaced by 2-oxoglutarate decarboxylase, converting 2-oxoglutarate to succinate semialdehyde, and a succinate semialdehyde:NAD(P)$^+$ dehydrogenase which converts succinate semialdehyde into succinate (*dashed grey lines*). Also represented here (*dotted black lines*) is a glyoxylate cyce, found *inter alia* in cyanobacteria. It is a means of completely oxidizing the acetyl moiety of acetyl CoA from the catabolism of stored lipid or exogenous acetate to CO_2 in the absence of a complete TCAC (Raven and Beardall 1990), a role which may be less widespread with the occurrence of the bypass of the block in the TCAC caused by the absence of isocitrate dehydrogenase (See text for more details)

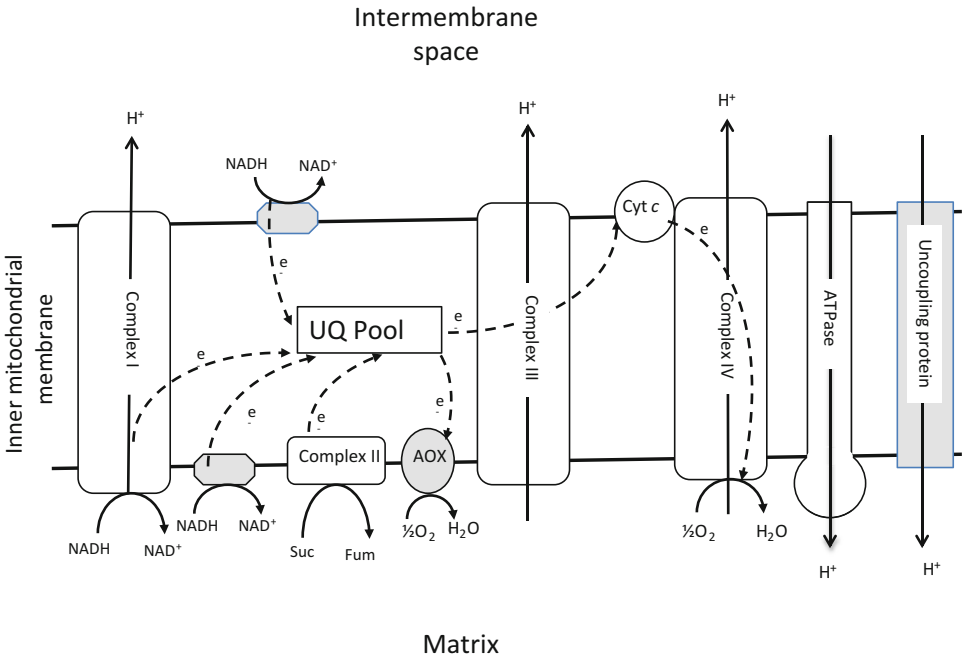

Fig. 2 Mitochondrial electron transport chain (ETC) in the inner mitochondrial membrane. Variations in the 'conventional' (unshaded complex) ETC are shaded, starting with a proton pumping (Complex I) NADH-UQ oxidoreductase oxidizing internal NADH, and a non-proton pumping NADH-UQ oxidoreductase oxidizing external NADH. Most algae also contain an alternate oxidase (AOX) that bypasses the proton-pumping Complexes II and IV. Dinoflagellates lack Complex I, using a non-proton pumping NADH-UQ oxidoreductase oxidizing internal NADH instead. The uncoupling protein performs the same functions as the AOX plus non-proton pumping NADH-UQ oxidoreductases, but is less well characterized in algae (See text for more details)

The mitochondria of *Euglena gracilis* lack 2-oxoglutarate dehydrogenase, the usual means of converting 2-oxoglutarate to succinyl CoA in mitochondria. Instead, they have 2-oxoglutarate decarboxylase, converting 2-oxoglutarate into succinate semialdehyde, and a succinate semialdehyde:NAD(P)$^+$ dehydrogenase which converts succinate semialdehyde into succinate (Shiheoka et al. 1986) (Fig. 1). Many cyanobacteria also lack 2-oxoglutarate dehydrogenase, and in a number of cases examined they also have the two-enzyme sequence found in *Euglena* replacing 2-oxoglutarate dehydrogenase (Zhang and Bryant 2011). The presence of this bypass negates the argument, discussed by Droop (1974), that the absence of a means of converting 2-oxoglutaratae to succinate could cause obligate photolithotrophy. The 2-oxoglutarate dehydrogenase – succinyl CoA thiokinase sequence generates, from the conversion of 2-oxoglutarate to succinate, not only NADH but also a 'substrate level' GTP or ATP, while the two enzyme bypass (Shiheoka et al. 1996) only generates the NAD(P)H. *Euglena gracilis* produces δ-aminolevulinic acid in its plastids for synthesis of chlorins and hemes from glutamate via the tRNA-dependent pathway five-carbon pathway, but mitochondrial hemes are produced via the 'animal' pathway involving glycine and succinyl CoA (Mayer and Beale 1992). Since *Euglena gracilis* does not produce succinyl CoA in its mitochondria from 2-oxoglutarate dehydrogenase, another source of succinyl CoA is required; this requirement is met by succinyl CoA synthetase, an enzyme which uses a nucleoside triphosphate such as GTP or, perhaps, ATP (Mayer and Beale 1992).

3.4 Mitochondria: Redox Chain (Complexes I–IV) and Proton Pumping from the Matrix

The redox chain in the inner mitochondrial membrane of algae (see Lloyd 1974; Cardol et al. 2005; Falkowski and Raven 2007; Raven and Ralph 2015) has not been subject to much experimental investigation, e.g. measurement of the proton to electron stoichiometry for electrons moving from NADH in the matrix through complexes I, III and IV to O_2. However, there are genomic and proteomic studies. As in most eukaryotes other than metazoa, there is an alternative oxidase as well as cytochrome oxidase, and an external (outer surface of the inner mitochondrial membrane) NAD(P)H dehydrogenase which transfers electrons to UQ without the proton pumping that occurs in complex I. Complex I takes electrons from matrix NADH to UQ, with the pumping of 4 (or perhaps only 3; Wikstrom and Hummer 2012) protons per pair of electrons. Analysis of the dinoflagellate genome (Danne et al. 2012; cf. Remakle et al. 2001) shows that these organisms lack the proton-pumping complex I of the inner mitochondrial membrane; this was lost in the base of the apicomplexan-dinoflagellate clade of alveolates. Instead of complex I there is a NADH-UQ oxidoreductase in the inner mitochondrial membrane that does not pump protons. The dinoflagellates have the typical non-proton-pumping complex II (succinate-UQ oxidoreductase) as well as the proton-pumping complexes III and IV which together transfer electrons from reduced UQ to O_2. This lack of complex I means that the number of protons pumped per electron from matrix NADH transferred to O_2 is decreased, in the conventional view, from 5 to 3 (Falkowski and Raven 2007), or from 4.5 to 3 if Wikstrom and Hummer (2012) are correct. In the conventional view this means a 40 % decrease in the protons transported, and hence in the ATP produced, per electron transferred from matrix NADH to O_2. Could this, at least in part, be the reason for the relatively high ratio of mitochondrial respiration to light-saturated photosynthesis in dinoflagellates compared to diatoms, green algae, prymnesiophytes and cyanobacteria (Table 1 of Geider and Osborne 1989)?

Most of the algae have a facultatively functioning alternative oxidase in the inner mitochondrial membrane (Prihoda et al. 2012). This oxidase accepts electrons from the complexes I or II, or the external NADH dehydrogenase, and transfers them to O_2 to produce H_2O, replacing complexes III and IV and cytochrome oxidase as the means of reducing O_2 to H_2O (Prihoda et al. 2012). This pathway does not pump protons from the matrix when succinate (via Complex II) or external NADH is the electron donor, and only pumps two protons per electron when internal NADH (via Complex I) is the electron donor; the protons:electron ratio decreases to 1.5, accepting the findings of Wikstrom and Hummer (2012). This oxidase has been suggested to be a leak for excess (and potentially damaging) reductant from illuminated plastids (Prihoda et al. 2012), and could also act as a means of balancing the synthesis of ATP and that of carbon skeletons during biosynthesis. The 'uncoupling protein' of the inner mitochondrial membrane constitutes a proton leak, and could also perform the two functions assigned to the alternate oxidase. This protein is considered below under proton movement into the matrix.

3.5 Mitochondria: Complex V (ATP Synthase) and Proton Movement into the Matrix

The ATP synthase in mitochondria of *Chlamydomonas* has different peptides in the stalk than occur in some non-algal ATP synthases; this probably has no effect on proton:ATP stoichiometry (Vázquez-Avecedo et al. 2006). The assumed stoichiometry of protons to ATP in the mitochondrial ATP synthase of algae is taken from work on better-investigated

organisms such as yeast. There is however a mismatch in yeast mitochondrial ATP synthase between the 'structural biology' predictions of stoichiometry and the observations. Yeast mitochondria have a measured proton: ATP of 2.9, which is lower than is predicted from the ratio of c subunits to alpha and beta subunits of 3.3 (Petersen et al. 2012; see Raven and Ralph 2015). It would be easier to account for the reverse situation, i.e. protons moving through the c subunits without contributing to ADP phosphorylation by the alpha and beta subunits in a single rotation.

The 'uncoupling protein' of the inner mitochondrial membrane constitutes a facultative proton leak into the matrix, in parallel with and short-circuiting the ATP synthase (Complex V). It has been less thoroughly examined in algae than has the alternate oxidase (Jarmuszkiewicz et al. 2010), but as indicated above it could perform the same functions as the alternate oxidase.

4 Functions of 'dark' Respiration: Maintenance and Growth

4.1 Maintenance Requirements

Maintenance of cells is needed when growth is not possible but the cells are not dormant, as well as during growth. The main respiratory product needed for maintenance is ATP. While ATP is also produced in the light by photosynthetic processes occurring in the thylakoid membrane, in the dark the respiratory pathways are the only source of ATP. A major consumer of ATP in maintenance is protein turnover. The best quantitation of this for comparison with respiratory ATP production is the work of Quigg and Beardall (2003). Here the experimental materials were growing cells of *Dunaliella* and *Phaeodactylum*, each grown at several irradiances. The maintenance protein turnover measured here would presumably include not only the breakdown and resynthesis of photodamaged components of PSII, mainly the D1 protein (*psbA* gene product), and (in diazotrophic cyanobacteria) nitrogenase, but also the turnover of other proteins, e.g. Rubisco, which may not be related to damage (Quigg and Beardall 2003; Quigg et al. 2006; Raven 2011, 2012a). Also included in turnover in growing cells would be the cyclin proteins that are produced and then broken down at specific stages in the life cycle. If individual amino acyl residues are damaged they must be broken down and replaced. Since the catabolic pathways differ from the anabolic pathways, there will be a need for carbon skeletons as well as reductant in the form of the catabolic NADPH rather than the NADH generated in catabolism. This catabolic NADH could be oxidised by the electron transport chain if it was in the mitochondrial matrix, or had access to the NADH dehydrogenase on the outer surface of the inner mitochondrial membrane. These two NADH locations involve the electron transport chain starting with, respectively, the internal NADH dehydrogenase feeding electrons into Complex I, and the external NADH dehydrogenase feeding electrons to UQ and hence Complex III.

The other major requirement for energy in maintenance relates to maintaining gradients of solutes across membranes in the face of leakage through the lipid membrane, or slippage through transporters (Raven and Beardall 1981a, 1982; Quigg et al. 2006). This requirement to recoup leaked solutes is not just a phenomenon of non-growing cells; like protein turnover, it also occurs during growth.

A factor that also applies to very light-limited growth, but is especially significant for long-term maintenance, concerns the increasing relative significance of proton leakage across membranes using protons as the driving ions in chemiosmotic energy transduction (Raven and Beardall 1981b, 1982). The argument relates to a lower rate of energy input (low light, or respiratory substrate limitation in long-term maintenance in the dark (or in light or dark in chemoorganotrophic algae) involving a greater fraction of the energy input being dissipated by proton leakage. This greater fractional dissipation of energy is based on constancy of the proton permeability on a membrane area basis, membrane area per unit biomass, and proton electrochemical driving force (= proton motive force) across the membrane.

Focussing on ATP generation by mitochondria, a possible solution is to decrease the functional area of inner mitochondrial membrane. In the short term this could be achieved by de-energizing some of the mitochondria, assuming there are several mitochondria in the microalga cell rather than the single mitochondrion found in very small cells (e.g. *Ostreococcus*) and in some larger cells (Raven 1984; Chrétionnot-Dinet et al. 1995). The lipophilic dye Rhodamine 123 is an in vivo probe for the electrical potential difference (by far the largest component of the proton electrochemical driving force) across the inner mitochondrial membrane, and it has been shown to operate in a marine diatom (Cid et al. 1996). Schaum and Collins (2014) used Rhodamine 123 to measure the electrical potential difference in mitochondria of the marine picoplanktonic prasinophycean *Ostreococcus*, and interestingly showed that the potential is smaller in faster-growing than in slower-growing strains. In the long term a decrease in mitochondrial volume and membrane area would serve to decrease the relative effect of proton permeability, but would not allow as rapid a return to growth upon resupply of energy,

4.2 Respiratory Requirements for Photolithotrophic Growth: Carbon Skeletons, ATP and NADPH

Carbon skeletons from the TCAC (and EMP/ED and OPP pathways) are needed for biosynthesis of lipids, amino acids

and nucleotides. While the function of the various respiratory pathways in producing NAD(P)H and ATP needed for these biosyntheses can in principle be provided in the light by the photosynthetic reactions located in the thylakoid membrane (Raven 1976a, b, 1984, 2013), there is no alternative to those parts of the EMP and OPP pathways not shared by the Calvin-Benson cycle, and the TCAC, to provide the carbon skeletons. Withdrawal of intermediates of the TCAC for biosynthesis requires replenishment of C_4 compounds by anaplerotic reactions (Raven 1974). This usually takes the form of anaplerotic fixation of HCO_3^- to produce oxaloacetate using either phosphoenolpyruvate or pyruvate as the acceptor molecule in a $C_3 + C_1$ carboxylation catalysed by phosphoenolpyruvate carboxylase or pyruvate carboxylase, respectively (Falkowski and Raven 2007). Such anaplerotic inorganic carbon assimilation is almost all related to the synthesis of nitrogen-containing compounds, with a requirement of 0.30–0.35 inorganic carbon molecule per molecule of nitrogen (as ammonium) assimilated (Raven and Farquhar 1990; Raven et al. 1990; Vanlerberghe et al. 1990; Granum and Myklestad 1999). For an organism with a C:N atomic ratio of 6.625 (the Redfield Ratio) this anaplerotic fixation amounts to about 5 % of net inorganic carbon assimilation during growth (Falkowski and Raven 2007). Mechanisms by which CO_2 from respiration can be routed to anaplerotic carboxylations using HCO_3^- have been suggested (Raven 2001) and experimentally examined, with some support (Giordano et al. 2003).

A possible alternative to anaplerotic $C_3 + C_1$ carboxylases in replenishing the TCAC during carbon skeleton withdrawal for biosynthesis is the glyoxylate cycle (Fig. 1). This pathway is used in the production of C_3 dicarboxylic acids for gluconeogenesis during fatty acid catabolism and assimilation of exogenous acetate (Droop 1974; Chautin et al. 2013; Knoop et al. 2013). Suggestions that it was needed as a way of effectively completing the TCAC in organisms, such as cyanobacteria, lacking 2-oxoglutarate dehydrogenase (Luinenberg and Coleman 1990, 1993) have been made less likely by the finding that many cyanobacteria (Zhang and Bryant 2011) resemble *Euglena* (Shigeoka et al. 1986) in having an alternative pathway from 2-oxoglutarate to succinate (Fig. 2). The possibility (Raven 1974) that the glyoxylate cycle could replace the $C_3 + C_1$ carboxylations as a means of replenishing oxaloacetate in the TCAC during biosynthesis (Fig. 2) is rendered less likely, at least for *Synechococcus* PCC 7942, by the findings of Luinenberg and Coleman (1990, 1993) on the essentiality of PEPC for growth of this cyanobacterium,

For microalgae growing photolithotrophically in a light-dark cycle it has been shown that there is a very large range of rates of net synthesis of nucleic acids, proteins and lipids in the scotophase relative to the photophase (Raven 1976a, b, 1984; Cuhel et al. 1984; Needoba and Harrison 2004). There is also a range, for microalgae, of ratios of CO_2 production over 24 h in "dark" respiration per increment in cell particulate organic carbon (Raven 1976b). As is discussed below, there are difficulties in measuring the rate of the "dark" respiratory reactions in illuminated photosynthetic cells. The assumption made by Raven (1976b) was that the respiratory rate in the light phase was that same as the rate measured immediately after the light was turned off; this rate is generally higher than that measured during the rest of the scotoperiod (Beardall et al. 1994). Raven (1976b) found that the "dark" respiration for chemoorganotrophic growth was higher than that for photolithotrophic growth. The lower dark respiration involved in photolithotrophic growth is often lower than that predicted from cell composition, consistent with the use of ATP and NADPH from the thylakoid reactions of photosynthesis to replace some of the respiratory ATP and NADPH which would be used in the dark for biosynthesis of cell materials from photosynthesis (Raven 1976a, b, 1984, 2013).

Raven (1976a, b, 1984, 2013) showed that there were energetic advantages associated with the direct use of some of the ATP and NADPH produced by illuminated thylakoids in the synthesis of nucleic acids, proteins, lipids and non-storage polysaccharides in the light rather, than in storing the carbohydrate products of overall photosynthesis for use in these synthetic processes in the dark phase to produce both the carbon skeletons and the ATP plus NADPH. These energetic arguments do not take into account the energy and other resources used in the synthesis of the enzymic and other catalysts of the syntheses. In addressing this point, Raven (1976a, b, 1984, 2013) points out that restricting the biosyntheses to the light phase of the diel cycle means that more of the machinery used in the biosynthesis, notably the enzymes and the RNAs used in protein synthesis (Nicklisch and Steinberg 2009; Flynn et al. 2010), is needed per cell to achieve the same growth rate over the 24 h than if biosynthesis occurred at a constant rate through the diel cycle. Production of the additional machinery needed to achieve in a shorter time (the photoperiod) the same total of biosyntheses as occur over 24 h in an organism with the same rate of biosynthesis in the dark as in the light requires additional energy input, offsetting at least in part the savings in energetic running costs of restricting biosynthesis to the photoperiod. There is also a need for more nitrogen and phosphorus for the proteins and RNAs involved in the biosynthesis, and if these two resources are present at low concentrations, there may be advantages in acquiring them over the complete diel cycle to provide additional machinery such as ribosomes which are needed to double the content of nucleic acid, proteins and lipids per cell prior to cell division.

These arguments about the need for different cell quotas of biosynthetic machinery depending on the rate of biosynthesis over the light-dark cycle also apply to respiratory machinery. The need for respiratory machinery to support maintenance processes in the dark phase may be rather higher if any of the

protein needed for the subsequent cell division is synthesised in the light phase, because the mean cell content of protein in the dark phase would be higher than with the other extreme assumption of a constant rate of protein synthesis in the light and in the dark phases (Raven 1976a, b, 1984, 2013). The growth related roles of respiration in generating carbon skeletons for biosynthesis regardless of whether biosynthesis continues into the dark phase, and of being the only source of ATP and NADPH for biosynthesis in the dark phase, means a greater cell quota of respiratory apparatus for carbon skeleton biosynthesis in the light phase is confined to that phase, with no growth-related use made of these mitochondria in the dark phase (Raven 1976a, b, 1984, 2013). By contrast, a steady increase in the per cell content of respiratory machinery would be sufficient if biosynthesis occurred at similar rates in the dark phase as it does in the light phase.

Analysis of the roles of 'dark' respiration in biosynthesis in the light and dark is difficult because of problems with estimating the rates of the various processes consuming O_2 and generating CO_2 in the light (Raven and Beardall 1981a, 2003, 2005; Beardall and Raven 1990; Cardol et al. 2008; Granum et al. 2009; Raven and Ralph 2015), and also the interpretation of the frequently observed phenomenon of enhanced post-illumination respiration (Beardall et al.1994). This is a very widely found process in macroalgae (Raven and Smith 1978; Raven et al. 1979) as well as microalgae (Vanlerberghe et al. 1990; Beardall et al. 1994; Xue et al. 1996; Quigg et al. 2012). It has been interpreted, with no supporting evidence, as a carry-over of biosyntheses occurring in the light into the dark period, with a corresponding requirement for ATP, NADPH and carbon skeletons (Raven and Smith 1978; Raven et al. 1979; Raven 1984). If this were the case, then it would not be expected to occur in cells with the same rate of biosynthesis in the dark as in the light; this does not seem to have been examined.

Microalgae in their natural environment, and in many biotechnological applications, are subject to higher frequency variations in PAR than the scotoperiod, alternating with the sinusoidal variation of solar photon input through the photoperiod (see Raven and Ralph 2015). In nature these are, for phytoplankton, variations as a result of entrainment with vertical water movements in a non-stratified upper mixed layer (= epilimnion), while in mass culture they relate to vertical mixing. Most of the studies have dealt with growth and photosynthesis (Falkowski and Raven 2007; Iluz et al. 2012; however, several publications show that there are also effects of fluctuating light of the rate of 'dark' respiration. In the absence of a large body of data on microalgae, we consider first the work of Kübler and Raven (1996) who examined fluctuating PAR effects on photosynthesis and respiration in the benthic marine red macroalgae *Lomentaria articulata* (lacking a CO_2 concentrating mechanism) and *Palmaria palmata* (expressing a CO_2 concentrating mechanism). Respiration after a light period was influenced by the frequency of fluctuations in the illumination period in *Palmaria*, but not in *Lomentaria*. However, in *Lomentaria* a single high PAR fleck at the end of the light period decreased post-illumination respiration, contrasting with the findings of Beardall et al. (1994) for marine phytoplankton.

Working on the planktonic cyanobacterium *Planktothrix agardhii* and the diatom *Stephanodiscus neoastraeae*, Fietz and Nicklisch (2002) examined the effects of either continuous or fluctuating (with a periodicity of 30 min) PAR superimposed on a 12 h light: 12 h dark diel cycle. They found that, compared to constant PAR through the photoperiod, fluctuating PAR with the same total light dose gave a slightly lower light-saturated photosynthetic rate and a halved rate of 'dark' respiration in *Planktothrix*, and a slightly higher maximum photosynthetic rate and no effect on the 'dark' respiration rate in *Stephano-discus*. The lower respiration rate in *Planktothrix* in fluctuating light suggests that either not all of the respiration in the constant PAR cultures is linked to growth and maintenance activities, or that more growth biosyntheses occur at the expense of the direct use of photoproduced ATP and NADPH in the photoperiod in the cultures exposed to fluctuating light than in the controls, thereby sparing the requirement for dark respiration (Raven 1976a, b, 1984).

Wagner et al. (2006) examined the effects of fluctuations in PAR during the photoperiod of the diel cycle relative to non-fluctuating controls on the rates of light-saturated photosynthesis and 'dark' respiration of cultures of *Chlorella* and *Phaeodactylum*. These workers supplied 19.1 mol m^{-2} PAR per day to control cultures in a sinusoidal variation in the photoperiod, and the same sinusoidal PAR supply interrupted 25 times by excursions down to zero PAR (mimicking entrainment in vertically mixing water in the upper mixed layer/epilimnion), providing a total PAR of 4.2 mol m^{-2} per day. The 24 h respiration is a larger fraction of the daily photosynthesis for both algae in the fluctuating than in the sinusoidal PAR regimes, though it is not clear how much of this difference is attributable to the fluctuations rather than the lower daily PAR. Iluz et al. (2012) considered respiration, and especially enhanced post-illumination respiration, as a factor in the enhancement of net photosynthesis and productivity by fluctuations in PAR between supersaturating and lower irradiance, with a mean PAR higher than the rate-limiting value, although their analysis did not consider experimental work on respiration rates in fluctuating PAR.

Most microalgae have effective CO_2 concentrating mechanism (CCMs) which suppress photorespiration, and thus CO_2 release, via the Photosynthetic Carbon Oxidation Cycle (PCOC) in the light. Nonetheless, some potential for PCOC activity is found in microalgae and cyanobacteria even when the CCM is expressed (Eisenhut et al. 2006, 2008). Microalgae and cyanobacteria contain a variety of PCOC pathways for metabolism of the glycolate produced by

Rubisco oxygenase activity in the light (Beardall and Raven 1990; Raven et al. 2000; Raven and Beardall 2005; Beardall et al. 2009). In Charophyceae, Chrysophyceae, Eustigmatophyceae, Glaucocytophyceae, Raphidophyceae, Xanthophyceae (= Tribophyceae) and Rhodophyceae it appears that glycolate is oxidized to glyoxylate using glycolate oxidase whereas in the Bacillariophyceae, Chlorophyceae, Prasinophyceae, Trebouxiophyceae and Cryptophyceae glycolate dehydrogenase is used (Beardall and Raven 1990; Betschke et al. 1992; Raven et al. 2000; Raven and Beardall 2005; Beardall et al. 2009). Other higher taxa of microalgae have been examined, but no glycolate-oxidising activity was detected. Although genomic data show the presence of genes encoding glycolate oxidase/dehydrogenase in *Emiliania huxleyi* (Prymnesiophyceae) (Read et al. 2013), the sequence does not permit distinction of oxidase from dehydrogenase. In diatoms there are two glycolate oxidizing enzymes – one in the mitochondria and one in peroxisomes (Kroth et al. 2008). Metabolism of glyoxylate to glycine and release of CO_2 and NH_3 as two glycine molecules become one serine, which permits recovery of C in glycerate, occurs in the mitochondria (see Kroth et al. 2008), In diatoms the urea cycle may play a role in reincorporation of CO_2 and NH_3 released from the glycine to serine conversion in the mitochondria; the carbamoyl phosphatase synthase enzyme occurs in the mitochondria of diatoms (Kroth et al. 2008; Prihoda et al. 2012), consistent with the equimolar consumption of CO_2 (as HCO_3^-) and NH_3, which is the ratio in which they are released in the photorespiratory carbon oxidation cycle (PCOC). However, it must be remembered that continuation of the PCOC requires that one NH_3 is used in the amination of glyoxylate to glycine for each NH_3 released in the two glycine, one serine step, with a stoichiometric transamination from serine (producing hydroxypyruvate) to glyoxylate (producing serine). These different pathways for the PCOC, glycolate disposal and recovery of glycerate have consequences for the overall energetics of net C assimilation (see Raven et al. 2000).

It is difficult to disentangle these various processes that occur in the light from mitochondrial "dark" respiratory metabolism via glycolysis and the TCAC. Various approaches have been used, using tracer labelled O_2 (Luz and Barkan 2000) or CO_2 (Carvalho and Eyre 2012), inhibitors or varying the external O_2 concentrations (Beardall et al. 2003, 2009), but these can alter the fraction of the gases measured that relate to mitochondrial functioning and thus add uncertainty to the significance of the data obtained.

4.3 Dissolved Organic Carbon Loss

The dissolved organic carbon loss from microalgae (Hellebust 1974) as a fraction of tracer inorganic carbon assimilated in the previous light phase under standard conditions varies with taxon and cell size (López-Sandorval et al. 2013; see Raven and Ralph 2015). Although dinoflagellates have species with a greater DOC loss than do other higher taxa tested and some species of pico- and micro-algae showed a greater loss than any species of nano-algae, the variation among species was so great that the mean values for DOC loss were not significantly different among higher taxa or among size classes (López-Sandorval et al. 2013). The greater DOC loss from dinoflagellates forms an interesting parallel with the higher rate of dark respiration per unit of maximum photosynthetic rate mentioned above, although at the moment there is not the mechanistic basis for the high DOC loss analogous to that suggested for the high respiration rates. There is more DOC loss from cells subjected to fluctuating PAR compared to algae with the same total light dose supplied as constant PAR (Cosper 1982).

A wide range of soluble organic compounds is lost from microalgae, with a range of consequences for the algae and their environment (Hellebust 1974; Engel et al. 2004; Raven et al. 2005; Raven 2010). One component of soluble organic carbon loss is glycolate from Rubisco oxygenase and phosphoglycolate phosphatase; some earlier work based on transient conditions showed a very large fraction of photosynthate lost from cells as glycolate, but later steady-state measurements showed much lower fluxes (Hellebust 1974; Raven and Beardall 1981a, 2003; Beardall and Raven 1990; Raven et al. 1990; Beardall et al. 2003; Raven 2012b). Larger molecules secreted by microalgae include toxins, siderophores in cyanobacteria and enzymes such as certain carbonic anhydrases, amino acid oxidase and alkaline phosphatase (Raven 2010). These compounds all have known roles. The final compound considered is transparent exopolymeric material (TEP): Engel et al. (2004) and Raven et al. (2005). It is less easy to see potentially fitness-enhancing roles for TEP than for the compounds mentioned above, but potentially damaging effects are known, e.g. increasing aggregation of particles including cells and binding of essential trace metals (Engel et al. 2004; Raven et al. 2005).

4.4 Chemo-organotrophy

Chemoorganotrophy can involve osmochemoorganotrophy and phagochemoorganotrophy, with the two processes potentially occurring in the same organism (Droop 1974; Flynn et al. 2013; Schmidt et al. 2013). Both variants can occur in potentially photosynthetic algae when they are grown in the dark with, respectively, dissolved and particulate organic carbon. For algae that have lost the capacity to photosynthesise, chemo-organotrophic growth is the only possibility (Droop 1974; Raven 1976a, b, 1984). Quantitation of the role of respiration in chemo-organotrophic algal growth has been analysed by Raven (1976a, b, 1984).

The assimilation of external soluble sugars requires active transport of the relevant sugar across the plasmalemma, and a kinase that can phosphorylate the sugar; the sugar phosphate can then be incorporated into core metabolism (Droop 1974; Raven 1984). For the assimilation of acetate, uncatalysed entry of the undissociated form is a possibility, while incorporation into core metabolism for biosynthesis requires the glyoxylate cycle (Droop 1974; Raven 1984; Fig. 1).

4.5 Mixotrophy

Mixotrophy involves the occurrence of photosynthesis and chemoorganotrophy in the same cell and (in the light) at the same time. It can involve osmochemoorganotrohy and phagochemoorganotrophy, with the two processes potentially occurring in the same photosynthetic organism (Droop 1974; Flynn et al. 2013; Schmidt et al. 2013). While mixotrophy in algae usually involves oxygenic photosynthesis combined with chemo-organotrophy, the definition could reasonably be extended to include phagoorganotrophs such as the dinoflagellate *Oxyrrhis marina* which lacks oxygenic photosynthesis but has a proton-pumping rhodopsin (Slamovitz et al. 2011; Janke et al. 2013) as well as sensory rhodopsin (Hartz et al. 2011; Slamovitz et al. 2011). While the sensory rhodopsin is in the plasmalemma (Hartz et al. 2011), the proton-pumping rhodopsin is in the endomembrane system (Slamovitz et al. 2011). The energy-transducing rhodopsin could function in the light in acidifying the food vacuoles, thus aiding phagoorganotrophy, sparing the use of ATP from respiration in operating the food vacuole proton-pumping V-ATPase and constituting mixotrophy. Although the rhodopsin proton pump has a low photon use efficiency relative to photosynthetic phosphorylation, or even photosynthesis followed by oxidative phosphorylation, the latter two processes are not an option in an organism lacking photolithotrophy, such as *Oxyrrhis marina* (Raven 2009). Furthermore, the rhodopsin system has a minimal iron cost relative to the photosynthetic or photosynthesis-plus-photorespiratory alternatives for proton pumping, so the rhodopsin system could be favoured in iron-deficient environments, even among photolithotrophs and mixotrophs for endomembrane or plasmalemma proton pumping (Raven 2009; Marchetti et al. 2012).

Kamjunke and Tittel (2009) suggest that osmochemoorganotrophy in photosynthetically competent (osmomixotrophic) algae can play a role under photolithotrophic growth conditions by recycling some of the leaked dissolved organic carbon (see above under Dissolved organic carbon loss). In agreement with this suggestion there was a lower rate of net dissolved organic carbon efflux in two osmomixotrophic green microalgae (*Chlamydomonas acidophila* and *Chlorella protothecoides*) than in the obligately photolithotrophic *Chlamydomonas segnis*,

5 Conclusions and Prospects

The dark respiratory processes in eukaryotic microalgae are generally similar to those of other eukaryotes. However, there are some interesting differences, e.g. the ED pathway replacing the EMP pathway in diatom plastids and an alternative pathway from 2-oxoglutarate to succinate in the TCAC of *Euglena* (and Cyanobacteria). A further difference is the dinoflagellates' lack of complex I (matrix NADH oxidation and UQ reduction driving proton pumping) of the inner mitochondrial membrane; complex I is replaced by a pathway that does not pump protons. All of the alternative pathways have a lower energetic efficiency than the 'conventional' pathways. Much more widespread among microalgae is the mitochondrial alternative oxidase; this is another pathway with a lower energetic efficiency than the conventional (complexes III and IV) pathway. Further work is needed to establish the limits of distribution among microalgae of these alternative pathways; "-omics" technologies have a vital role here.

There are several areas of microalgal respiration where energetic efficiency is poorly understood, e.g. the proton: electron ratio in the various segments of the conventional mitochondrial redox pathways, and the proton:ATP ratio of the mitochondrial ATP synthase. These stoichiometries deserve investigation; among other things this would help in modeling the requirement for organic carbon conversion to carbon dioxide in maintenance and growth.

As indicated above, the main functions of respiration are maintenance processes (using ATP) and growth processes (using NADPH, ATP and carbon skeletons). In both cases the ATP and NADPH can in part be supplied in the light in photolithotrophic cells by the thylakoid reactions. Further work is needed to establish the extent to which such replacement occurs. Additional work is needed to tackle the problems of estimating the rate of "dark" respiratory pathways in illuminated photosynthetically-competent cells.

Significant net carbon losses from microalgae also occur as soluble organic carbon, with the exception of cells growing osmochemoorganotrophically, where there is net entry of dissolved organic carbon. Further work on dissolved organic carbon loss is needed to establish the phylogenetic distribution, the extent in photolithotrophic relative to phagochemoorganotrophic and phagomixotrophic growth, and the functions, of this process.

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Part III

Nutrients and Their Acquisition

Combined Nitrogen

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1 Definition

By combined nitrogen is meant nitrogen covalently bonded to one or more elements other than nitrogen, so this Chapter deals with all nitrogen sources other than N_2.

2 N Sources

2.1 Inorganic N Sources

Some cyanobacteria are capable of diazotrophy, i.e. the production of ammoniacal N (NH_3/NH_4^+) from molecular N (N_2) (e.g. Sohm et al. 2011; Zehr 2011). Eukaryotic algae are capable of using molecular N_2 only when associated with symbiotic diazotrophic bacteria (Usher et al. 2007; Rai et al. 2003; Schmidt et al. 2013). The combined nitrogen used for the rest of metabolism comes, in these cases, from the ammonium product of diazotrophy.

The most common forms of inorganic N used by eukaryotic algae and many cyanobacteria are, however, combined N compounds, mostly NO_3^- (+5) and NH_4^+ (−3), occasionally NO_2^- (+3) (Galván et al. 1996; Falkowski and Raven 2007; Martiny et al. 2009). In the ocean NO_3^- is the main source of combined N for microalgae in upwelling zones yielding high productivity. A smaller fraction of the production depends on NO_3^- in the rest of the ocean, where most of the productivity involves recycled N (organic N and NH_4^+). This N is generated from chemoorganotrophic metabolism of the products of primary production, with little intervention by nitrification in surface waters or eddy diffusion of NO_3^- from the mineralising and nitrifying microorganisms in the deep ocean (Falkowski and Raven 2007; Raven et al. 1992). Photosynthetic primary productivity in oceanic waters is often limited by the availability of combined N, with inorganic combined N concentration frequently well below 10 mmol m^{-3} (Falkowski and Raven 2007). In freshwater, although algal elemental stoichiometry is rather similar to that of their marine counterpart, the ambient N:P ratio is much higher than in the ocean and primary production is typically controlled by P. This is mainly due to the higher abundance of diazotrophic organisms and to the higher precipitation of P in insoluble complexes with particulate matter, whose concentration is orders of magnitude higher in freshwater habitats than in the ocean (Falkowski and Raven 2007). In cultivated soils and in some desert soils, NO_3^- concentrations are often several orders of magnitude higher, with implications for those terrestrial algae, including lichenised algae, with access to soil nutrients (minerotrophic) rather than relying entirely on rain for N and other nutrients (ombrotrophic).

External NH_4^+ is chemically stable in oxygenated as well as in anoxic environments. In oxygenated environments chemolithotrophic (nitrifying) archaea and bacteria convert NH_4^+ to NO_2^- and then NO_3^- (Voss et al. 2013). In anoxic environments, with NO_2^- as co-substrate, NH_4^+ is converted by a limited range of chemolithotrophic bacteria to water and dinitrogen in the anammox reaction, while in hypoxic and anoxic environments bacterial denitrification converts

The following paper is relevant to this chapter, especially Sects. 5.1 and 6.1.
Glibert PM, Wilkerson FP, Dugdale RC, Raven JA, Dupont CL, Leavitt PR, Parker AE, Burkholder JM, Kana TM (2015) Pluses and minuses of ammonium and nitrate assimilation by phytoplankton and implications for productivity and community composition, with special reference to nitrogen-enriched conditions. Limnol Oceanogr. doi:10.1002/lno.10203 (in press)

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NO_3^- and NO_2^- to N_2O and N_2 (Voss et al. 2013). The anammox and denitrification reactions lead to loss of combined N from the water body, together with sedimentation of organic N; where these processes are conspicuous, inputs of combined N are required if primary photosynthetic productivity is to be maintained (Falkowski and Raven 2007; Voss et al. 2013). These inputs include lightning (providing NO_x and HNO_3), rainfall, and rivers, and biological N_2 fixation in the water body. Combined N input to the ocean from the breakdown of algae, grazers, parasite and decomposers growing in the ocean is, of course, recycled from combined N in marine primary producers (see above; Voss et al. 2013).

NH_4^+ (Raven et al. 1992) and, to a much smaller extent, NH_3 are important sources of nitrogen for photosynthetic organisms in the ocean. The analysis by Raven et al. (1992) shows that, globally, more N is assimilated by primary producers as N_2, organic N, NH_4^+ and NH_3 than as NO_2^- and NO_3^- in aquatic environments.

High concentrations of NH_4^+ may inhibit growth of photosynthetic organisms; this has been attributed to various causes (Allen and Smith 1986). One possibility (which requires growth) is the excretion of H^+ produced in NH_4^+ assimilation into organic matter (discussed in more detail below); this ratio is at least 1 H^+ per NH_4^+. If these protons are excreted into a spatially limited or poorly buffered medium, then acidity damage to the organism could occur; this is not likely to occur in phytoplankton, where photosynthesis will offset the NH_4^+ effect on external pH, at least for NH_4^+ assimilation in the light (Flynn et al. 2012). An alternative mechanism of ammonium toxicity is 'futile cycling' of ammonium in the concentration range of low-affinity transport system (LATS; see below), involving aquaporin-like NH_3 channels (Kronzucker et al. 2001; Coskum et al. 2013). The necessary high NH_4^+ concentrations are unlikely to occur in algal habitats apart from high intertidal rockpools (decomposition of uric acid from avian excreta) or sewage lagoons. A further complication is competition of high NH_4^+ concentrations with K^+ discussed below Sect. 5.1.

2.2 Organic N Sources

A variety of dissolved organic N sources are consumed by algae: examples are amino acids, urea, purines, pyrimidines and glycine betaine, and, possibly, peptides and proteins (Neilsen and Lewin 1974; Moore et al. 1987; Ricketts 1990; Tsay et al. 2007; Fernandez et al. 2009; Näsholm et al. 2009; Bhargava et al. 2011; Schmidt et al. 2013). Genomic data have shown that a very large number of amino acids transporters exist (Rentsch et al. 2007). In most cases, acquisition of organic N is inhibited by the presence of NH_4^+ (Fernandez et al. 2009). For phagomixotrophic algae, i.e. those that carry out both photosynthesis and the ingestion of organic particles, there is an additional supply of a range of organic nitrogen compounds (Flynn et al. 2013; Mitra et al. 2014).

3 Nitrogen in Algae

Average stoichiometries of algae show that N is among the most abundant component of photosynthetic cells (Giordano 2013; see also Ho et al. 2003; Quigg et al. 2003, 2011), being fourth after H, C and O by atoms, and third after C and O by mass, in the organic components. The energy cost of the assimilation of oxidised forms of N, estimated on these stoichiometries is not trivial (Table 1). Under energy limitation, competition may occur among energy use among C, N and S assimilation (Ruan 2013; however, the expected energetic advantage of using NH_4^+ rather than NO_3^- as N source under low irradiances is frequently not observed (Thompson et al. 1989; Raven et al. 1992). Also the requirements for Fe, Mo and P vary as a function of the form of combined N. NO_3^- reductase requires Fe and Mo, while NO_2^- reductase requires Fe, in addition to any other requirements for Fe and Mo in the assimilation of NH_4^+ and organic N, and more general N metabolism; it is noteworthy that the additional Fe or Mo costs for assimilation of oxidised N are almost as large as that of N_2 fixation, at least (for Mo) in the most common form of nitrogenase (Mo-Fe, rather than V-Fe or Fe-Fe) (Raven 1988; Kustka et al. 2003). The additional P requirement is related to the need for RNA to synthesise the additional proteins (NO_3^- and NO_2^- transporters; NO_3^- and NO_2^- reductases) involved in assimilation of oxidised N compared to assimilation of reduced N such as organic N and NH_4^+, although this additional requirement is not as great as that for the additional proteins and their turnover, in the case of N_2 fixation (Raven 2012, 2013a).

The assimilation of NO_3^- and NO_2^- into cell material generates about 0.7 OH^- per N assimilated, while assimilation of NH_4^+ generates about 1.3 H^+ per N assimilated (Raven and Smith 1976; Raven 1985, 1986, 2013b). These values assume that the C source for photolithotrophy is CO_2, so that if inorganic C enters as HCO_3^- there is a 1:1 stoichiometry of HCO_3^- entry and H^+ influx or OH^- efflux and includes the 2 OH^- produced in the assimilation of 1 SO_4^{2-} with an organic N:organic S of 15. Since the great majority of microalgae live in aquatic habitats, the disposal of the excess H^+ or OH^- is by efflux from the cell to the medium.

N is an essential components of catalysts and intermediates of primary metabolism; it is found in amino-acids and hence proteins, in nucleotides (including ADP/ATP and $NAD(P)^+$/NAD(P)H) and hence in nucleic acids, in vitamins and, in phototrophs, chlorophylls and phycobilin chromophores, as well as glutathione involved removing reactive oxygen species. These essential components are not constant with environmental changes, e.g. qualitative and quantitative changes in the transcriptome and proteome.

Table 1 Comparison of photon costs of inorganic C assimilation and nitrate reduction in microalgae

Mechanism of inorganic C acquisition in air-equilibrated solution	Photon requirement for conversion of 1 mol external inorganic C to carbohydrate	Photon requirement for conversion of NO_3^- to NH_4^+ per mol gross inorganic C assimilation for 9.94 mol gross C per mol N	Fraction of total photons used in growth that are used for NO_3^- reduction (column 3/ columns 2 + 3)
Diffusive CO_2 entry followed by glycolate metabolism back to carbohydrate using a ribulose-1,5-bisphosphate carboxylase-oxygenase with very high carboxylase: oxygenase ratio; range for two mechanisms	9.92–9.96 mol photons per mol CO_2 converted to carbohydrate	1.81 mol photons per mol NO_3^-	0.153–0.154
CO_2 concentrating mechanisms; range for five mechanisms, allowing for leakage of CO_2 equal to the rate of photosynthesis	10–11 mol photons per mol inorganic C converted to carbohydrate	1.81 mol photons per mol NO_3^-	0.141–0.153

Minimum photon costs per mol CO_2 converted to carbohydrate assume air-equilibrium solutions, 1 electron moved from plastocyanin/cytochrome c_6 to ferredoxin/flavodoxin in photosystem I (PSI) per photon absorbed with the excitation energy arriving at PSI, 0.8 electron per moved from water to plastoquinone through photosystem II (PSI) per photon absorbed with the resulting excitation energy arriving at PSII, and various assumptions about diffusive CO_2 entry or CO_2 concentrating mechanism (Raven et al. 2014). To determine the appropriate C:N ratio to apply in determining the photon cost of NO_3^- assimilation as a fraction of that involved in CO_2 assimilation it is necessary to determine the quantity of organic C last in respiratory decarboxylations necessary for growth and maintenance relative to the organic C accumulated in biomass for a given C:N ratio in the biomass (Raven 1976). This value is taken as 0.5 CO_2 per C in the biomass (Raven 1976), and includes the energy cost of transport of N sources (NH_4^+, NO_2^-, NO_3^-, organic N) into cells as well as assimilation of NH_4^+ or organic N into cell constituents, i.e. all aspects of N metabolism apart from NO_3^- assimilation. It is assumed that the photon cost of the growth-related processes other than C skeleton synthesis, i.e. ATP and NADPH production, that must involve respiration in the dark, is the same as the alternative source of ATP and NADPH in the light, i.e. the use of photoproduced ATP and NADPH in addition to that required in carbohydrate production (Raven 1976, 2013c; Neeboba and Harrison 2004). For the Redfield C:N ratio in biomass, 106 × 1.5/16 or 9.94 CO_2 converted to carbohydrate (or their photon equivalent for direct use of photoproduced NADPH and ATP) per 1 N

There are also roles for N in processes that do, or may, not occur in all algae. Growth substances such as indoleacetic acid and cytokinins are involved in development in some algae. N also plays a role in response mechanisms to changes of environmental conditions, both abiotic and biotic. These responses include production of N-containing osmolytes such as glycine betaine and proline in high osmolarity environments, in heavy metals chelators such as metallothioneins and phytochelatins when exposed to toxic concentrations of the heavy metals, in compounds defending the organisms against biophages, both parasites and grazers, and protection against UV involving compounds such as mycosporine-like amino acids in some eukaryotes and scytonemin in cyanobacteria (Andrews et al. 2013; Raven 2015). The requirement for these acclimation responses to abiotic and biotic changes in the environment may bear on the demand for N. Some N-deficient algae can replace N-containing by non N-containing compounds in carrying out a particular role; an example is replacement of the N-containing osmolyte glycine betaine by the N-free osmolyte dimethylsufoniopropionate (Keller et al. 1999; Giordano and Prioretti 2016; Giordano and Raven 2014; Raven 2015).

Nitrogen is often the proximate limiting nutrient in the sea (Falkowski 1997), whereas it is more rarely controlling growth in freshwaters (Maberly et al. 2002; Giordano et al. 2005; Elser et al. 2007). There is evidence of codon bias, sparing C and S, but not N use in, the highly expressed ribosomal proteins of bacteria including freshwater and marine cyanobacteria (Bragg et al. 2012). This is consistent with C and S deficiency rather than N deficiency having been more significant in the evolution of these organisms. However, the data are also consistent with explanations related to ribosome function rather than elemental availability. The absolute and relative (to other nutrients) availability of N is rather different in freshwater, marine and terrestrial environment (Elser and Hasset 1994; Giordano et al. 2005). This, in combination with an overall similar elemental cell stoichiometry (Quigg et al. 2003, 2011; Giordano 2013), may be the origin of some differences in the N metabolism of terrestrial, marine and freshwater organisms.

In plants, NO_3^- is also used as an osmoticum; this does not seem to be the case in microalgae where any NO_3^- in vacuolated organisms such as larger diatoms is best regarded as a storage pool in temporally variable environments and as a means of transporting N in cells undergoing periodic vertical migration in stratified habitats with very low combined N at the surface and higher combined N (as NO_3^-) at the thermocline (Raven 1987, 1997; Raven and Doblin 2014).

4 Sensing and Signalling

In algae, NO_3^- acts as a signal for the activation of a number of genes involved in its own acquisition and assimilation (Bouguyon et al. 2012; Fernandez et al. 2009 and references therein). Initiation of the expression of NO_3^- assimilation-related genes depends on the intracellular NO_3^- concentration; consequently, some NO_3^- transporters (specifically system I, see below) are needed for this signal transduction pathway. NIT2 is the only regulatory gene of N assimilation so far known from photosynthetic eukaryotes, e.g. *Chlamydomonas* (Camargo et al. 2007); this protein is a transcription factor whose expression is increased in the absence of NO_3^- and is negatively regulated by NH_4^+, although further clarification of the NH_4^+ effect is needed (Fernandez et al. 2009 and references therein). It is noteworthy that NIT2 is not present in the thermo-acidophilic red alga *Cyanidioschizon merolae*[1] or the flowering plant *Arabidopsis* (Imamura et al. 2009), suggesting that other transcription factors related to NO_3^- assimilation occur in photosynthetic organisms. In *C. merolae*, at least in vitro, CmMYB1, a member of the myeloblastosis (MYB) transcription factor family, is able to bind to promoter regions of N assimilation genes and may play a role similar to NIT2 in *Chlamydomonas* (Imamura et al. 2009).

NO_3^- also acts as a signal on a broader scale, possibly reflecting the complex interaction of N metabolism with other pathways (e.g. Huppe and Turpin 1994; Stitt 1999; Krouk et al. 2010a, b; Hockin et al. 2012; Raven 2012). In algae, the signaling role of NO_3^- has mostly been studied in *Chlamydomonas reinhardtii*. In this green alga, NO_3^- regulates both metabolism and developmental processes (e.g. gametogenesis) (Fernandez et al. 2009). NO_3^- and the product of the regulatory gene NIT2 have been shown to be involved in the regulation of C allocation to storage pools (i.e. carbohydrates and triacylglycerides); this is not surprising in view of the very tight coupling of C and N metabolism in algal cells (Huppe and Turpin 1994). In *C. reinhardtii*, NH_4^+ sensing is mostly involved in the negative regulation of NO_3^- assimilation genes and is apparently mediated by CDP1, a cysteine-rich protein with no homology with other known proteins, and CYG56, a NO-inducible guanylate cyclase (see below for NO signaling). The synthesis of cyclic GMP is believed to be a key step in the perception of NH_4^+ and in the NH_4^+ signal transduction chain (de Montaigu et al. 2011).

In algae a variety of pathways leading to NO production have been found (see Fröhlich and Durner 2011). A NO synthase (NOS) with about 45 % sequence similarity to human NOS occurs in the green alga *Ostreococcus tauri* (Foresi et al. 2010). In *Chlamydomonas*, it has been proposed that NR catalyses NO production in the cytosol from NO_2^- (Sakihama et al. 2002). Since NO inhibits the mitochondrial electron transfer, under high cell concentration of NO_2^-, the provision of ATP from the mitochondrial transfer chain may be hampered. AOX1, one of the two AOX genes encoding for mitochondrial alternative oxidases, is regulated by NO_3^- and may be critical to maintain ATP synthesis when cell NO_2^- concentration is high. In the green trebouxiophycean alga *Chlorella*, AOX may be directly involved in NO production by catalyzing NO_2^- reduction to NO; in this organism NR is not involved in NO production (Tischner et al. 2004).

Sanz-Luque et al. (2013) proposed that the well known inhibitory effect of NH_4^+ on the expression of several genes involved in N assimilation in *Chlamydomonas reinhardtii* (Fernandez et al. 2009) is mediated by NO, reversibly regulating NR activity and high affinity uptake systems of both NH_4^- and NO_3^-/NO_2^-. An intact cell is needed for the operation of this system, perhaps because different organelles are involved in the signal transduction pathway.

5 N Acquisition

5.1 Inorganic N

A variety of high (HANT) and low (LANT) affinity transport systems have been identified in eukaryotic algae as well as in plant. These transport systems are typically energized by H^+ or Na^+ cotransport (2 H^+ or 2Na^+: 1 NO_3^-), and evidence consistent with Na^+:NO_3^- cotransport in the marine diatom *Phaeodactylum tricornutum* is provided by Rees et al. (1980) (Table 2). Electrophysiological evidence for the marine diatom *Coscinodiscus wailesii* (Boyd and Gradmann 1999) gives strong evidence for 2Na^+:NO_3^- cotransport, and further shows that the NO_3^- influx from 1 mmol m^{-3} NO_3^- is more than adequate to support the observed growth rate (Table 2). Cyanobacteria use an ABC transporter for NO_3^- that is directly energized by ATP and does not involve cotransport (Kobayashi et al. 1997), although there is evidence of Na^+ dependence of NO_3^- influx (Lara et al. 1993).

NRT1 transporters have been identified in the genome of *Chlamydomonas*. Other NRT1 have been characterized as low affinity transporters (Huang et al. 1999; Zhou et al. 1998). NRT2 transporters are widely distributed among prokaryotes and eukaryotes (Fernandez et al. 2009 and references therein). As frequently found for anion transporters, NRT2s contain 12 transmembrane helices, with a loop after domain 3 and cytosolic regions at both C and N termini

[1]Wherever possible the currently accepted names for species are used. The name used in the paper cited is also indicated. For details of names see chapter "Systematics, Taxonomy and Species Names: Do They Matter?" of this book (Borowitzka 2016).

Table 2 Microalgae for which there is evidence consistent with the influx of a combined N source by cotransport (symport) with Na^+

Alga	N form	Evidence of Na^+ symport	References
Cyclotella cryptica	$CH_3NH_3^+$	Na^+ dependence: $K_{1/2}$ 10 mol m^{-3}	Wheeler (1980)
Phaeodactylum tricornutum	$CH_3NH_3^+$	Na^+ dependence: $K_{1/2}$ 14 mol m^{-3}	Wright and Syrett (1983)
Phaeodactylum tricornutum	NO_3^-	Na^+ dependence: $K_{1/2}$ 2.6 mol m^{-3}	Rees et al. (1980)
Coscinodiscus wailesii	NO_3^-	Na^+ dependence; net positive charge entry during NO_3^- influx	Boyd and Gradmann (1999)
Synechococcus elongatus	NO_3^-	Na^+ dependence	Lara et al. (1993)
Cyclotella cryptica	Alanine	Na^+ dependence: $K_{1/2}$ 15 mol m^{-3}	Hellebust (1978)
Cyclotella cryptica	Arginine	Na^+ dependence: $K_{1/2}$ 10 mol m^{-3}	Hellebust (1978)
Cyclotella cryptica	Glutamate	Na^+ dependence: $K_{1/2}$ 15 mol m^{-3}	Hellebust (1978)
Tetraselmis subcordiformus	Alanine	Na^+ dependence	Qafaiti and Stephens (1989)
Tetraselmis subcordiformis	Serine	Na^+ depencence	Qafaiti and Stephens (1989)
Desmonostoc (Nostoc) muscorum	Proline	Na^+ dependence	Bhargava et al. (2011)
Phaeodactylum tricornutum	Urea	Na^+ dependence: $K_{1/2}$ 71 mol m^{-3}	Rees et al. (1980)
Phaeodactylum tricornutum	Guanine	Na^+ dependence: $K_{1/2}$ 8.25 mol m^{-3}	Shah and Syrett (1982)
Aphanothece halophytica	Glycine betaine	Na^+ dependence	Moore et al. (1987)
Desmonostoc (Nostoc) muscorum	Glycine betaine	Na^+ dependence	Bhargava et al. (2011)

Based on Table 7.6 of Raven (1984)

(Forde 2000). In *Chlamydomonas* (as well as in plants), the cytosolic C terminus domain of NRT2 is especially long (Fernandez et al. 2009) and NRT2 is sometimes flanked by an additional protein NAR2 (nitrate assimilation-related protein 2). The presence of such protein is believed to be necessary for high affinity transport, at least in *Arabidopsis* and *Chlamydomonas*, (Fernandez et al. 2009 and references therein).

A variety of high (HANT) and low (LANT) affinity NO_3^- transporters are present in *Chlamydomonas* (Fernandez et al. 2009 and references therein). In this green alga, at least four different systems have been characterized: three of these systems (I, II, III) are induced by NO_3^- and are under the regulatory control of NIT2 protein, are inhibited by NH_4^+ but not by Cl^- and are maximally active at high CO_2 (see Fernandez et al. 2009 and references therein for details). System IV, shows a rather different behaviour from the others; it is probably a member of NRT2, but the gene that encodes for it has not been identified (Rexach et al. 1999; Navarro et al. 2000). It is capable of transporting both NO_3^- and NO_2^-, both with a Km of 30–40 mmol m^{-3}. Distinguishing it from the other three *Chlamydomonas* NRTs, systems IV does not require NO_3^- for its expression, is not inhibited by NH_4^+ but is sensitive to Cl^-, and is maximally active at low CO_2 (Galván et al. 1996; Rexach et al. 1999). This regulatory pattern is compatible with it being a HCO_3^- transporter.

A third group of NO_3^-/NO_2^- transporters characterized in *Chlamydomonas* are the NAR1 (NO_3^- assimilation related component 1) transporters. The NAR1.1 (and possibly the NAR1.2) gene product is a chloroplast NO_2^- transporter, regulated by C availability. The NAR1.1 gene is located in the NO_3^- cluster, linked to NIA1 (the gene encoding NO_3^- reductase) and it is co-regulated with the other genes in the cluster, with which it shares inhibition by NH_4^+ under NIT2 control. The NAR1.1 gene belongs to the FNT (formate nitrite transporters) family, which has not been found in plants or animals (Mariscal et al. 2006). In *Chlamydomonas*, some NAR1 proteins are not plastidial: NAR1.5 seems to be mitochondrial, and Nar1.3, Nar1.4 and NAR1.6 are believed to be plasmalemma transporters (Fernandez et al. 2009 and references therein).

NAR1 genes have been found in *Ostreococcus* (single copy) and other algae, fungi and protozoa (Fernandez et al. 2009). The NAR1 gene products are also present *Saccharomyces* ad *Plasmodium*, although these organisms do not assimilate NO_3^-/NO_2^-; it is therefore possible that, also in algae, these proteins are involved in the transport of other monovalent ions besides NO_2^- (e.g. HCO_3^- and $HCOO^-$). The *Chlamydomonas* NAR1.2 gene responds to inorganic C availability and is under the control of CCM1 gene, which regulates the activation of the CO_2 concentrating mechanisms (Giordano et al. 2005), reinforcing the hypothesis of a role for some NAR1 gene in inorganic C transport. When expressed in *Xenopus* oocytes, NAR1.2 protein transports HCO_3^- (as well as NO_2^-; Mariscal et al. 2006). NAR1.1 and NAR1.6 are inhibited by NIT2 and respond to N sources; they therefore are most probably real NO_2^- transporters. Other products of NAR1 genes are controlled by neither NIT2 nor CCM1 and are thus putatively involved in transport processes not directly related to N and C acquisition and trafficking.

The acquisition of NH_4^+ in algae (as in plants), mostly involves transporters of the AMT/MEP family (Andrews et al. 2013; Fernandez et al. 2009). The AMT transporters of photosynthetic organisms were thought to function entirely as NH_4^+ uniporters (Ludewig et al. 2007), with accumulation of ammonium in the cytosol occurring up to the value indicated by the Nernst equation by the inside-negative electrical potential difference across the plasmalemma; a 59 mV potential difference could give an equilibrium accumulation of tenfold the external concentration (Raven 1984). Earlier work on the giant-celled alga *Chara* showed that ^{14}C-methylammonium (as a labelled proxy for ammonium) influx equalled positive charge entry under a voltage clamp (injection of negative current to maintain the electric potential difference at a predetermined value), consistent with NH_4^+ uniport (Walker et al. 1979). However, the molecular characteristics of the *Chara* transporter have not been examined.

The work of Ortiz-Ramirez et al. (2011) has shown that a plant (*Phaseolus* vulgaris) AMT transporter, PvAMT1;1s, functions in symport of NH_4^+ and H^+ with a 1:1 stoichiometry. It seems that NH_3, rather than NH_4^+, is the species transported (Ludewig et al. 2007) and that the stoichiometry of the transport is 1 NH_3 for 2 H^+; this, inside the cell, after equilibration of NH_3 with NH_4^+ at the pH of the cytosol (about pH 7.4: Raven and Smith 1976), this would explain the overall 1 NH_4^+:1 H^+ stoichiometry that is attributed to this transporter by Ortiz-Ramirez et al. (2011) (See Andrews et al. 2013 for a thorough discussion of this topic). This 2:1 H^+:NH_4^+ stoichiometry allows a much greater accumulation ratio for NH_4^+ than does NH_4^+ uniport, as would the Na^+:(methyl)NH_4^+ cotransport in the marine diatoms *Cyclotella cryptica* (Wheeler 1980) and *Phaeodactylum tricornutum* (Wright and Syrett 1983) (Table 2). Boyd and Gradmann (1999) showed that the NH_4^+-related current entry was sufficient to support the N requirements for growth even from 1 mmol m^{-3} NH_4^+, in the marine diatom *Coscinodiscus wailesii*. With such a low external concentration, the equilibrium NH_4^+ concentration in the cytosol of *Coscinodiscus wailesii* would only be 20–30 mmol m^{-3} at the resting electrical potential across the plasmalemma of this diatom if NH_4^+ entered by uniport. This concentration is only marginally sufficient to account for N assimilation by glutamine synthetase; however, the data of Boyd and Gradmann (1999) do not appear to rule out Na^+:NH_4^+ cotransport which would allow a greater accumulation of NH_4^+ in the cytosol.

The transport of NH_3 by an AMT transporter also ensures that these channels are highly selective for N and do not also transport K^+, which has a similar hydrated radius to that of NH_4^+. It is also possible that low affinity NH_4^+ transport takes place via K^+ channels. There is a large AMT1 gene family in *Chlamydomonas*, with members distributed throughout the nuclear genome. Some of these genes (AMT1.1–6) are closely related to the AMT transporters of plants, whereas AMT1.7–8 is more distantly related. Such large number of genes encoding AMT1 transporters could be the result of functional redundancy, but it may also reflect adjustments to changing N availability. This is consistent with the various modes of regulation of the AMT transporters in the presence of different N source and amounts, and regulation of exchanges between cell compartments and with the medium (Fernandez et al. 2009 and references therein). The involvement of *Chlamydomonas* NH_4^+ transporters in a chemotactic response to dissolved NH_4^+ has been proposed by Ermilova et al. (2004).

NH_4^+ transport at the molecular level is not as well characterised in other algae as it is in *Chlamydomonas*. The green alga *Micromonas* (Prasinophyceae) and *Chlorella* (Trebouxiophyceae) and the chromoalveolates studied so far contain AMT2 genes of bacterial origin, in addition to the AMT1 genes (McDonald et al. 2010), suggesting diverse origins for NH_4^+ transporters in algae.

Diatoms respond in a species-specific manner to growth at high NH_4^+ concentrations; for instance, while *Cylindrotheca closterium* (=*Ceratoneis closterium*) is unable to grow at 10 mol m^{-3} NH_4^+, *Phaeodactylum tricornutum* thrives in such condition (see fig. 4 in Giordano 2013). Experiments conducted in Mario Giordano's laboratory showed that the high NH_4^+ effect on *Ceratoneis* is substantially decreased when the concentration of K^+ in the growth medium is increased; however, the addition of K^+ to *Phaeodactylum* has no effect (Norici et al. unpublished). This suggests the existence of different NH_4^+ uptake systems in the two algae, with the toxic effect of high NH_4^+ in *Ceratoneis* possibly due more to K^+ starvation than to NH_4^+ toxicity: in this species, K^+ and NH_4^+ may be taken up through for the same channels, with high NH_4^+ out-competing K^+. Two very similar AMT transporters have been cloned for *Cylindrotheca fusiformis*, which are most highly expressed in N-starved cells, with less expression in NO_3^- grown cells and least in NH_4^+-grown cells (Hildebrand 2005).

Futile cycling of NH_4^+ through low affinity NH_4^+ transporters (Kronzucker et al. 2001; Britto and Kronzucker 2006) may provide an explanation of some aspects of NH_4^+ toxicity. Another possibility is that toxicity is a result of NH_3 movements at the plasmalemma and the tonoplast by an aquaporin-related channel combined with NH_4^+ transport at these membranes by AMT, inwardly-rectifying K^+ channels and non-selective cation channels (Coskum et al. 2013).

Chlamydomonas is among the few organisms in which both AMT transporters and the related *Rhesus* (Rh) proteins have been identified (Fernandez et al. 2009). Although the Rh protein may act as a bidirectional NH_3 channel, there are evidences that in *Chlamydomonas* it is involved in CO_2 permeation at the plastid envelops (Soupene et al. 2002, 2004).

5.2 Dissolved Organic N

5.2.1 Amino Acids

The use of amino acid as the N source is widespread among algae. One mechanism, in the model species *Chlamydomonas reinhardtii* and in marine members of the Dinophyta and Haptophyta (Palenik and Morel 1990a, b), is deamination of a diversity of L-amino acids outside the cells (Muñoz-Blanco et al. 1990), catalysed by a periplasmic amino acid oxidase, with varying expression in different water bodies (Mullholland et al. 1998). This enzyme is induced (with gametogenesis in *Chlamydomonas*) by N starvation (Vallon et al. 1993). NH_4^+ is then acquired and assimilated, while the oxoacid derived from the deamination is not taken up (Muñoz-Blanco et al. 1990).

This mechanism is relatively more important in oligotrophic areas of the ocean (Mullholland et al. 1998). An operon-like cluster of genes involved in urea/arginine uptake and assimilation has been identified in *Ostreococcus tauri* (Derelle et al. 2006) and *Chlamydomonas* (Fernandez et al. 2009) genomes. The only specific amino acid transporter found so far in *Chlamydomonas* is for asparagine (Kirk and Kirk 1978).

The marine diatom *Cyclotella cryptica* can take up acidic, neutral and basic amino acids: influx of an example of each of these groups, i.e. glutamate, alanine and arginine, show Na^+ dependence, consistence with Na^+ cotransport (Hellebust 1978) (Table 2). The marine chlorodendrophyte *Tetraselmis subcordiformis* shows Na^+ dependence of alanine and serine influx for $K_{1/2}$ of the influx, but not for the amino acid-saturated rate of uptake (Qafaiti and Stephens 1989). The Na^+:amino acid ratio for the putative cotransport is 2 (Qafaiti and Stephens 1989).

5.2.2 Betaines

Glycine betaine can be taken up by several cyanobacteria using a Na^+-dependent mechanism (Moore et al. 1987; Bhargava et al. 2011). Glycine betaine uptake has also been demonstrated in three marine phytoplankton species, *Amphidinium carterae*, *Emiliania huxleyi* and *Thalassiosira pseudonana*, but with no indication of the effects of varying Na^+ (Keller et al. 1999).

5.2.3 Purines

A number of algae can take up and metabolise purines (Neilsen and Leewin 1974). The Na^+ dependence of guanine influx in the marine diatom *Phaeodactylum tricornutum* is consistent with Na^+:guanine cotransport (Shah and Syrett 1982) (Table 2).

5.2.4 Urea

A large number of algal species are able to grow on urea, both in the case of exogenous urea and in the case of recycling of internal organic N (e.g. from catabolism of arginine or purines. Neilsen and Lewin 1974; Solomon et al. 2010; Witte 2011; Werner et al. 2013). In *Chlamydomonas reinhardtii*, urea is taken up by an energy-dependent system (Williams and Hodson 1977). Active transport of urea into cells of the freshwater giant-celled charophycean green alga *Chara australis* (= *Chara corallina*) involves 2 Na^+:1 urea co-transport (Walker et al. 1993). The Na^+-dependence of urea influx in the marine diatom *Phaeodactylum tricornutum* (Rees et al. 1980) is consistent with Na^+:urea cotransport (Table 2).

5.2.5 Conclusions

In summary, there is a prevalence of H^+ or Na^+ symport systems for influx of combined N forms in which there is net positive charge on the transported cation:combined N complex or, for cationic N forms, a greater positive charge. This allows a large energy input per mol combined N, and hence a large accumulation ratio (cytosol: medium). The resulting high internal concentration of combined N is in some cases necessary to achieve the flux of N through the assimilation system required for cell growth (Raven 1980, 1984; Raven et al. 2008).

6 Assimilation

6.1 Inorganic N

Regardless of the oxidation number of the chemical form in which N is acquired, it is incorporated into organic compounds as ammonium-ammonia (-3), the most reduced forms of N in nature.

The assimilation of NO_3^- starts with the cytosolic enzyme nitrate reductase (NR), which catalyses the initial reduction of NO_3^- to NO_2^-, in most cases using NADH as the electron donor. NR is regulated at the transcriptional and post-translational level. NR was also reported to be regulated by phosphorylation in the red macroalga *Gracilaria chilensis* (Chow and Cabral de Oliveira 2008), although the mechanisms of such regulation appears to be different from that of terrestrial plants. In *Chlamydomonas,* and possibly in most other algae (Berges 1997), the phosphorylation site through which 14-3-3 protein operates is not present. In *Chlamydomonas reinhardtii*, the enzyme is not subjected to phosphorylation-dephosphorylation and it appears to be

under a redox control centred on the plastoquinone pool of the chloroplast, with a higher NR activity when the PQ pool is more reduced (Giordano et al. 2005). It is noteworthy that in plants, NR activity is stimulated when the electron transfer chain is more oxidized (Sherameti et al. 2002). The redox regulation of NR in *C. reinhardtii* is also associated with the presence of NO_3^-: NR becomes inactive in the absence of NO_3^- and can be reactivated by NO_3^- resupply and, in vitro, by ferricyanide oxidation (Fernandez et al. 2009 and references therein). It has been proposed that plastidial malic dehydrogenase is involved in regulating reductant utilization for NO_3^- reduction, via its involvement in the malate/oxalacetate shuttle and thus the modulation of the redox state of the chloroplast and the cytosol (Quesada et al. 2000).

The rhodophytan marine macroalga *Gracilaria tenuistipitata* has a circadian oscillation of NR abundance and activity even in continuous light, suggesting the operation of an endogenous clock at the mRNA level (Falcão et al. 2010). However, the congeneric *G. chilensis* uses no such endogenous clock (Chow et al. 2004).

An unusual mode of regulation was found in the NR of *Heterosigma akashiwo* (Coyne 2010). In this raphidophycean, the NAR1 gene encoding NR is constitutively expressed, even in the absence of NO_3^- and in the presence of NH_4^+. An apparent biphasic expression pattern of NAR1 was observed upon addition of NO_3^- to N-starved cultures. The presence of NR activity in the presence of NH_4^+ was also observed in a cyanobacterium *Synechococcus* sp UTEX 2380, which also showed biphasic kinetics of NR (Ruan 2013). More recently, Stewart and Coyne (2011) found that a 2/2 hemoglobin (2/2Hb) domain exists in the hinge-2 region of the NR of the raphidophyceans *H. akashiwo* and of *Chattonella subsalsa*. The 2/2Hbs of *Heterosigma* and *Chattonella* have a quite high similarity to the mycobacterial 2/2Hbs, which are known to catalyse the conversion of NO to NO_3^-. Stewart and Coyne (2011) proposed a dual function for these NRs, which would be capable of converting NO to NO_3^- and subsequently operate a reductive NO_3^- assimilation.

NO_3^- reductase seems to be absent in the culturable strains of the cyanobacterial genus *Prochlorococcus* (Lopez-Lozano et al. 2002; Moore et al. 2002; Martiny et al. 2009); only some low light ecotypes are able to reduce nitrate. Martiny et al. (2009) performed a metagenomic analysis that thus included non-culturable strains; they discovered a number of *Prochlorochoccus* lineages that do possess the ability for NO_3^- assimilation, among both high-light and the low-light adapted strains. The distribution of the NO_3^- assimilation genes also had a defined regional distribution, with a higher frequency in the Caribbean Sea and in the Indian Ocean. Martiny et al. (2009) related this inhomogeneity in the metagenomic data to NO_3^- availability and, consequently, attributed the presence or absence on NR and other NO_3^- assimilation genes to differences in selective pressures in different regions of the oceans. According to this study, the *Prochlorococcus* NR genes form a distinct phylogenetic clade related to marine *Synechococcus* strains. The fact that in these strains the genomic location of the NO_3^- assimilation genes is different from that in marine *Syneochococcus* suggests that these sequences were not recently transferred from *Synechococcus*.

Nitrite reductase (NiR) appears overall rather similar in plants and algae, both structurally and in terms of regulation (it is plastidial, mostly constitutive and highly active in both cases). An interesting and direct link between sulfate and nitrate assimilation was observed in the extremophile red alga *Cyanidioschyzon merolae*, which inhabits acidic hot springs (Imamura et al. 2010). In this organism, no typical NiR was identified in the genome. However, two genes that closely resemble sulfite reductase (SiR) were found and one of these clustered close to nitrate-related (NAR) genes. The similarity between SiR and NiR is well known, but in all photosynthetic organism, the two genes are distinct; the specificity of Sir for NO_2^- can be dramatically increased by a single mutation (Nakayama et al. 2000) and NiR has been shown to function as a Sir in *Mycobacterium tuberculosis* (Schnell et al. 2005). The product of *C. merolae* SiR gene accumulates in the chloroplast when the alga is grown in the presence of nitrate and is repressed by ammonium; furthermore, *C. merolae* SiR functionally complements a NiR-deficient mutant of the cyanobacterium *Leptolyngbya boryana* (Imamura et al. 2010). No major differences in the glutamine synthetase/glutamine-oxoglutarate aminotransferase (GS/GOGAT) system have been identified. We shall therefore not deal with this portion of the N assimilation pathway, in this review.

6.2 Organic N

6.2.1 Aminoacids

It appears that the pathways of amino acid metabolism in algae are, as far as present knowledge goes, not greatly different from that in other organisms. Thus, for glycine and serine, at least in the light, the photorespiratory carbon oxidation cycle (2-phosphoglycolate – glycolate – glyoxylate – glycine – serine) constitutes an alternative for their synthesis to the conversion of 3-phosphoglycerate (from the Benson-Calvin cycle or from glycolysis) to serine and glycine, either through the phosphorylated pathway (3-phosphoglycerate – 3-phosphohydroxypyruvate – 3-phosphoserine – serine – glycine), or the non-phosphorylated pathway (3-phosphoglycerate – glycerate – hydroxypyruvate –serine – glycine). The phosphorylated pathway of glycine and serine synthesis is present in six species of cyanobacteria (Colman and Norman 1997). Some algae can grow on N-free organic carbon compounds in the dark (Nielsen and Lewin

1974), when the photorespiratory carbon oxidation cycle cannot be involved in glycine and serine synthesis; the same is true for amino acid synthesis needed for protein synthesis in the dark phase of a light-dark cycle (Needoba and Harrison 2004). Glycolate oxidation is essential in cyanobacteria, as shown by the lethal effect of knocking out all three mechanisms of glycolate metabolism. This essentiality is despite the very low flux through 2-phosphoglycolate and glycolate as a result of the presence of a CO_2 concentrating mechanism that increases the intra-carboxysome CO_2 concentration, such that the oxygenase activity of ribulose-1,5-bisphosphate carboxylase-oxygenase is greatly decreased (Eisenhut et al. 2008). This essentiality of glycolate metabolism does not necessarily concern glycine and serine synthesis, since there are three means of glycolate removal (apart from efflux to the medium), in cyanobacteria: not only the photorespiratory carbon oxidation cycle via glycine and serine, the tartronate semialdehyde (= glycerate) pathway, and the complete oxidation to CO_2 via oxalate (Eisenhut et al. 2008). Any one of these can handle the glycolate flux, so the pathway via glycine and serine is not essential (Eisenhut et al. 2008).

6.2.2 Urea

Urea taken up by algae can be used to generate NH_4^+ by deamination, with the catalysis of either urease or ATP-urea amidolyase (UALase). UALase, among the algae tested, is only found in green algae of the Chlorophyceae and the Trebouxiophyceae (Leftley and Syrett 1973; Bekheet and Syrett 1977; Dagestad et al. 1981). UALase catalysis involves a two-step process: an initial ATP-dependent carboxylation of urea to produce allophanate and a subsequent hydrolytic reaction that generates $2NH_3$ and $2HCO_3^-$ from allophanate. Differently from yeasts, in which UALase is a single enzyme derived from the fusion of a urea carboxylase and an allophanate hydrolase, in *C. reinhardtii*, the two activities are allocated on distinct proteins (Solomon et al. 2010). In other algae, urea is also be broken down by urease (urea amidohydrolase); this cytosolic enzyme catalyzes the hydrolytic cleavage of urea to ammonia and carbamate; carbamate then spontaneously generates another molecule of NH_3 and carbonic acid (Solomon et al. 2010). Urease occurs in green algae of the Charophyceae (and their descendants, the embryophytic plants), Prasinophyceae and Ulvophyceae, as a well as cyanobacteria, Rhodophyta, Dinophyta, Haptophyta and, in Ochrophyta of the classes Bacillariophyceae, Fucophyceae, Pelagophyceae and Tribophyceae (Leftley and Syrett 1973; Bekheet and Syrett 1977; Fan et al. 2003; Dyhrman and Sanderson 2003; Solomon et al. 2010; Agostoni and Erdner 2011).

As well as metabolising exogenous urea, diatoms and haptophytes are also capable of synthesising urea (Bowler et al. 2010; Allen et al. 2011; Bender et al. 2012). The diatom urea biosynthetic pathway involves ornithine, citrulline and arginine. Similar intermediates are used in the final steps of arginine synthesis, even if the enzymes are different, and the enzyme that cleaves arginine to produce urea and regenerate ornithine is unique to the urea cycle (Bowler et al. 2010; Allen et al. 2011; Bender et al. 2012). The urea cycle is believed to facilitate integration of the metabolism of carbon and nitrogen (Bowler et al. 2010; Allen et al. 2011; Bender et al. 2012).

7 Conclusion

Many of the conclusions come from work on relatively few species; this makes generalizations difficult.

Algae can use a wide range of combined N sources; all of them can use NH_4^+ and can probably all use urea and amino acids N forms, and most can use NO_2^- and NO_3^-.

The prevalence of H^+ or Na^+ symport systems for influx of combined N, in which there is net positive charge on the transported cation:combined N complex or, for cationic N forms, a greater positive charge can be related to an increased accumulation ratio of the combined N, allowing an increased assimilation rate per unit assimilatory enzyme.

Apparently, N metabolism is more directly under redox control in algae than in plants. This is especially true for the initial steps of the pathway (i.e. the reaction catalysed by nitrate reductase).

Molecules such as NO and NO_3^- are important component of cells signalling systems and their role in modulating metabolism and responses to environmental changes is probably great, although little studied.

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Nutrients and Their Acquisition: Phosphorus Physiology in Microalgae

Sonya T. Dyhrman

1 Introduction

Phosphorus is fundamental to life, serving an integral role in aspects of cellular metabolism ranging from energy storage, to cellular structure, to the very genetic material that encodes all life on the planet. Weathering of phosphorus rich rocks is the major source of new phosphorus into aquatic environments (Benitez-Nelson 2000; Paytan and McLaughlin 2007). This phosphorus is utilized and transformed by cyanobacteria and eukaryotic algae driving complex metabolic and biogeochemical dynamics. For reviews on the biogeochemical dynamics of phosphorus see (Benitez-Nelson 2000; Paytan and McLaughlin 2007). Dissolved organic phosphorus and its cycling in marine systems is comprehensively reviewed in (Karl and Björkman 2002; Karl 2014), and in Karl 2014 there are recent summaries of marine cellular phosphorus dynamics, stress responses, and interactions with the marine phosphorus cycle (Karl 2014).

This chapter focuses on phosphorus physiology in microalgae including cyanobacteria and eukaryotic groups. Many of the examples come from studies with marine species, so care should be applied when extrapolating to freshwater taxa, although many of the responses and underlying themes are consistent. This chapter also does not focus on phosphorus in macroalgae. There are many reviews focused on phosphorus physiology or metabolism in eukaryotic algae, and cyanobacteria which should be referred to for additional details on all of the topics highlighted in the following sections (Grossman 2000; Beardall et al. 2001; Grossman and Takahashi 2001; Dyhrman et al. 2007; White and Metcalf 2007; Dyhrman 2008; Scanlan et al. 2009; Villarreal-Chiu et al. 2012; McGrath et al. 2013; White and Dyhrman 2013).

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Knowledge about cellular phosphorus dynamics in microalgae has been rapidly advancing with new methods and more sensitive approaches. This chapter builds upon the rich literature highlighted above with a primary focus on findings leveraged from technical developments in cell sorting, molecular 'omic tools, and advances in ^{31}P NMR, and mass spectrometry. The chapter focuses on how these advances have expanded understanding in the following sections; (2) Phosphorus in the cell, (3) Inorganic phosphorus utilization, (4) Organic phosphorus utilization, (5) Phosphorus stress responses, (6) Methodological advances, and (7) Emerging themes and ongoing challenges.

2 Phosphorus in the Cell

Phosphorus is of course a critical nutrient, and required for all cyanobacteria and eukaryotic algae for growth (Fig. 1). Phosphorus is used as an energy currency in signaling and driving reactions, and it is also a building block in biochemicals as critical to life as nucleic acids and lipid membranes (Merchant and Helmann 2012). There are two primary ways to think about phosphorus in the cell; the presence of phosphorus–rich biochemicals and the type of phosphorus bond in phosphorus-containing compounds.

2.1 Phosphorus Biochemicals

The major biochemical pools in a typical cell are: protein 52 %, polysaccharide 17 %, RNA 16 %, lipid 9.4 %, DNA 3.2 %, other <3 % (Karl 2014). This composition can vary considerably between taxa, and as a function of physiology. Of these pools, the largest phosphorus sink is nucleic acids (Fig. 1), making phosphorus essential for the storage expression of genetic information (Merchant and Helmann 2012). In fact, Van Mooy and Devol 2008 found that RNA synthesis was the largest biochemical sink for phosphate, accounting

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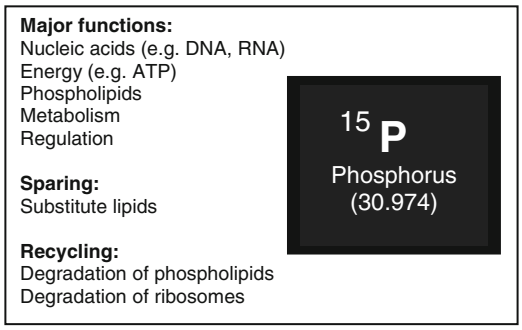

Fig. 1 An overview of the roles phosphorus plays in algae and known sparing or recycling mechanisms (Adapted from: Merchant and Helmann 2012)

for about half of the total phosphate uptake, in North Pacific plankton communities dominated by *Prochlorococcus* (Van Mooy and Devol 2008). In this system phospholipids synthesis accounted for about, 20 % of the phosphate uptake, with the remainder (30 %) of phosphate uptake likely being accounted for by DNA, phosphorus biochemicals (e.g. ATP), and or abiotic adsorption (Van Mooy and Devol 2008). Other studies have also shown that synthesis of genomic DNA may compose as much as half of the total phosphorus demand for picocyanobacteria (Bertilsson et al. 2003). These contributions may not be consistent across all taxa, or physiological states, nevertheless they underscore the importance of phosphorus to the cellular lipid and nucleic acid pools of algae. It is worth noting that in a typical eukaryotic cell the RNA pool consists of about 80–85 % ribosomal RNA (28S, 18S, 5S), while 10–20 % is made up of a variety of a low molecular weight species (tRNAs, mRNA etc.). Thus there is a large demand for phosphorus associated with rRNA, that can be modulated to recycle phosphorus in some cases (Fig. 1).

Although the pool may be small, phosphorus is a major component in nucleoside triphosphates (Fig. 1) like ATP and GTP (Merchant and Helmann 2012). These critical biochemicals serve as the universal energy currency in the cell with many biosynthetic processes fueled directly or indirectly by their hydrolysis (Merchant and Helmann 2012). In this context the critical role that phosphorus plays in driving cellular metabolism in algae cannot be over stated. While measurements of specific phosphorus containing biochemicals like RNA or ATP are feasible, and valuable (both for cellular modeling studies and understanding phosphorus physiology and phosphorus cycling in field populations) direct measurements of these biochemicals in cultures or field populations of cyanobacteria and eukaryotic algae are uncommon (Karl 2014).

2.2 Phosphorus Bond Classes

Phosphorus in algae can be characterized by bond form, often utilizing ^{31}P NMR to assess the chemical shift made by different bond forms to diagnose their presence and concentration in samples from eukaryotic algae and cyanobacteria. These bond forms include, phosphomonoester (P-O-C), phosphodiester (C-O-P-O-C), phosphonate (C-P), and polyphosphate (P-O-P-O-P) (Fig. 2). Surveys of cyanobacteria and algae suggest that the relative percentage of phosphorus in different bond forms is intrinsically variable between taxa, and can also vary with physiology and growth conditions. There is also some likely variability derived from the screening method used, as some forms do not extract well, and there can be biases around the specific biochemicals the bond type is contained in (Cade-Menun et al. 2005). Further, NMR approaches are not equally sensitive for all bond classes and materials, and sample processing could release enzymes or induce other alterations in the cellular phosphorus composition (Cade-Menun et al. 2005). A typical algal cell appears to be dominated by phosphate, phosphoester (monoester and diester) and polyphosphate (Clark et al. 1999; Dyhrman et al. 2009; Cade-Menun and Paytan 2010). The detection of phosphonate in eukaryotic algae or cyanobacteria is rare, although it may be present as trace amounts, or higher in some cases. For example, some studies have identified the presence of low amounts of phosphonate (<0.5 % total particulate phosphorus) using biochemical assays (Kittredge et al. 1969; Kittredge and Roberts 1969; Clark et al. 1999), and strains of the cyanobacterium *Trichodesmium erythraeum*[1] had a peak consistent with the presence of phosphonate which was detected using ^{31}P NMR (Dyhrman et al. 2009). In the following sections, the properties, metabolic function and synthesis of polyphosphate, as well as phosphoester, and phosphonate bound organic matter are highlighted.

2.2.1 Polyphosphate

Polyphosphate compounds consist of three to thousands of orthophosphate groups linked together in a chain by phosphoanhydride (P-O-P) bonds (Fig. 2). Cellular polyphosphate can be found in many forms such as highly condensed storage granules, nucleotides such as adenosine triphosphate (ATP), and in inorganic chains (tripolyphosphate), which can range in length from three to thousands of residues long (Kornberg et al. 1999). The form of polyphosphate may differ in how it can be detected. For example, storage granules can be imaged through microscopy and staining techniques, and there are fluorometric methods for dissolved and particulate measurements of polyphosphate (Diaz and Ingall 2010; Mazard et al. 2012; Martin and Van Mooy 2013). Polyphosphate has also been detected in eukaryotic algae and cyanobacteria with ^{31}P NMR (Dyhrman et al. 2009;

[1]Wherever possible the currently accepted names for species are used. The name used in the paper cited is also indicated. For details of names see chapter "Systematics, Taxonomy and Species Names: Do They Matter?" of this book (Borowitzka 2016).

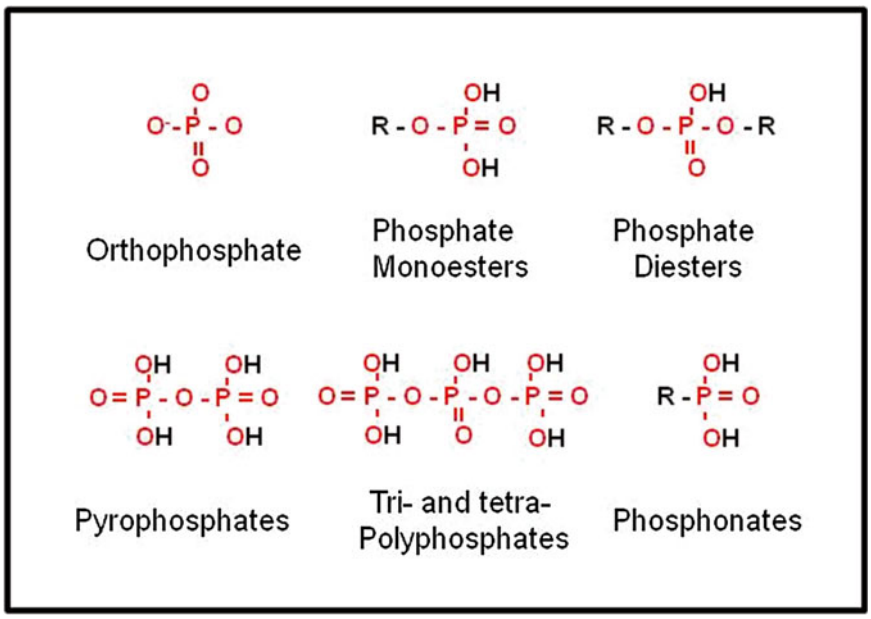

Fig. 2 Cellular phosphorus forms identified by bond type

Orchard et al. 2010b). Some polyphosphate containing biochemicals can be detected directly like ATP (Björkman and Karl 2001). There is no single perfect method for polyphosphate detection, because they are all biased and or prone to matrix effects and background to some degree. For example, polyphosphate chain length can bias the fluorometric method (Diaz and Ingall 2010), and there can be interference depending on the extraction procedure (Martin and Van Mooy 2013; Martin et al. 2014). Polyphosphate is intensively studied in the context of waste water treatment, but these methodological constraints have in part constrained studies of polyphosphate dynamics and metabolism in cyanobacteria and eukaryotic algae.

Polyphosphate is found in all major groups of life, but its function is varied, and in many regards remains unclear (Kornberg et al. 1999). With regards to the eukaryotic algae and cyanobacteria, polyphosphate has been examined in *Chlamydomonas*, *Skeletonema*, *Thalassiosira*, *Synechocystis*, *Nostoc*, *Calothrix*, *Synechococcus and Trichodesmium* among others (Romans et al. 1994; Capone et al. 1997; Gomez-Garcia et al. 2003; Mateo et al. 2006; Nishikawa et al. 2006, 2009; Diaz et al. 2008; Orchard et al. 2010b; Mazard et al. 2012). With its common presence, polyphosphate is largely thought to be ubiquitous (Raven and Knoll 2010). With this broad distribution, the metabolic functions of polyphosphate are variable and highly diverse. Cellular polyphosphate has been variously attributed to a stationary phase adaptation, an energy storage compound, a metal chelator, an osmotic regulator, a buffer against alkali conditions, a factor in DNA competency (as part of a DNA channel), and in phosphorus homeostasis (as a phosphate storage compound) among other potential functions (Kornberg 1995; Kornberg et al. 1999). It is largely this link between polyphosphate and phosphate storage that has driven research on polyphosphate dynamics as a function of phosphorus physiology in algae (see below). However, studies of polyphosphate function and biosynthesis are further complicated by the many varied factors that appear to influence polyphosphate concentrations and granule formation within cells; polyphosphate can accumulate in microbes as a function of growth phase, phosphorus supply, cation and metal concentrations, pH, or temperature (Kornberg 1995; Kornberg et al. 1999).

The production of polyphosphate in cyanobacteria is typically controlled by the *ppK* gene encoding a polyphosphate kinase (*ppK*) that reversibly adds phosphate to the end of the polyphosphate chain. For example, this enzyme would act to add or remove the gamma phosphate of ATP. The gene is common to all cyanobacteria that have been examined (Scanlan et al. 2009). The gene encoding a polyphosphate polymerase (Vtc4) has only recently been identified (Hothorn et al. 2009) in eukaryotes. This gene encodes a protein that interacts with the vacuole membrane and generates polyphosphate from the gamma phosphate in ATP in a phosphotransfer reaction to form polyphosphate chains. Polyphosphate polymerases and specific homologs of Vtc4 are present in the diatom *Thalassiosira pseudonana* (Hothorn et al. 2009), the pelagophyte *Aureococcus anophagefferens* (Wurch et al. 2011b), and the coccolithophore *Emiliania huxleyi* (Dyhrman et al. 2006b), but its distribution in other algae is not well known. Studies leveraging gene expression to examine trends in polyphosphate biosynthesis are hampered by the reversible nature of the biosynthesis enzymes like the polyphosphate kinase. However, upregulation of the Vtc4 polyphosphate polymerases has been observed in a number of studies (Dyhrman et al. 2006b, 2012). This may

be to mobilize polyphosphate stores, but the expression of the genes does not appear to be linked to a dramatic reduction in cellular polyphosphate (Dyhrman et al. 2012).

There are two basic processes significant to polyphosphate dynamics related to phosphorus supply or phosphorus physiology (Eixler et al. 2006). The first, termed luxury uptake, is the storage of excess phosphate as polyphosphate when phosphorus is abundant. Luxury uptake of phosphorus has been documented in culture experiments with marine and freshwater algae (Bertilsson et al. 2003; White et al. 2006; Diaz et al. 2008). Luxury uptake has also been extensively studied in waste water treatment scenarios, where phosphorus is removed from activated sludge through luxury uptake and stored as polyphosphate in microbes (Pauli and Kaitala 1997; Crocetti et al. 2000). In algae, luxury uptake is likely to occur where phosphate is in excess relative to other resources like nitrogen, and algae are able to store this excess phosphate as polyphosphate for future utilization. Luxury uptake could thus drive the accumulation of polyphosphate in systems or areas where phosphate is in excess, like the coastal zone. Diaz et al. (2008) measured polyphosphate concentrations of ~7 % in coastal diatoms (*Skeletonema* spp.) under nutrient replete conditions and hypothesized a luxury uptake response. However, it is worth noting that for most algae the process of so called luxury uptake and formation of polyphosphate is counter intuitive as only a small fraction of a cell's ATP requirement could be met by phosphorylation of ADP, even with a large "luxury" polyphosphate store. Polyphosphate takes up less volume than phosphate, so there may be size dependent influence over a given species' production of polyphosphate when phosphorus is in excess. Raven and Knoll 2010 suggests this may be an important consideration for small cyanobacteria, but arguably less so when cells are larger (Raven and Knoll 2010). Another consideration is the potential for polyphosphate to increase cell density. The ballasting effect of polyphosphate in causing cells to sink is calculated to be greater if orthophosphate is stored as polyphosphate (Raven and Knoll 2010). Formation of polyphosphate under conditions of environmental excess, may be driven by factors other than phosphorus storage, which could in part explain the variability seen in cellular polyphosphate cycling.

The other process significant to polyphosphate dynamics is the overplus response, where phosphorus deplete cells accumulate polyphosphate in response to an increase in phosphate supply to levels greater than needed to meet phosphorus demand (Jacobson and Halmann 1982; Bolier et al. 1992). The overplus response is not particularly well studied in algae, but it has been hypothesized that overplus could drive the cellular accumulation of polyphosphate in low phosphorus systems where phytoplankton are phosphorus deficient, but experience variations in their phosphate environment (Karl and Björkman 2001, 2002). In fact, a large fraction of cellular phosphorus is found as polyphosphate in cyanobacteria from the genus *Trichodesmium* collected from the low phosphorus (<15 nM) Sargasso Sea. In this study, Orchard et al. 2010b hypothesized that this large allocation of phosphorus to polyphosphate could be the result of an overplus-like response (Orchard et al. 2010b). Clearly, the dynamics of cellular polyphosphate production is variable in both cultures and field populations. These polyphosphate dynamics clearly warrant further study to fully appreciate the role that polyphosphate plays in algal metabolism, phosphorus homeostasis, and influence over phosphorus biogeochemistry in different systems.

2.2.2 Phosphoester

Phosphorus containing organic matter with an ester bond is some of the most commonly observed, because phosphomonoester bonds (P-O-C), and phosphodiester (C-O-P-O-C) bonds are present in a number of important phosphorus-rich cellular biochemicals; these bonds are common in DNA, RNA, ATP, and lipids to name a few (Fig. 2). In studies utilizing ^{31}P NMR to identify bond class, ester bond phosphorus is typically the major pool of organic phosphorus in algae (Dyhrman et al. 2009; Cade-Menun and Paytan 2010). Cade-Menun and Paytan 2010 showed that phosphoester in several algal species averaged across numerous control cultures was ~108 μmol g^{-1}, representing about 25 % of the total cellular phosphorus in this bond class alone (Cade-Menun and Paytan 2010). The importance and abundance of ester bond phosphorus in cells is also reflected in the dissolved organic matter pool. For example, in typical marine systems high molecular weight dissolved organic phosphorus (DOP) is ~75 % (Clark et al. 1998) and in the larger fraction of DOP observed by Young and Ingall 2010, 80–85 % was phosphoester (Young and Ingall 2010).

Because this bond type is present in such diverse biochemicals, there is not a single specific gene or pathway that controls phosphoester biosynthesis. Rather, these pathways are as diverse as the biochemicals that contain ester bonds. The specific dynamics of ester bond organic matter have also not been studied in a comprehensive manner. Cade-Menun and Paytan 2010 examined the average phosphoester content in specific categories based on chemical shift with ^{31}P NMR in suite of algae. Although there were subtle shifts in phosphoester composition as a function of light, temperature and phosphorus concentration, these were highly averaged responses (Cade-Menun and Paytan 2010). The ^{31}P NMR approach could mask what are substantial intracellular rearrangements between different biochemicals, that do not resolve as a difference in the bulk phosphoester pool. More work is required to examine the dynamics of this bond class in algae.

2.2.3 Phosphonate

Phosphonate bond organic matter has a direct C-P linkage (Fig. 2), and unlike esters, phosphonates are not found in required cellular biochemicals like ATP, or nucleic acids. As a result, phosphonates were often considered a relic of a prebiotic age where oxygen concentrations were likely low, and organophosphonates, rather than organophosphoesters might have predominated (McGrath et al. 2013). For example in the late 1960s phosphonates were detected in the Murchison meteorite, suggesting a prebiotic origin (McGrath et al. 2013). However, increased scrutiny in recent years has built on early observations of phosphonates in microbes and invertebrates (Kittredge and Roberts 1969; White and Metcalf 2007), highlighting the potential significance of phosphonate in algae, and the importance of phosphonate cycling in aquatic systems (Karl 2014). Biochemicals with a phosphonate bond produced by microbes include 2-aminoethylphosphonate (2-AEP), phosphonoacetic acid, fosfomycin and methylphosphonate (Metcalf et al. 2012; Villarreal-Chiu et al. 2012). Only 2-AEP has been specifically detected in eukaryotic algae, including dinoflagellates and coccolithophores (Kittredge et al. 1969). Screening for phosphonate using ^{31}P NMR, which requires additional analyses to identify specific compounds, has rarely detected phosphonate in cyanobacteria or eukaryotic algae (Cade-Menun et al. 2005; Cade-Menun and Paytan 2010). The major exception to date, is the presence of an apparent phosphonate chemical shift in ^{31}P NMR profiles of *T. erythraeum* strains (Dyhrman et al. 2009). The phosphonate bond can be present in a diverse set of cellular biomolecules including lipids, proteins and antibiotics (McGrath et al. 2013). However, the presence of specific phosphonate biomolecules has not been examined in algae, and this warrants further investigation.

The metabolic roles of phosphonates in algae have not been directly examined, and of course depend on the biomolecules which contain the carbon phosphorus bond. In microbes, phosphonates like fosfomycin are antibiotics, and phosphonates like 2-AEP can be found as side groups on exopolysaccharides or glycoproteins, or in the polar head groups of membrane phosphonolipids (McGrath et al. 2013 and references therein). 2-AEP is similar to non phosphonate containing ethanolamine phosphate and is likely present in phosphonolipids (McGrath et al. 2013 and references therein). It has been suggested that phosphonolipids increase structural rigidity or protect against enzymatic degradation, relative to their ester bond counterparts, since the C–P bond is stronger, and not subject to hydrolysis by phosphatases (McGrath et al. 2013 and references therein). Characteristically, phosphonates are more resistant to chemical hydrolysis, thermal decomposition, enzymatic degradation and photolysis than similar compounds that contain phosphoester linkages (McGrath et al. 2013).

The initial step of most phosphonate biosynthesis is thought to begin with the reversible interconversion of phosphoenolpyruvate (PEP) or carboxyphosphoenolpyruvate (CPEP) to phosphonopyruvate or carboxyphosphonopyruvate via a PEP Mutase (Seidel et al. 1988) or CPEP Mutase (Hidaka et al. 1990) respectively. PEP Mutase typically acts with a phosphonopyruvate decarboxylase to catalyze the C-P bond formation, while the mechanism, or other enzymes, coupled to the CPEP Mutase are unknown (White and Metcalf 2007). One known exception to the PEP or CPEP Mutase biosynthesis pathways is the biosynthesis of methylphosphonate by the bacterium *Nitrosopumilus maritimus* (Metcalf et al. 2012). Whether phosphonate production in algae is mediated by these enzymes is unknown, as none of these enzymes have been specifically examined or characterized in cyanobacteria or eukaryotic algae.

Given the dearth of phosphonate biosynthesis and characterization studies in algae, the dynamics or regulation of phosphonate production is equally poorly understood. Some of these studies are constrained by the challenges in tracking low concentrations of the phosphonate bond, or specific phosphonate biochemicals like 2-AEP. There are no detailed studies of how cellular 2-AEP varies with growth phase, nutritional physiology or other factors. Using ^{31}P NMR, Dyhrman et al. (2009) showed that phosphonate equivalents derived from an 18 ppm chemical shift co-varied at a roughly constant proportion (10 %) of the total cellular phosphorus. This suggests that phosphonate bond organic matter cycling within the cell was not related to phosphorus physiology (Dyhrman et al. 2009). However, there can be chemical interference with this chemical shift, and the relatively constant percentage could mask considerable rearrangement or cycling of phosphonate into different biomolecules. With the many recent discoveries regarding phosphonates in algae, studies of phosphonate compounds in algae are likely to continue to advance our understanding of their role in algal phosphorus physiology.

3 Inorganic Phosphorus Utilization

Phosphate is widely accepted to be the preferred form of phosphorus for growth, and is largely considered the only inorganic phosphorus source, although that view is changing. In the classical Monod model, growth rate would depend on the external concentration of phosphate (see Morel 1987). This model was altered to allow for internal storage, for example inorganic polyphosphate accumulation (Droop 1973). In the Droop model growth rate increases with increasing cell quota, so that growth rate is dependent on previous nutrient uptake as well as phosphate concentration in the environment. Many studies have illustrated the complexities involved in understanding nutrient uptake and

growth (Morel 1987), as quotas are variable as a function of physiology, and that physiology can result in different spectrums of bioavailable phosphorus. Even more recently, the realization that phosphate is not likely the sole bioavailable inorganic phosphorus source to algae is driving renewed focus on this topic. This section focuses on phosphate, polyphosphate and phosphite utilization by eukaryotic algae and cyanobacteria.

3.1 Phosphate Uptake

Phosphate uptake is controlled by transporters in the cell membrane, and their form and abundance influences the kinetics of that uptake. Eukaryotic algae typically have multiple phosphate transporters, which has been observed in genome studies (Gobler et al. 2011; Read et al. 2013). The coccolithophore *E. huxleyi*, is one of only a few eukaryotic algae to have multiple strains sequenced, in this case the different isolates have different copy numbers of phosphate transporters (Read et al. 2013) which hints at the potential role of phosphorus physiology in driving genome differentiation. Although the eukaryotic algae have genes with clear homology to phosphate transporters, which have been studied in detail (Li et al. 2006, 2012), it is rare for them to be functionally characterized.

Phosphate uptake in the cyanobacteria is also controlled by phosphate transporters, which drive the affinity and rate of phosphate uptake. Some cyanobacterial genomes including strains of *Crocosphaera watsonii* (=*Cyanobium waterburyi*) and a single strain of *Synechococcus* (RS9916) appear to carry homologs of the *E. coli pitA* low affinity phosphate transporter (Dyhrman and Haley 2006; Scanlan et al. 2009; Bench et al. 2013). In *E. coli* this transporter mediates phosphate uptake in high phosphorus environments. In *C. watsonii* WH 8501 this gene does not appear to be regulated by phosphorus supply in contrast to the *pstS* component the *pstSCAB* phosphate uptake system (see Sect. 5.3) which is regulated by phosphorus supply (Dyhrman and Haley 2006). The *pitA* gene is rarely found in *Synechococcus* and is not apparently present in the *Prochlorococcus* genomes studied to date (Scanlan et al. 2009). It has been hypothesized that these groups may use the *pstSCAB*, high affinity phosphate uptake system, to mediate phosphate uptake regardless of phosphate concentration (Scanlan et al. 2009).

The kinetics of phosphate uptake typically behave with Michaelis-Menten kinetics where they have been observed in both pure culture and field populations, often using ^{33}P or ^{32}P radiotracers (Perry 1976; Casey et al. 2009; Laws et al. 2011). Specific uptake kinetic values and patterns of uptake include both monophasic and multiphasic uptake patterns (Chisholm and Stross 1976). Monophasic uptake suggests the presence of one transport system, and multiphasic kinetics the presence of more than one. For example, in the diatom *T. weisflogii* both V_{max} (maximal uptake rate) and K_m (affinity) increased with decreasing phosphate availability indicating the potential induction of a high affinity phosphate transport system and multiphasic kinetics (Donald et al. 1997). Similarly, *Euglena gracilis* exhibited multiphase phosphate uptake (Chisholm and Stross 1976). In *Chlamydomonas*, V_{max} increases with phosphorus starvation, with the apparent presence of both low and high affinity phosphate transport systems. The high affinity systems control phosphate uptake at low phosphorus (Grossman and Takahashi 2001).

The kinetics of phosphate uptake have also been extensively studied in cultures and field populations of cyanobacteria. In culture, studies of phosphate uptake suggest that there is considerable variability in K_m and V_{max} between even strains of the same species (Fu et al. 2005). There is also considerable variability in these kinetic parameters as a function of phosphorus physiology. These observations are dependent on the types of transport systems each strain carries, and how their expression is modulated. For example, in the colony forming cyanobacterium *Trichodesmium*, K_m does not appear to change in response to phosphorus physiology, however V_{max} increases with decreasing phosphate availability (Fu et al. 2005), and higher V_{max} was observed in *Trichodesmium* collected from low phosphorus systems relative to higher phosphorus systems (Orchard et al. 2010a). This could indicate that the same transport system is used regardless of phosphorus physiology, but that the number of transporters increases when cells are in a low phosphate environment. In *Synechococcus* WH7803 K_m and V_{max} both increased when phosphate was lowered indicating the potential induction of a high affinity phosphate transport system (Donald et al. 1997). *Synechococcus* PCC6803 has two complete *pstSCAB* systems one with low affinity and high velocity, the other with high affinity and low velocity, thus mediating different maximum phosphate uptake rates (V_{max}) and half saturation constants (Pitt et al. 2010). In a last example, *Prochlorococcus* MED4 has a small cell-specific V_{max} in culture (Krumhardt et al. 2013), and this value ranges from <0.02 in the Sargasso Sea (Casey et al. 2009) to between 5 and 20 amol P cell^{-1} day^{-1} in the higher phosphorus North Pacific (Duhamel et al. 2012). It can still compete in these environments however, because culture studies with *Prochlorococcus* MED4 indicate it has a high specific affinity for phosphate (Krumhardt et al. 2013). Measurements of uptake kinetics are valuable for modeling field populations. However, the detection of specific V_{max}, K_m, and phases is dependent on a number of factors including (1) the energy steps during transport like the use of ATP, (2) phosphorus bioavailability per cell, (3) the nutritional history of the cell, (4) the cell quota (which can shift based on physiology), and (5) the experimental substrate among other potential factors

(Jansson 1988), and so specific patterns and values are not always directly comparable.

3.2 Polyphosphate Utilization

The bioavailability of polyphosphate to eukaryotic algae and cyanobacteria has not been studied in great detail. Inorganic polyphosphate is likely bioavailable if it is dissolved and in forms that are hydrolyzable by surface associated enzymes, or small enough to be taken up directly. There are limited studies on the bioavailability of pyrophosphate or polyphosphate in the eukaryotic algae, but ongoing work suggests that inorganic polyphosphate in chain lengths up to 120 residues is readily assimilated by a diversity of eukaryotic taxa (Diaz et al. 2015). The gene pathways mediating polyphosphate bioavailability are not well known.

Short (three residue) polyphosphate appears to be bioavailable to representative cyanobacteria, including *Synechococcus* and *Prochlorococcus* (Moore et al. 2005). Whether longer chain polyphosphate is bioavailable is not well known. In whole water samples from station ALOHA in the North Pacific that are likely dominated by cyanobacteria, polyphosphate was similar to glycerol phosphate, and other DOP compounds in its bioavailability (Björkman and Karl 1994). The mechanisms controlling extracellular polyphosphate metabolism in the cyanobacteria are not well understood. The genes for polyphosphate metabolism including a *ppK* and *ppX*, and the pyrophosphatase *ppA* can all act to break down polyphosphate into shorter chain lengths, but it is unclear if they are acting on exogenous polyphosphate. The *ppA* and *ppK* genes have been shown to be regulated by phosphorus physiology in *Synechocystis* strain PCC 6803 (Gomez-Garcia et al. 2003), but this is not the case for *Synechococcus* WH8102, or *Microcystis aeruginosa* (Tetu et al. 2009; Harke and Gobler 2013), suggesting that this pattern is not strongly consistent over different cyanobacterial groups.

3.3 Phosphite Metabolism

There is no evidence to date that any of the eukaryotic algae can use phosphite as a sole phosphorus source. However, a striking finding in recent years, is the discovery that *Prochlorococcus* strains can use phosphite as a sole phosphorus source (Feingersch et al. 2012; Martinez et al. 2012). One gene cluster implicated in phosphite metabolism is encoded by transport related genes (*ptxABC*) and a NAD-dependent phosphite dehydrogenase (*ptxD*). This gene cluster appears to be present in several cyanobacterial genomes including, *Prochlorococcus* MIT9301, MIT9303, *Cyanothece* sp. ATCC51142, and *Trichodesmium erythraeum* ISM101 *Cyanothece* CCY0110, *Nostoc* sp. PCC7120, *Nostoc punctiforme* PCC73102 and *Nodularia spumigena* CCY9414 (Martinez et al. 2012). Martinez et al. (2012) showed that *Prochlorococcus* MIT9301 can use phosphite as a sole phosphorus source, and that the *ptxD* gene complements *E. coli* phosphite utilization mutants in vivo. Martinez et al. (2012) also utilize metagenome and metatranscriptome data to show that phosphite utilization genes are expressed in low phosphorus waters and that their overall abundance is elevated in low phosphorus environments relative to high phosphorus environments. Heterotrophic bacteria can generate energy through the reduction of phosphite, which suggests that the prevailing concept of a lack of a phosphorus redox cycle in nature should be revisited (White and Metcalf 2007). Last, the apparent inability of the eukaryotic algae to metabolize phosphite, suggests this phosphorus source could drive niche separation between the eukaryotes and the cyanobacteria, at least in low phosphorus systems.

4 Organic Phosphorus Utilization

While inorganic phosphate is generally regarded as the most bioavailable form of phosphorus, it is increasingly recognized that organic phosphorus is a critical phosphorus source in aquatic environments. Although surprisingly little is known about the distribution and concentration of specific chemical constituents of the DOP pool in aquatic environments (see Karl and Björkman 2002), understanding of the bioavailability of specific forms of DOP has benefited dramatically from developments in genomics in particular. These studies have highlighted the diversity of bioavailable compounds as well as how variability in the gene distribution between strains and taxa could implicate phosphorus as an important driver of microbial niche partitioning. This section will review the presence and distribution of enzymes for the utilization of (1) phosphoester and (2) phosphonate bond organic matter. There are a number of sources that review the distribution and bioavailability of these bond classes (Karl and Björkman 2002; Dyhrman et al. 2007; White and Metcalf 2007; Villarreal-Chiu et al. 2012; Karl 2014).

4.1 Phosphoesterases

The concentration of phosphoester in the DOP pool is likely to be higher than phosphate in many systems such as the Sargasso Sea, because the DOP dominates dissolved inorganic phosphate, and phosphoester is the largest fraction of DOP (Jakuba et al. 2008; Young and Ingall 2010). As such, the presence and distribution of phosphoesterases likely plays a significant role in meeting algal phosphorus

demand across many aquatic systems. Phosphoesterases are also likely important in phosphorus cycling and recycling in the cell as they hydrolyze phosphate from lipids, nucleic acids, and ATP among other biochemicals. Three important classes of phosphoesterases highlighted in the subsequent sections are (1) alkaline phosphatase, (2) phosphodiesterase, and (3) 5′ nucleotidase.

4.1.1 Alkaline Phosphatase

Alkaline phosphatase is arguably the most well-studied of the enzymes used by algae to hydrolyze ester form DOP. The enzyme is commonly present in eukaryotic algae and cyanobacteria where it hydrolyzes phosphate from phosphomonoesters for assimilation by the cell (Fig. 3). The enzyme is typically regulated by low phosphorus (Cembella et al. 1984a, b; Quisel et al. 1996; Lin et al. 2013). Although a primary inverse relationship to environmental phosphate is often observed, alkaline phosphatase activity can be detected in relatively high phosphate environments, and has even been observed to be induced by cyanobacterial toxins like cylindrospermopsin (Bar-Yosef et al. 2010), and cell-cell signaling (see Sect. 7.1.2) among other potential factors. Alkaline phosphatases are diverse and encoded by many different genes, often with many per genome, and the concurrent expression of many putative alkaline phosphatases in the transcriptome (Scanlan et al. 2009; Dyhrman et al. 2012; Read et al. 2013). It is possible that the multiple genes encode enzymes with different substrate specificities, metal cofactors or regulation patterns. For example, some cyanobacteria appear to carry both the *phoX* and *phoA* type genes (Orchard et al. 2009). Although homologs of the canonical *E. coli phoA* gene (Zn dependent) are present in some cyanobacteria and upregulated under low phosphorus conditions (Fuszard et al. 2010), recent research suggests that the *phoX* (thought to be Ca dependent) type alkaline phosphatase may be much more prevalent, at least in the marine cyanobacteria (Sebastian and Ammerman 2009; Kathuria and Martiny 2011). This finding was attributed to the fact that Zn concentrations in the aquatic systems like the ocean are low, whereas Ca is plentiful. However a recent study by Yong et al. (2014) determined that the PhoX enzyme has a novel Fe-Ca cofactor. Given that Fe is low in many regions of the ocean, Fe could limit the ability of marine cyanobacteria to hydrolyze phosphomonoesters in regions where phosphate was also low, like the North Pacific Subtropical Gyre. With Zn and Fe both at low concentrations in the ocean it is unclear why the Fe requiring *phoX* is more broadly distributed. The *phoX* type alkaline phosphatase is present in a diverse suite of marine and freshwater cyanobacteria, including the genera *Trichodesmium*, *Synechococcus*, *Prochlorococcus*, and *Cylindrospermopsis* (Orchard et al. 2009; Scanlan et al. 2009; Kathuria and Martiny 2011; Sinha et al. 2014). The *phoX* gene is upregulated by low phosphate conditions, in the cyanobacteria in which it has been examined (Orchard et al. 2009; Kathuria and Martiny 2011). Although the prevalence and importance of *phoX* is increasingly widely accepted, the study of these alkaline phosphatases is evolving as new tools (e.g. mass spectrometry proteomics) become available. For example, a recent study focused at the protein level found that the PhoA protein was much more abundant than the PhoX protein in phosphorus-starved *Synechococcus* WH8102 (Cox and Saito 2013).

Since the alkaline phosphatase encoding genes are not well characterized in the eukaryotic algae, the extent to which different alkaline phosphatases present in the eukaryotic algal genomes have different substrates, regulation patterns or co-factors is not comprehensively understood. Three examples from the eukaryotic algae, where the alkaline phosphatase protein has been purified and characterized include the green alga *Chlamydomonas reinhardtii* (Quisel et al. 1996), the dinoflagellate *Prorocentrum minimum* (=*Prorocentrum cordatum*) (Dyhrman and Palenik 1997), and the coccolithophore *E. huxleyi* (Xu et al. 2006). Putative alkaline phosphatase encoding genes have been identified in studies of the diatom *T. pseudonana* (Dyhrman et al. 2012), and the pelagophyte *A. anophagefferens* (Wurch et al. 2011b), where – like with the cyanobacteria – they are upregulated by low phosphate. This suggests that the enzyme is transcriptionally regulated in at least in this subset of examples. The metal co-factors associated with the alkaline phosphatases of the eukaryotic algae have not been widely studied. However, the coccolithophore *E. huxleyi* has an alkaline phosphatase that is sensitive to Zn, and possibly Co availability (Xu et al. 2006; Jakuba et al. 2008). Further, the diatom *Phaeodactylum tricornutum* has a phosphorus-regulated alkaline phosphatase that is not Zn dependent, but Ca activated more akin to the cyanobacteria (Lin et al. 2013).

Alkaline phosphatase activity is particularly well studied in algae because there are many substrate analogs for field studies (Dyhrman 2005). Phosphatase activity has been shown to be broadly present in both marine and freshwater environments (Duhamel et al. 2010 and references therein), where the activity is often (although not always) inversely related to the phosphate or total dissolved phosphorus concentration (Cembella et al. 1984a, b; Jansson et al. 1988; Karl and Björkman 2002; Karl 2014). There is also a fluorogenic substrate for use in cell-specific enzyme labeled fluorescence (ELF) assays (González-Gil et al. 1998). This substrate will tag cells with the enzyme activity with a fluorescent product (see below). Many studies have used this tool in both marine and freshwater systems to examine the distribution of alkaline phosphatase activity in both eukaryotic algae and cyanobacteria (González-Gil et al. 1998; Dyhrman and Palenik 1999; Rengefors et al. 2001, 2003; Dyhrman et al. 2002; Nedoma et al. 2003; Lomas et al. 2004; Ruttenberg and Dyhrman 2005; Dyhrman and Ruttenberg

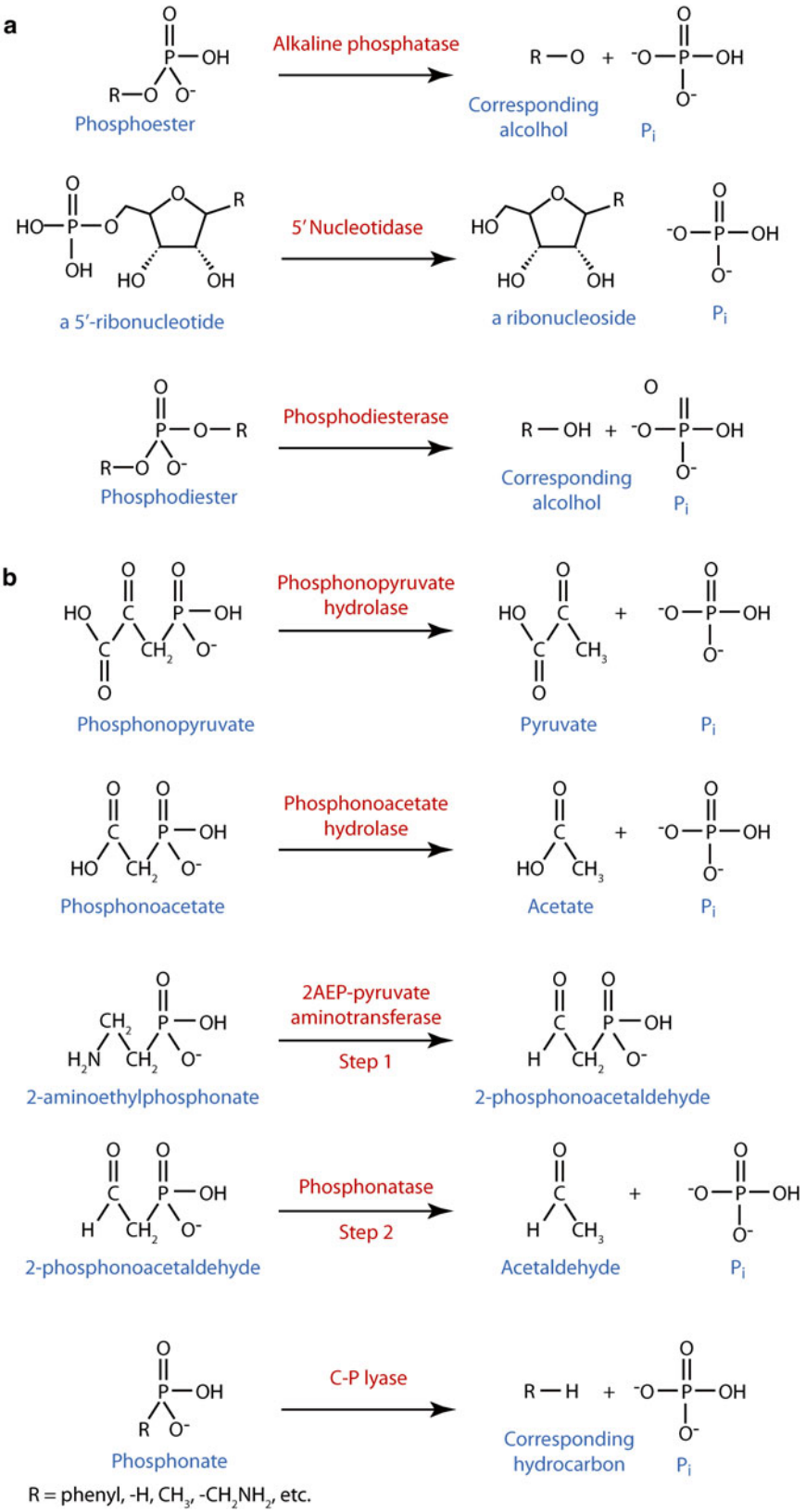

Fig. 3 A schematic of the common phosphohydrolytic enzymes and the reactions they catalyze. (**a**) Key enzymes for the hydrolysis of phosphoester found in eukaryotic algae and cyanobacteria. (**b**) Critical enzymes for the hydrolysis of phosphonate, some of which may be present in cyanobacteria. Many of the transcripts for many of these enzymes are induced by phosphorus stress in eukaryotic algae or cyanobacteria

2006; Nicholson et al. 2006; Hynes et al. 2009; Ranhofer et al. 2009; Duhamel et al. 2010; Mackey et al. 2012; Girault et al. 2013; McLaughlin et al. 2013). These studies suggest that alkaline phosphatase is widely present in field populations of algae, underscoring the importance of this enzyme in algal metabolism of phosphomonoesters.

4.1.2 Phosphodiesterase

The phosphodiesterases are not as well studied as alkaline phosphatases, however, the phosphodiesterase enzyme is also likely important for the metabolism of both exogenous and intracellular phosphodiesters. Phosphodiesterases act to break phosphodiester bonds in compounds like DNA, RNA, cyclic nucleotides, and lipids among others (Fig. 3). The genes encoding enzymes with phosphodiesterase activity are not well characterized relative to alkaline phosphatase, and there are likely many present in algae with a range of substrate specificities. For example cyclic nucleotide phosphodiesterase activity is related to nucleotide metabolism and broadly present in cyanobacteria including *Synechocystis* PCC6803, *Arthrospira platensis*, and *Anabaena cylindrica* (Sakamoto et al. 1991). General phosphodiesterase activity assayed with fluorometric substrates is also widespread among the eukaryotic algae including several species of *Chaetoceros*, *Dytilum brightwellii*, *T. pseudonana*, and the raphidophyte *Heterosigma akashiwo* (Yamaguchi et al. 2014). A phosphodiesterase was also partially purified and characterized as cell-surface associated in the diatom *P. tricornutum* (Flynn et al. 1986).

Phosphodiesterases may be regulated by a number of different factors, depending on their specificity and role in the cell. In the eukaryotic algae this activity appears to be increased when cells are starved for phosphate (Yamaguchi et al. 2014), and work in the diatom *T. pseudonana* suggests this enzyme is transcriptionally controlled (Dyhrman et al. 2012). Some eukaryotic algae, including diatoms and raphidophytes, have been shown to grow on phosphodiester as a sole phosphorus source, suggesting that the enzyme may act on exogenous sources (Yamaguchi et al. 2014). Culture studies have additionally identified the bioavailability of phosphodiesters such as cAMP in certain strains of *Prochlorococcus* and *Synechococcus* (Moore et al. 2005). This has been corroborated in field studies where the community is dominated by *Prochlorococcus*; here phsophodiester was a broadly available phosphorus source to the community (Björkman and Karl 1994). It is also thought that phosphorus-regulated phosphodiesterases could be involved in the breakdown of phospholipids seen in both marine algae and cyanobacteria under low phosphorus (Dyhrman et al. 2012).

Environmental measurements of phosphodiesterase activity are not common, but a recent study with samples spanning the North and South Pacific Oceans identified enhanced phosphodiesterase activity at the lowest (<10 nM) phosphate concentrations, and phosphodiesterase activity in the dissolved fraction was even higher than that of alkaline phosphatase activity (Sato et al. 2013). These results are consistent with culture studies and further emphasize the likely importance of this enzyme to DOP utilization by algae.

4.1.3 5′ Nucleotidase

The enzyme 5′ nucleotidase is also broadly present in algae where it hydrolyzes phosphate form 5′ nucleotides like ATP (Fig. 3). The genes encoding this enzyme are not well characterized, but putative 5′ nucleotidases are common in algae and detected in the genomes of the cyanobacteria and eukaryotic algae examined to date. 5′ nucleotidases are increasingly identified in transcriptome profiling studies, particularly with the eukaryotic algae like the pelagophyte *A. anophagefferens*, and the diatom, *T. pseudonana* among others (Dyhrman et al. 2006b, 2012; Wurch et al. 2011b). In these two cases, genes encoding 5′ nucleotidases were upregulated in transcriptomes from phosphorus-starved cells relative to replete, suggesting a transcriptional level regulation by phosphate. This differs from the lack of phosphorus regulation typically observed in heterotrophic bacteria (Ammerman and Azam 1985). A putative 5′nucleotidase transcript was also upregulated in phosphorus-depleted *Synechococcus* WH8102 (Tetu et al. 2009). The enzyme was partially purified and characterized as cell-surface associated in the diatom *P. tricornutum* (Flynn et al. 1986) and the coccolithophore *E. huxleyi* (Dyhrman and Palenik 2003). In both cases the enzyme activity was increased when cells were phosphorus-depleted (Flynn et al. 1986; Dyhrman and Palenik 2003). Last, a 5′ nucleotidase protein was also more abundant in a phosphorus-stressed proteome relative to a replete proteome in *A. anophagefferens* (Wurch et al. 2011a). Clearly this enzyme is broadly present and serves an important role in the metabolism of phosphorus, particularly when phosphorus is low.

It is common in algae to be able to grow on nucleotides as a sole phosphorus source, and the bioavailability and uptake phosphorus from exogenous ATP and AMP is well documented in cultures (Krumhardt et al. 2013) and field populations (Ammerman and Azam 1991; Björkman et al. 2012). The extent to which nucleotides are processed outside the cell versus taken up and then hydrolyzed inside the cell, is not well understood, but the studies available to date suggest that at least some nucleotidase activity is localized to the cell surface in eukaryotic algae (Flynn et al. 1986; Grossman and Takahashi 2001; Dyhrman and Palenik 2003; Wurch et al. 2011a), while cyanobacteria may be able to take up nucleotides directly.

Field measurements of bulk 5′ nucleotidase activity are rare, but do not appear to vary as a function of phosphate concentration, perhaps reflecting a lack of phosphorus

regulation in heterotrophic bacteria (Ammerman and Azam 1985, 1991). Studies of ATP uptake and hydrolysis are increasingly common on flow sorted cyanobacterial and even small eukaryote populations. These studies suggest that utilization of ATP can potentially meet a large fraction of phosphorus demand in field populations of *Prochlorococcus*, *Synechococcus*, *Trichodesmium*, and picoeukaryotes, particularly when inorganic phosphate is low (Casey et al. 2009; Orchard et al. 2010a; Björkman et al. 2012; Duhamel et al. 2012).

4.2 Phosphonate

Phosphonates were generally considered to be an unavailable form of phosphorus for algal growth until the release of the marine cyanobacterial genomes revealed genes putatively involved in phosphonate metabolism (Palenik et al. 2003; Dyhrman et al. 2006a). These early observations have led to an expansion of work in this area, which collectively is demonstrating that many cyanobacteria have the ability to metabolize phosphonates through a diverse suite of enzyme systems (Scanlan et al. 2009; Martinez et al. 2010). Enzyme systems for the hydrolysis of phosphonates include substrate-specific enzymes like phosphonoacetaldehyde hydrolase, as well as the broad specificity C-P lyase (White and Metcalf 2007; Villarreal-Chiu et al. 2012; McGrath et al. 2013) (Fig. 3). Notably, clear pathways for phosphonate metabolism have not been identified in the eukaryotic phytoplankton, nor is there direct evidence from culture studies. If this finding is borne out by further scrutiny, a potentially significant component of the DOP pool is unavailable to the eukaryotes and may drive community composition changes where DOP is an important phosphorus source.

4.2.1 Substrate-Specific Phosphonate Hydrolases

Many of the phosphonate hydrolases are well characterized in heterotrophic bacteria, and their presence, distribution, and regulation increasingly so for the cyanobacteria. For comprehensive reviews see the following syntheses (White and Metcalf 2007; Villarreal-Chiu et al. 2012; McGrath et al. 2013). Substrate-specific enzymes include phosphonopyruvate hydrolase, phosphonoacetate hydrolase, and phosphonoacetaldehyde hydrolase (phosphonatase) among potential other less well-characterized enzymes (McGrath et al. 2013) (Fig. 3).

Phosphonoacetate hydrolase is encoded by the *phnA* gene (Villarreal-Chiu et al. 2012). It is a Zn metalloenzyme that hydrolyzes phosphonoacetate to form acetate and phosphate (McGrath et al. 2013) (Fig. 3). This enzyme would be a potential route for 2-AEP metabolism. Phosphonopyruvate hydrolase is encoded by *palA* and is also a metalloenzyme (Fig. 3). The latter is often encoded together with genes related to phosphonate transporter, but is not always regulated by phosphate (McGrath et al. 2013). Screens of ocean metagenomic data suggest that both genes are present, although *phnA* is much more abundant; present in ~11 % of genomes sampled in the Global Ocean Survey relative to ~0.1 % for *palA* (Villarreal-Chiu et al. 2012). Their oceanic distribution hints at the importance of these pathways of phosphonate metabolism in marine systems, however the distribution of these genes in cyanobacteria has not been established.

Phosphonoacetaldehyde hydrolase (phosphonatase) is encoded by *phnX*, and in the degradation of 2-AEP is linked to a 2-AEP pyruvate aminotransferase encoded by *phnW* (Fig. 3). These are present in some cyanobacteria including freshwater *Synechococcus* strain OS-B′ (Adams et al. 2008) and marine *Synechococcus* WH8102 (Su et al. 2003). The activity encoded by these genes can be induced by phosphorus deficiency (Villarreal-Chiu et al. 2012), or alternatively be substrate inducible (Adams et al. 2008), and further work is required to confirm their presence and regulation more broadly in the cyanobacteria.

Ongoing work in this area is contributing to a rapidly changing understanding of the phosphonate hydrolayses. Martinez et al. (2010) identified the presence of a 2-oxoglutarate dioxygenase, *phnY*, and a possible phosphonohydrolase, *phnZ*, in *Prochlorococcus* strains (MIT9303, MIT9301), which were sufficient to allow utilization of 2-AEP as the sole phosphorus source in *E. coli*. Interestingly, the frequency of the *Prochlorococcus phnY* and *phnZ* genes was significantly higher in the phosphorus-depleted surface waters of the Sargasso Sea compared with the North Pacific subtropical gyre (Coleman and Chisholm 2010; Martinez et al. 2010). However, the presence of these genes did not clearly confer the ability for *Prochlorococcus* MIT9301 to grow on 2-AEP as a sole phosphorus source, and the genes may be related to phosphite metabolism (Martinez et al. 2012). It is also important to note that evidence for phosphonate metabolism in the cyanobacteria has been identified in the absence of characterized gene pathways, suggesting there are other potential enzymes yet to be identified (Gomez-Garcia et al. 2011). In short, new pathways for phosphonate metabolism are still being identified and work remains in order to characterize these enzymes and the role they serve in cellular phosphorus metabolism for algae.

4.2.2 Broad Specificity C-P Lyase

In contrast to the substrate-specific phosphonate hydrolayses, there is a broad specificity enzyme complex called a C-P lyase, which can hydrolyze a diverse suite of phosphonate compounds (Fig. 3). The C-P lyase is encoded by a suite of genes denoted *phnGHIJKLM* (White and Metcalf 2004b, 2007). These genes are often linked to those for phosphonate transport denoted *phnCDE* (White and Metcalf 2007). The

transport genes are broadly present in both marine and freshwater cyanobacteria (Scanlan et al. 2009; Bench et al. 2013; Harke and Gobler 2013). Conversely the C-P lyase encoding genes are less common (Dyhrman et al. 2006a; Scanlan et al. 2009). The *phnJ* gene is typically used as a marker for the C-P lyase enzyme, and it is present in *Synechococcus* sp. isolated from microbial mats (Adams et al. 2008), *Trichodesmium* (Dyhrman et al. 2006a), *Cylindrospermopsis* (Sinha et al. 2014), *Nostoc* PCC7120 (Dyhrman et al. 2006a), and *Nodularia spumigena* (Voss et al. 2013) to name a few. This gene set has not been observed in the marine picocyanobacteria like *Prochlorococcus* and *Synechococcus* to date (Scanlan et al. 2009). It is worth emphasizing that all of the C-P lyase containing cyanobacteria identified here are brackish, or freshwater except *Trichodesmium*. As such, *Trichodesmium* appears to occupy a unique niche with regard to phosphorus metabolism among the other marine cyanobacteria, which may explain why *Trichodesmium* is so successful in low phosphorus environments.

Expression of the C-P lyase genes is typically phosphorus controlled in *E. coli* and expression studies in the cyanobacteria suggest this is the case (Dyhrman et al. 2006a; Adams et al. 2008). Further, the expression of these genes has been seen in both marine and freshwater field populations (Dyhrman et al. 2006a; Gomez-Garcia et al. 2011). Although there are not fluorogenic substrates available for assaying C-P lyase activity, the enzyme activity can be tracked by the evolution of methane in the presence of methylphosphonate (Beversdorf et al. 2010). Using this type of assay, the *Trichodesmium* C-P lyase activity has been measured in both cultures and field populations, substantiating the gene expression results (Beversdorf et al. 2010). The composition of phosphonates is largely unknown, but the presence of a broad specificity enzyme may confer an advantage for growth on a broader spectrum of DOP in a select few cyanobacteria.

5 Phosphorus Stress Responses

Phosphorus deficiency has long been recognized as an important driver of algal physiological ecology in freshwater systems (Schindler 1977), and is increasingly recognized as a major driver of marine ecosystems (Karl 2014), influencing microbial genetic diversity (Coleman and Chisholm 2010) and global oceanic primary production (Benitez-Nelson 2000). For example, there is growing evidence that phosphorus limits marine primary production in the subtropical North Atlantic (Mather et al. 2008; Lomas et al. 2010), and other major ocean systems (Paytan and McLaughlin 2007), thus influencing the magnitude and rate of phosphorus and carbon export over modern and geological time-scales (Benitez-Nelson 2000; Paytan and McLaughlin 2007; Diaz et al. 2008). Often phosphorus deficiency, starvation, stress, and limitation are used interchangeably, but there are many subtleties to how these terms may be interpreted. Here the term phosphorus stress is used to mean a physiological response to low phosphorus (distinct from a stress response to high phosphorus), the extent to which the phosphorus stress response is able to recover phosphorus for the cell will dictate whether cellular growth is limited, or arrested by phosphorus. To cope with low phosphorus in nature, both cyanobacteria and eukaryotic algae have evolved an inducible, sophisticated, and multi-faceted, phosphorus stress response involving the following major strategies. These phosphorus stress responses include (1) robust sensor response control of phosphorus stress induced transcription, (2) phosphorus sparing or recycling (3) high affinity or increased phosphate transport, and (4) a switch to the utilization of alternative phosphorus forms.

5.1 Phosphorus Stress Signaling

In cyanobacteria, phosphorus stress responses are controlled by a sensor response system that results in the transcription of the set of genes cells need to respond to phosphorus deficiency. The genes making up this phosphorus stress response are often referred to as the pho regulon, after the term described for *E. coli* (Torriani-Gorini 1987; Wanner 1996). Transcription of the cyanobacterial pho regulon is thought to be controlled by a two component sensor response system (*phoR*, *phoB*), where PhoR senses phosphorus availability and activates the transcriptional regulator PhoB (Fig. 4). PhoB binds to specific regions of DNA upstream of pho regulon genes, called pho boxes. Pho boxes have been identified upstream of a number of putative pho regulon genes in marine cyanobacteria like *Prochlorococcus*, *Synechococcus* and *Trichodesmium* (Su et al. 2007). For example there is a putative pho box upstream of the *phoX* gene in *Trichodesmium*, which encodes an alkaline phosphatase induced by phosphorus stress (Orchard et al. 2009), as well as pho boxes present upstream of other phosphorus-regulated genes in *Trichodesmium* and *Crocosphaera* (Dyhrman et al. 2006a; Dyhrman and Haley 2006; Su et al. 2007; Orchard et al. 2009). This canonical *phoB*/*phoR* model is supported by studies in *Synechococcus* strain WH8102 where expression analyses of *phoB*/*phoR* mutants confirmed that these genes either directly or indirectly controlled transcription of pho regulon genes (Tetu et al. 2009). The similar *sphS*/*R* system in *Synechocystis* also controls the phosphorus stress response (Suzuki et al. 2004). In the cyanobacteria, there are also additional signaling genes to consider in the phosphorus stress response (Fig. 4). For example, strains of *Synechococcus* and *Prochlorococcus* have the *ptrA* gene, a paralog of the global nitrogen regulator *ntcA* (Scanlan et al. 2009). This

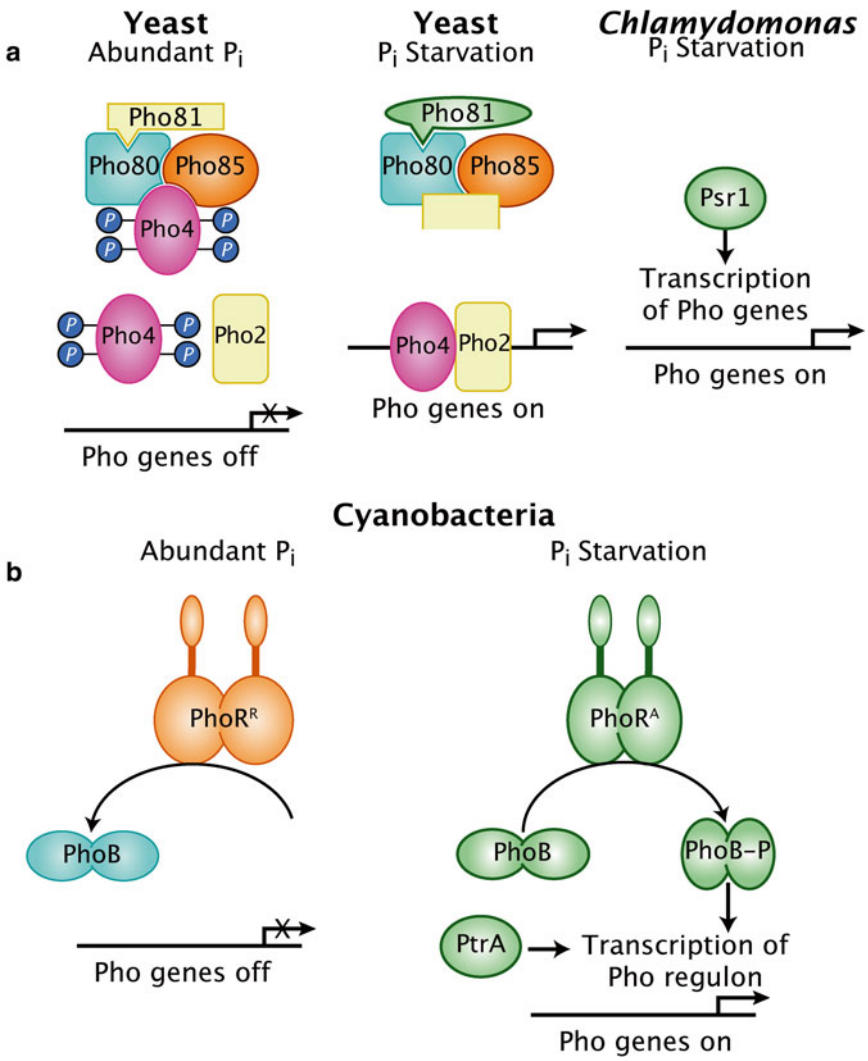

Fig. 4 The putative systems that control sensing and responding to phosphorus in algae. (**a**) The sensor response system for yeast involves the kinase (Pho81) which controls the phosphorylation or dephosphorylation of Pho4, which in turn controls transcription of the Pho genes (Lenburg and O'Shea 1996). The degree of phosphorylation on Pho4 can control the degree of transcription (Springer et al. 2003). In *Chlamydomonas*, a putative phosphorus regulatory protein Psr1, is also involved in regulating the transcription of phosphorus-responsive genes (Grossman and Takahashi 2001). The extent to which this model is broadly applicable in eukaryotic algae is poorly understood. (**b**) The putative sensor response system in cyanobacteria, is thought to be similar to *E. coli*, where PhoR is activated by low phosphorus, phosphorylating PhoB. PhoB controls transcription of the Pho regulon genes, with some exceptions (Su et al. 2007). In cyanobacteria like *Synechococcus* the PtrA protein has also been shown to have a regulatory role on sensing and responding to phosphorus stress (Ostrowski et al. 2010). *Green* indicates genes or proteins that have been observed to increase with phosphorus stress in the eukaryotic algae and cyanobacteria

gene is upregulated in response to phosphorus stress in *Prochlorococcus* strain MED4 along with *phoR* and *phoB* (Martiny et al. 2006; Reistetter et al. 2013), and *Synechococcus* strain WH8102 *ptrA* mutants have reduced inducible alkaline phosphatase activity relative to the wild type (Ostrowski et al. 2010).

The threshold for when this signal transduction cascade would occur is not well defined. It likely differs between isolates, and would potentially be a function of both exogenous phosphorus supply and intracellular phosphorus pools. In some cyanobacteria there is evidence of some pho regulon genes (e.g. fast and slow *pstSCAB* sets) being induced before others (Pitt et al. 2010), and it may be that there are components of the phosphorus stress response that are controlled by a different signaling cascade, or by changes in the *phoB*/*phoR* encoded mechanism that allow a graded response. For example, in *Synechococcus* WH 8102 there appears to be two controllers, with PhoB-dependent induction of high-affinity phosphate transporters, followed by the PtrA-dependent induction of phosphatases (Ostrowski et al. 2010).

The mechanisms controlling the phosphorus stress signaling cascade have been examined in *Chlamydomonas* (Grossman and Takahashi 2001), but are not well explored in many of the other eukaryotic algae. The *Chlamydomonas* signaling response appears to be controlled in part by the Psr1 protein (Fig. 4), which functions as a transcription regulator that influences transcription of the phosphorus stress response genes (Wykoff et al. 1999; Grossman 2000). Psr1 is the first regulator of phosphorus metabolism in eukaryotic algae to be identified, and it is related to regulators in *Arabadopsis*, not yeast (Wykoff et al. 1999). These findings suggest that phosphorus metabolism in *Chlamydomonas* and possibly other algae is regulated in a way that is different from that of nonphotosynthetic eukaryotes. However, recent analyses of phosphorus stress transcriptomes suggest that phosphorus stress signaling in some groups could be more akin to what is observed in yeast (Fig. 4). In yeast, the cyclin kinase system encoded by pho85 and pho80 acts to phosphorylate and block transcriptional activation of the phosphorus stress genes by pho4, a transcriptional activator (Lenburg and O'Shea 1996). The degree of phosphorylation

may be tuned to the degree of phosphorus stress, allowing yeast to finely tune their stress responses (Komeili and O'Shea 1999; Springer et al. 2003). In low phosphorus conditions pho81 is upregulated and inhibits phosphorylation of pho4, allowing transcription of the phosphorus stress response genes (Komeili and O'Shea 1999; Wykoff and O'Shea 2001). In the pelagophyte *A. anophagefferens* putative pho81 and pho4 are upregulated under phosphorus stress (Frischkorn et al. 2014). Although certainly not definitive, this observation is suggestive of possible differences in phosphorus stress signaling between algal lineages, and this warrants closer scrutiny.

5.2 Phosphorus Sparing or Recycling

It has been widely observed that cyanobacteria and eukaryotic algae have the ability to modulate their phosphorus requirement (quota) thus reducing cellular phosphorus (Krauk et al. 2006). The mechanisms driving this phosphorus sparing are thought to generally fall into three main areas, reduction of phosphorus rich biochemicals, substitution of phosphorus rich biochemicals, and bypasses of phosphorus rich metabolic reactions.

5.2.1 RNA Recycling

Nucleic acids and lipids both represent major phosphorus reservoirs in phosphorus replete algae (Fig. 1). The cellular phosphorus found as RNA typically accounts for at least 50 % of the non-storage phosphorus in algae and plants (Raven 2013). It has been hypothesized (the growth rate hypothesis) that sustained rapid growth requires high concentrations of ribosomes. Since ribosomes are rich in phosphorus, this would predict growth rate and phosphorus content to be positively correlated (Flynn et al. 2010). Although the applicability of this hypothesis to algae is debatable (Flynn et al. 2010), this concept is consistent with a reduction in ribosomes and rRNA when phosphorus is deficient. When phosphorus is depleted studies have observed a decrease in RNA per cell (Grossman 2000) (Figs. 1 and 5), probably largely reflected in a decline in rRNA as protein translation slows, allowing this source of phosphorus to be recycled. In *Chlamydomonas* there is a reduction of the number of ribosomes in phosphorus-limited cells (Grossman 2000). In addition, global transcriptomic and proteomic studies in both cyanobacteria and eukaryotic algae have observed a down regulation of transcripts and ribosomal proteins in phosphorus-stressed cultures relative to replete controls (Tetu et al. 2009; Dyhrman et al. 2012). Although some of these responses may be common in any stressor that reduces growth rate, a reduction in the cellular rRNA pool would conserve this phosphorus for other uses in the cell.

5.2.2 Phospholipid Substitution

Phospholipids are also a major phosphorus reservoir in algae. There is an increasingly rich literature spanning both cyanobacteria and eukaryotic algae that indicates many groups can substitute the non-phosphorus containing sulfolipid sulphoquinovosyldiacylglycerol (SQDG) for the phosphorus-rich phospholipid phosphatidylglercerol (PG) (Van Mooy et al. 2009; Merchant and Helmann 2012) (Fig. 5). This substitution is common in the marine cyanobacteria like *Synechococcus* and *Trichodesmium*, and eukaryotic algae including diatoms and coccolithophores (Van Mooy et al. 2009). For example, in *Chlamydomonas*, phosphorus deficiency reduces the phospholipids phosphatidylglercerol (PG) roughly 50 % in concert with an increase in sulfolipids (Merchant and Helmann 2012). In phosphorus-stressed cultures of eukaryotic algae Van Mooy et al. 2009 also observed that non-phosphorus containing 'betaine' lipids were substituted for phosphorus containing phosphatidylcholine. Van Mooy et al. (2009), suggesting that remodeling of the lipid membrane may be common but there are many subtleties to the lipids that are modulated when phosphorus is depleted. Studies in a representative diatom suggest this lipid substitution happens rapidly upon phosphorus stress, and the ratio of SQDG:PG also quickly reverts if stressed cells are refed with phosphorus (Martin et al. 2011). This is increasingly recognized as an important phosphorus sparing mechanism, with a reduction in phospholipids sparing between 10 % and 30 % of the phosphorus quota for model diatoms and coccolithophores (Van Mooy et al. 2009; Martin et al. 2011).

A protein putatively involved in sulfoplipid biosynthesis (a UDP-sulfoquinovose synthesis protein (encoded by *sqdB*)), was identified as upregulated in low phosphorus transcriptomes or proteomes of both *A. anophagefferens* and *T. pseudonana*, which both shift their SQDG:PG ratio under phosphorus stress (Wurch et al. 2011a; Dyhrman et al. 2012). However, in marine *Prochlorococcus* MED4 the *sqdB* transcript was not upregulated by P stress (Reistetter et al. 2013), nor was it upregulated in freshwater *Microcystis aeruginosa* (Harke and Gobler 2013). The production of betaine lipids is controlled by BTA1, a betaine lipid synthase, in *Chlamydomonas* (Riekhof et al. 2005), but this gene has either not been examined or detected in most of the other genomes from eukaryotic algae. For example, there is not a clear homolog of BTA1 in the *T. pseudonana* genome, or phosphorus-stressed transcriptomes and proteomes, even though this diatom is known to produce betaine lipids in response to phosphorus deficiency (Van Mooy et al. 2009; Dyhrman et al. 2012). Although substitution of phospholipids in a common phosphorus sparing mechanism, in many cases linking the substitution to the dynamics of specific genes has not been examined in detail. Collectively, these findings underscore the importance of phospholipid substitu-

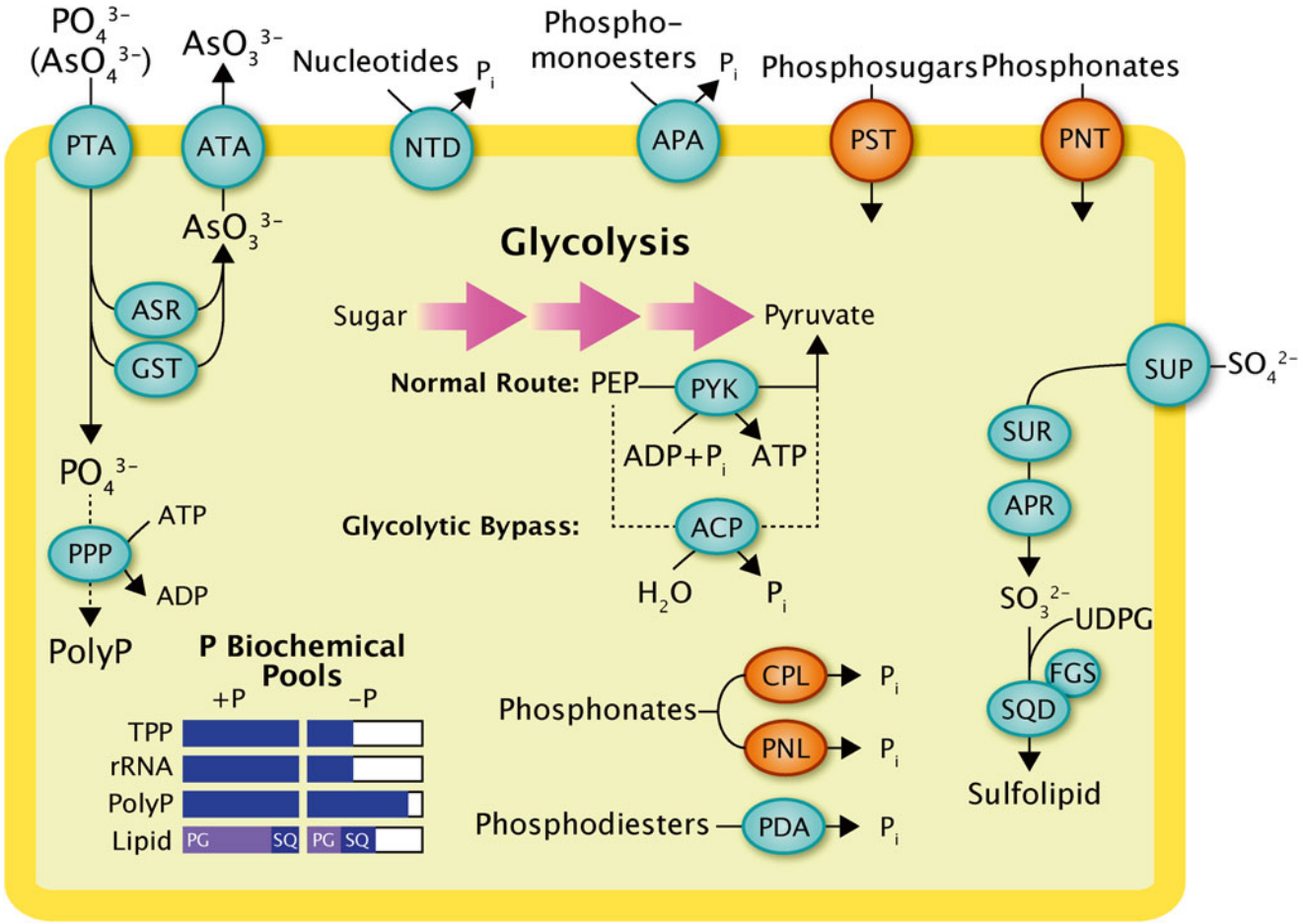

Fig. 5 A cell model illustrating common phosphorus stress responses in algae. Proteins in *blue* are found in both eukaryotic algae and cyanobacteria, while *orange* proteins are to date only found in cyanobacteria. Localization of the depicted proteins is for clarity and is not meant to represent actual cellular localization. *Bars* indicate changes in phosphorus containing biochemical pools or cellular inventories. *ACP* Acid phosphatase, *APA* Alkaline phosphatase (phosphomonoesterase), *APR* Adenosine-5′-phosphosulfate reductase, *ASR* Arsenate reductase, *ATA* Arsenite translocating ATPase, *CPL* Phosphonate (C-P) lyase, *FGS* Ferredoxin-dependent glutamate synthase, *GST* Glutathione S Transferase, *NTD* 5′ Nucleotidase, *PDA* Phosphate diesterase, *PEP* Phosphoenolpyruvate, *PG* Phospholipid, *PNL* Generic phosphonate lyase, *PNT* Phosphonate transporter, *PolyP* Polyphosphate, *PPP* Polyphosphate polymerase, *PST* P sugar transporter, *PTA* Phosphate transporter, *PYK* Pyruvate kinase, *rRNA* Ribosomal RNA, *SQ* Sulfolipid, *SQD* SQD1 (sulfolipid biosynthesis protein 1), *SUP* Sulfate permease, *SUR* Sulfate reductase, *TPP* Total particulate phosphate, *UDPG* Uridine diphosphate glucose

tion but also emphasize that the molecular underpinnings of these responses are not fully understood.

5.2.3 Polyphosphate Dynamics

As discussed previously (Sect. 3.2), polyphosphate can serve as a storage compound that accumulates during luxury uptake in phosphorus-replete environments, and can be mobilized for growth in phosphorus deplete environments when cyanobacteria or eukaryotic algae experience phosphorus stress (Fig. 5). With this canonical understanding of polyphosphate, the cellular polyphosphate pool would be expected to decrease in phosphorus-stressed cyanobacteria and eukaryotic algae, although this is increasingly being shown to be more complicated than this canonical view.

It has been hypothesized that cells inducing a phosphorus stress response could experience a temporary excess of phosphorus that could repress continued phosphorus uptake. The upregulation of a polyphosphate polymerase or polyphosphate kinase to produce polyphosphate during phosphorus stress conditions could enable the creation of a sink of readily accessible phosphorus while also circumventing repression of phosphorus scavenging (Ogawa et al. 2000). In brief, it is thought that an increase in polyphosphate, or at least an increase in the ratio of polyphosphate to total particulate phosphate (polyP:TPP) may avoid transient phosphate accumulation and the down regulation of the phosphorus stress response. There is some evidence to support this hypothesis in algae. For example, the Vtc4 polyphosphate polymerase

in *E. huxleyi*, *T. pseudonana*, and *A. anophagefferens* (Dyhrman et al. 2006b, 2012; Wurch et al. 2011b), and the *ppK* gene in *Synechocystis* PCC6803 (Gomez-Garcia et al. 2003), are all upregulated in phosphorus-stressed cultures relative to replete phosphorus controls. This observation is supported by an increase in polyP:TPP in phosphorus-stressed cultures of *T. pseudonana*, *T. erythraeum*, *and Synechococcus* WH8102 (Orchard et al. 2010b; Dyhrman et al. 2012; Martin et al. 2014). Although there could be an absolute decrease in polyphosphate with phosphorus stress, this pool may not decrease very much, such that the polyP:TTP ratio increases dramatically with phosphorus-stress.

Recent field work in the low phosphorus Sargasso Sea supports this observation. Martin et al. (2014) observed higher polyP:TPP in particulate matter dominated by cyanobacteria in the Sargasso Sea relative to the higher phosphorus regions (Martin et al. 2014). The supporting data implied that this observation was not necessarily the result of a strictly defined overplus scenario, but rather was the result of chronically low phosphorus and the phosphorus physiology of cyanobacteria in this region (Martin et al. 2014). This study, focused on a total cellular polyphosphate size fraction likely dominated by cyanobacteria, but the results are also consistent with taxon-specific measurements. Orchard et al. (2010a) saw similar increases in the ratio of polyphosphate to total particulate phosphorus in Sargasso Sea populations of the cyanobacterium *Trichodesmium*, higher than phosphorus replete culture controls (Orchard et al. 2010b). Further, trends in the picocyanobacterium *Synechococcus* from the same system, were also consistent with this trend (Martin et al. 2014).

In freshwater, lowering of phosphate in a eutrophic river, did not result in a lowering of algal community polyphosphate despite other evidence of phosphorus stress (Bolier et al. 1992). Taken together these observations emphasize the complicated dynamics of polyphosphate, and that the typical view that polyphosphate would be mobilized and largely drawn down by cells under phosphorus starvation is not necessarily the case. Polyphosphate may be drawn down, and or extensively cycled under phosphorus stress, but to date the reduction of total particulate phosphorus appears to largely be driven by changes in other phosphorus biochemicals (Fig. 5).

5.2.4 Phosphorus Bypasses

A last example of potential phosphorus sparing in algae involves bypassing of phosphorus rich metabolic reactions (Fig. 5). One example of a phosphorus rich metabolic pathway is glycolysis, where the conversion of one molecule of glucose into two molecules of pyruvate, requires two molecules of phosphate. Glycolysis in higher plants can be modulated by phosphorus stress in order to bypass those reactions that demand phosphate (Plaxton 1996). For example phosphoenolpyruvate carboxylase (PEPC) can serve as a glycolytic bypass enzyme by diverting phosphoenolpyruvate (PEP) to oxaloacetate (OAA) and releasing phosphate. OAA can then be converted to malate through the activity of malate dehydrogenase and eventually to pyruvate through a malic enzyme, thus completing the bypass of the ADP-requiring step of converting PEP directly to pyruvate catalyzed by pyruvate kinase (Plaxton 1996). Some evidence suggests that algae have this bypass (Theodorou et al. 1991; Wurch et al. 2011a; Dyhrman et al. 2012). Wurch et al. (2011a) identified a possible glycolytic bypass in *A. anophagefferens* using data from a low phosphorus proteome, and Dyhrman et al. (2012) found evidence of a glycolytic bypass in the diatom *T. pseudonana*, also from a low phosphorus proteome. However the extent to which this is true a phosphorus conservation strategy, and its presence in algae from other groups not highlighted here is largely unknown.

5.3 High Affinity or Increased Phosphate Transport

A common feature of the phosphorus stress response across both cyanobacteria and eukaryotic algae is the upregulation of phosphate transport systems when cells are phosphorus-stressed (Fig. 5). In some cases high affinity transporters are induced which would result in a decrease in K_m and a change in the type or number of transporters would change V_{max}. These potential shifts can be seen in culture studies or in the kinetic patterns for specific taxa as a function of phosphorus in the field (see Sect. 3.1). For example, phosphorus-stressed cultures of two different *Trichodesmium* strains had up to six times higher maximum phosphate uptake rates (V_{max}) than the rates observed in phosphorus replete cultures (Fu et al. 2005).

In cyanobacteria, high affinity phosphate transport is controlled by *pstSCAB*, which includes a high affinity binding protein (PstS) and an ATP-driven transport complex (PstCAB) (Scanlan et al. 2009). These genes are common in all the cyanobacteria examined to date from both marine (Scanlan et al. 2009) and freshwater systems (Harke et al. 2012; Sinha et al. 2014). The regulation of this system by phosphorus stress is variable between species and related isolates (Martiny et al. 2006; Fuszard et al. 2010), although many studies have observed phosphorus stress upregulation of at least one copy of *pstS* (Martiny et al. 2006; Orchard et al. 2009; Harke et al. 2012). In some cases the full *pstSCAB* gene cassette is upregulated under phosphorus stress (Martiny et al. 2006), in others it appears that the *pstCAB* is constitutively expressed and only certain copies of *pstS* or certain *pstSCAB* sets are upregulated (Pitt et al. 2010). There are also examples of one copy of *pstSCAB* being an early responder to low phosphorus, while a second copy of the gene group is only induced later under extreme phosphorus

stress (Pitt et al. 2010). In particular, the dynamics of *pstS* appear to track with aspects of P biogeochemistry. For example, *Prochlorococcus pstS* is overrepresented in genomes from the low phosphorus Sargasso Sea relative to the comparatively higher phosphorus North Pacific Subtropical Gyre (Coleman and Chisholm 2010), and both *Synechococcus* and *Prochlorococcus* PstS was detected in a metaproteome from the Sargasso Sea (Sowell et al. 2009). In summary it is common for cyanobacteria to modulate phosphate uptake as a function of phosphorus stress, and this is largely controlled through differential expression of *pstS* and or *pstSCAB*.

The upregulation of phosphate transporters is a common feature of studies in eukaryotic algae under phosphorus stress (Chung et al. 2003; Dyhrman et al. 2006b, 2012; Wurch et al. 2014). In *Tetraselmis*, there is a high affinity phosphate transporter that is upregulated when phosphorus is depleted (Chung et al. 2003), and this has been observed in the transcriptomes of a diverse array of other algae including two strains of *A. anophagefferens* (Wurch et al. 2011b; Frischkorn et al. 2014), the coccolithophore *E. huxleyi* (Dyhrman et al. 2006b), the prymnesiophyte *Prymnesium parvum* (Beszteri et al. 2012) and the diatom *T. pseudonana* (Dyhrman et al. 2012) among others. Many of these are Na^+-dependent phosphate transporters, such as those characterized in plants (Dyhrman et al. 2012; Rubio et al. 2004). This pattern of high-affinity phosphate transporters being induced when phosphorus is low is also evident in changes in protein abundance (Dyhrman et al. 2012), and uptake kinetics (Perry 1976). In *A. anophagefferens* strain 1984, the transcription of phosphate transporter (PTA) is tightly controlled by phosphorus, as it is induced when exogenous phosphate is depleted, and the transcript signal is rapidly lost within just 2 h of phosphorus being re-supplied to phosphorus-stressed cells, the transcript is not even detectable within 24 h (Wurch et al. 2011a). Notably the protein does not turn over as quickly, making the transcript a potentially better indicator of instantaneous phosphorus stress, and the protein data suggesting that phosphorus stress induced changes in phosphate uptake, may extend a division or more after the cell is no longer phosphate deplete (Wurch et al. 2011a). The timing and turnover of transcript, protein and activity are important considerations when screening for these different signals in field populations.

5.4 Utilization of Alternative Phosphorus Forms

Arguably the most important and commonly observed phosphorus stress responses in cyanobacteria and eukaryotic algae is the induction of enzymes and transporters for the metabolism of alternative phosphorus forms (Fig. 5). These alternative phosphorus forms are typically organically complexed with either an ester, diester, or phosphonate bond (see Sect. 4). Other forms of phosphorus that appear to be bioavailable are polyphosphate, and phosphite (see Sects. 3.2 and 3.3). The specific substrates, genes, and enzymes involved in the utilization of alternative phosphorus forms is area of intense scrutiny and novel and surprising findings in recent years (see Sects. 3.2, 3.3, and 4). Much of the details of these pathways are discussed in other sections of this chapter. As was emphasized in those sections the majority of enzymes, transporters and metabolic pathways that control the utilization of alternative phosphorus forms, are upregulated when phosphorus drops below critical threshold levels, the presence of the substrate does not typically cause upregulation of the enzyme or pathways.

With their phosphorus stress induced regulation, the enzymes involved in the utilization of alternative phosphorus forms are often used as biomarkers of phosphorus physiology (Dyhrman 2008), the most common of which is the enzyme alkaline phosphatase. This marker of phosphorus stress can be extremely useful, but it is best employed when there is a comprehensive understanding of its activity, regulation, the turnover of the marker in question (e.g. activity, protein, or transcript), as well as the species or community under study (Dyhrman 2008).

A striking feature of phosphorus metabolism research in the last decade is the expansion of the alternative phosphorus forms known to be bioavailable. The suite of known phosphorus compounds that will support the growth of algae is growing rapidly, and is likely to continue to do so. In some cases knowledge about the environmental chemistry of particular substrates is lagging behind bioavailability studies (e.g. phosphite), and interdisciplinary studies that track specific compounds in the environment as well as their metabolism by algae will be important areas of future work.

6 Methodological Advances

A number of recent advances and approaches have developed which are allowing phosphorus metabolism, and phosphorus stress responses in the eukaryotic algae and cyanobacteria to be studied in new and powerful ways (Beardall et al. 2001; Dyhrman 2008; McLean 2013; Wagner et al. 2013). In the context of phosphorus metabolism, the new methodologies that have arguably had the most impact fall into two broad categories; (1) Cell or taxon-specific approaches and (2) Molecular 'omic methods (Table 1).

6.1 Cell or Taxon-Specific Approaches

The value of screening physiology and cellular metabolism at a microbially-relevant scale is increasingly recognized

Table 1 Methodological advances and tools that can be used to study phosphorus metabolism in algae

Term	Definition	Assay technology	Example publication
Genome	Composed of DNA that encodes the full set of an organism's genes, the genome is the physiological blueprint of an organism	DNA sequencing	Armbrust et al. (2004)
Metagenome	The complete amalgam of DNA from a microbial community in a given environment, e.g. sea water	DNA sequencing	Venter et al. (2004)
Transcriptome	The complete set of an organism's transcribed genes. Typically assayed on cultured isolates	RNA sequencing	Dyhrman et al. (2012)
Metatranscriptome	The complete amalgam of RNA from a microbial community; it provides a snapshot of all genes expressed at the time of sampling	RNA sequencing	Gifford et al. (2011)
Proteome	The complete set of proteins present within an organism, typically a cultured isolate. The proteome represents all transcribed and translated genes at a given time	Mass spectrometry	Wurch et al. (2011a)
Metaproteome	The complete amalgam of proteins from a microbial community	Mass spectrometry	Morris et al. (2010)
Metabolome	All of the low molecular weight metabolites present within an organism	Mass spectrometry	Kujawinski et al. (2009)
Lipidome	The total profile of all lipids present within an organism	Mass spectrometry	Martin et al. (2011)

(Azam and Malfatti 2007). Bulk approaches can miss subtlety in physiological responses to phosphorus availability, and how individual taxa compete for phosphorus among other variables. At a single-cell level, the cell-specific enzyme labeled fluorescence (ELF) substrate (Paragas et al. 1997) for detecting alkaline phosphatase activity has been widely applied (Dignum et al. 2004). This substrate is colorless and soluble when unreacted but when the enzyme hydrolyzes phosphomonoester the resulting reaction product is both fluorescent and insoluble; the product precipitating at the site of the enzyme activity. There have been many studies utilizing this substrate to examine enzyme localization (Dyhrman and Palenik 1999; Dyhrman et al. 2012), culture activity and physiology (González-Gil et al. 1998), and perhaps most commonly to look at the activity within or between species in field populations (Dyhrman 2008). The tool has been broadly applied to algae in both marine and freshwater environments to examine alkaline phosphatase activity and its regulation (see Sect. 4.1.1). One of the striking features of many of these studies is the cell to cell variability in the detection of enzyme activity from the same environment. This variability has been confirmed to not be an artifact through direct antibody labeling of the enzyme in the dinoflagellate *P. minimum* (Dyhrman and Palenik 2001), and could indicate the importance of small scale interactions between algae and their geochemical microenvironment, or their interactions with other cells (see Sect. 7.1.2). In summary, this methodological advance has been instrumental in expanding the study of both the enzyme, and algal physiological ecology at a microbially relevant spatial scale.

Single-cell genomics is of course a powerful tool to link metabolic capacity to specific, and typically uncultured, cells from the environment (Macaulay and Voet 2014). As was highlighted above, application of this tool is also serving to emphasize the diversity of both genotypes and the importance of the microenvironment. There are still few examples of its application in eukaryotic algae or cyanobacteria, but work by Kashtan et al. (2014) found that *Prochlorococcus* ecotypes are really a group of unique sub-populations of different genotypes consisting of "core" genes, and a set of "flexible" genes that are sometimes present. Notably, cells from different subpopulations carry similar flexible phosphonate related gene sets (Kashtan et al. 2014), suggesting that the metabolism of phosphonate can act as a niche defining feature. Here again a single-cell methodological advance is emphasizing the importance of phosphorus metabolism to the evolution of cyanobacterial genotypes at a fundamental level.

Flow cytometers capable of high fidelity and rapid sorting of cyanobacteria and picoeukaryotes take advantage of these groups' intrinsic properties to sort populations based on size and pigmentation. This technology has been used to examine the uptake of radiolabeled phosphorus compounds and phosphate into specific populations (Björkman and Karl 1994; Casey et al. 2009; Duhamel et al. 2012). For example Casey et al. (2009) incubated Sargasso Sea populations with ^{33}phosphate and ^{33}ATP, then sorted cells into the picoeukaryote, *Synechococcus* and *Prochlorococcus* fractions with the flow cytometer and assayed isotope incorporation into the target populations (Casey et al. 2009). This general approach has been used in several recent studies (Casey et al. 2009; Björkman et al. 2012; Duhamel et al. 2012) to look at phosphorus uptake kinetics, competition between groups, and in particular to assay DOP utilization with proxy compounds like ATP. This major advance is allowing researchers to investigate how different populations of eukaryotic algae and cyanobacteria compete for phosphorus, and to what extent different phosphorus compounds are used to meet phosphorus demand.

6.2 Molecular 'omic Approaches

Classic approaches to algal nutrition have most often used either cultured isolates or community level assays to examine nutrient uptake (Kudela and Cochlan 2000), nutrient ratios (Anderson et al. 2002 and references therein), and enzyme activity (Mulholland et al. 2003), among other approaches. These types of studies are very valuable, providing a basic understanding of phosphorus metabolism, much of which has been outlined above. However, advances in molecular approaches (Table 1) are expanding our understanding of how algae metabolize phosphorus in both cultures and field populations (Dyhrman 2008). In short, we are able to move beyond the outward responses of the cells to nutrients (e.g. changes in elemental composition or growth) to the underlying mechanisms that drive those responses. This level of molecular detail not only allows the promise of better predictive power with regards to a species' response to phosphorus, but also provides the technological capability to specifically monitor that response in field populations. This section focuses on how (1) genomics and metagenomics, (2) transcriptomics and metatranscriptomics, (3) proteomics and metaproteomics, and (4) other molecular approaches (Table 1) have led to new insight into phosphorus metabolism and the role of phosphorus in the evolution and ecology of algae.

6.2.1 Genomics and Metagenomics

Genome sequencing was initially done in the cyanobacteria (Palenik et al. 2003; Rocap et al. 2003), and has now resulted in a handful of genomes for the eukaryotic algae (Rynearson and Palenik 2011). This has been one of the most dramatic advances driving research into the physiology of these groups in several decades (Table 1). Genomes can be screened for homologs to phosphorus-related genes in other organisms, and the genome sequence can serve as a framework for the development of other molecular applications like transcriptome profiling. The availability of genome sequences has resulted in the identification of genes that encode aspects of phosphorus physiology that were not considered with growth studies or other approaches, particularly with regard to studying microalgae metabolism of dissolved organic phosphorus. This was the case for the discovery of the C-P lyase gene complex in the *Trichodesmium* IMS101 genome (Dyhrman et al. 2006a). Phosphonate bound organic matter was largely thought to be refractory and not bioavailable to cyanobacteria until these genome observations. Now, these genome observations have resulted in a growing literature focused on the pathways, regulation, and bioavailability of phosphonates to cyanobacteria (Villarreal-Chiu et al. 2012). In one other example, analysis of the genomes for several strain of the coccolithophore *E. huxleyi*, found surprising variability in gene content with a set of core genes found in all strains, and a set of variable genes that were distributed unevenly across even closely related strains from the same environment (Read et al. 2013). Core genes included multiple copies of phosphate transporters and a high-efficiency alkaline phosphatase (Read et al. 2013), which underscores the importance of phosphorus acquisition to the ecophysiology and evolution of this organism. However, the numbers of phosphate transporters and alkaline phosphatases varied between strains, which is suggestive of the role genome variability plays in this organism's capacity to thrive in a wide range of phosphorus regimes (Read et al. 2013). In brief, genome sequencing is dramatically changing views about phosphorus metabolism and the role of phosphorus in the selection and evolution of genotypes.

With the smaller genome sizes of cyanobacteria metagenome sequencing of field populations is also an emerging tool in studying the distribution of phosphorus metabolism genes in the field (Gilbert et al. 2011) (Table 1). Metagenome sequencing has been highly informative at the two oceanographic time-series sites; Station ALOHA in the subtropical North Pacific Gyre and the BATS Station in the Sargasso Sea region of the western North Atlantic. Comparing metagenome profiles of *Prochlorococcus* between the two sites Coleman and Chisholm (2010) discovered a number of phosphorus metabolism or scavenging genes were more abundant in the low phosphorus Sargasso Sea than in the higher phosphorus North Pacific subtropical Gyre. The authors suggest that this differential presence of key phosphorus genes is reflective of the selective role the environment plays in the evolutionary processes driving genome content. However, metagenome sequencing is still rarely used for gene discovery in the eukaryotic algae because of their large genomes and non-coding regions. Metagenome sequencing was recently used to do a partial reconstruction of an uncultured, flow-sorted haptophyte (Cuvelier et al. 2010). Although this and related studies have not used this approach to identify metabolic genes related to phosphorus, the approach does hold promise for future applications, particularly for important taxa that are not in culture.

6.2.2 Transcriptomics and Metatranscriptomics

Transcriptome profiling of a cell's mRNA (Table 1) continues to evolve as a powerful approach for examining the responses of algae to phosphorus availability, and thus also in identifying the mechanistic drivers of phosphorus metabolism and scavenging. Particularly with the availability of genomes for model species, microarrays or high throughput RNA sequencing (e.g. RNA-seq) have been used to survey aspects of phosphorus metabolism in algae. This is even more critical for eukaryotic algae where the sequencing of nuclear eukaryotic algal genomes is challenging and they are still reasonably expensive to assemble, and annotate.

Researchers have increasingly been leveraging transcriptome sequencing, assembly and annotation as an alternative for deriving information on metabolic capacity and even genes expression patterns in the algae particularly for the eukaryotes. Transcriptomics allows the rapid and efficient characterization of the expressed genes without the intergenic regions, introns, and repetitive DNA common to eukaryotes. This powerful tool is increasingly used for gene discovery in the eukaryotic algae, and for identifying the regulation pattern of those transcripts in both cyanobacteria and eukaryotic algae.

Gene discovery is poised to make extraordinary progress with the ongoing sequencing and analysis of marine eukaryotic algae through the MMETSP (Marine Microbial Eukaryote Transcriptome Sequencing Project – http://marinemicroeukaryotes.org/). The MMETSP initiated and funded by the Gordon and Betty Moore Foundation, will result in roughly 700 transcriptomes from ~17 Phyla. Although this is dramatically expanding our understanding of the molecular underpinnings driving algal physiology, there are some caveats to note about this dataset. One caveat is that the dataset still represents a relatively select group that is dominated by cultured isolates, many of which are from brackish or coastal environments. Furthermore, many of the samples were run on a single culture condition (typically in replete medium) or if there were conditions, they rarely included a phosphorus stress treatment. Many transcripts involved in the phosphorus stress response are expressed at very low levels (<5 reads per million) in replete, and are only abundant (~5,000 reads per million) when cells are phosphorus-starved (Dyhrman et al. 2012). It is possible that for some organisms, the transcriptome data does not include some of the phosphorus metabolism genes, because phosphorus-responsive transcripts were at such low expression levels they were not assembled in a typical replete transcriptome. Regardless of these caveats, the transcriptome data is advancing understanding of the molecular level pathways driving these cell's physiological responses to phosphorus (Frischkorn et al. 2014). Recent assembly and low phosphorus profiling of the *A. anophagefferens* strain CCMP 1850 transcriptome, identified the transcripts putatively controlling phosphate uptake, organic phosphorus metabolism, and arsenate detoxification; determining important aspects of this organisms' phosphorus stress response (Frischkorn et al. 2014). Further, transcriptome assembly resulted in the identification of the Vtc4 polyphosphate polymerase which was missed in the modeling of the strain CCMP 1984 genome (Frischkorn et al. 2014), highlighting the value of these approaches for gene discovery.

Beyond gene discovery, transcriptome profiling with microarrays or sequencing-based approaches have examined the transcripts differentially abundant in eukaryotic algae and cyanobacteria that are phosphorus-stressed (Dyhrman et al. 2006b, 2012; Erdner and Anderson 2006; Tetu et al. 2009; Wurch et al. 2011b, 2014; Harke and Gobler 2013). Although qRT-PCR based screening of genes of interest is quite valuable (Wurch et al. 2014), these global approaches are useful for identifying novel aspects of the phosphorus stress response without a priori knowledge about the most interesting targets. For example, a transcriptome study in *T. pseudonana* identified candidate genes that may be involved in the restructuring of the lipidome (see Sect. 5.2.2) that occurs under phosphorus stress (Dyhrman et al. 2012). This study also identified a number of phosphorus-responsive transcripts mapping to the genome where no gene model was predicted (Dyhrman et al. 2012). In this latter case, such global screening can highlight the regulation patterns of genes of unknown function, and genes that were not predicted in the gene modeling.

There is also a growing literature focused on profiling global (all transcripts) responses of algae in the field, so called metatranscriptomics (Table 1). This is most typically approached with either high throughput sequencing (Dyhrman et al. 2012) or arrays (Shilova et al. 2014). Using a sequencing based-approach to sample bacterioplankton from the coastal waters of the southeastern United States, Gifford et al. (2011) used an internal standard to make absolute (per liter) estimates of transcript numbers (Gifford et al. 2011). In the two coastal samples tested, the expression of genes encoding high affinity phosphate transport (*pstSCAB*), polyphosphate kinase (*ppK*), and low affinity phosphate transport (*pitA*) were all detected (Gifford et al. 2011). Notably, alkaline phosphatase (*phoX*, *phoA*), phosphonate enzymes (*phnWXYZ*) including genes for the C-P lyase (*phnJ*) were not consistently observed over the detection limit in coastal waters, perhaps underscoring that organic phosphorus cycling is not prevalent in this system (Gifford et al. 2011). In a related approach, undertaken in a low phosphorus lake (Vila-Costa et al. 2013), the authors observed expression of genes related to the metabolism of different DOP forms, pointing to a major role for DOP in controlling this ecosystem. Although these approaches were not directed to cyanobacteria specifically, they point to the utility of the approach in the context of understanding key aspects of phosphorus metabolism in situ. Metatranscriptome profiling in the eukaryotic algae has not been frequently employed nor used to examine aspects of phosphorus metabolism (Marchetti et al. 2012). This is likely to change soon as data from the MMETSP allows for more robust mapping and identification of sequence reads across a greater range of both species and environments.

6.2.3 Proteomics and Metaproteomics

Proteomics involves the identification of all of the proteins and their abundance in the cell (Table 1). Advances in mass spectrometry and the availability of increasing sequence databases from genome and transcriptome studies, is making proteomics an important new tool for examining phos-

phorus metabolism in algae. Queries made at the protein level have the major advantage of examining the molecules actually mediating phosphate transport etc., but assaying proteins of course comes with associated caveats in that, the method is not as comprehensive as assaying transcripts, some proteins do not extract well, and their identification is heavily reliant on gene modeling and sequence databases. In the context of phosphorus metabolism, global protein profiling has been used to study how the proteome is modulated by fluctuations in phosphate supply (Wurch et al. 2011a), and by phosphorus stress (Dyhrman et al. 2012) in eukaryotic algae with genomes. Recent work has also used proteomics to examine phosphorus stress responses and metal phosphorus interactions in isolates of *Synechococcus* (Mazard et al. 2012; Cox and Saito 2013). Here, Cox and Saito (2013) found that zinc levels modulated the proteins associated with the phosphorus stress response, suggesting that Zn and phosphorus metabolisms are linked in this strain. Interestingly, PhoX (Ca requiring – see Sect. 4.1.1) was less abundant than PhoA (Zn requiring – see Sect. 4.1.1), which goes against the prevailing thought that PhoX is most important in marine systems (Cox and Saito 2013).

Like proteomics, metaproteomics is a powerful approach for screening for the presence and abundance of proteins in the environment (Table 1). This tool will likely gain increasing utility for cyanobacteria and eukaryotic algae with the MMETSP, and increased numbers of genomes facilitating protein identification. Metatranscriptome profiling has been applied in both the South Atlantic (Morris et al. 2010) and the Sargasso Sea (Sowell et al. 2009), where both studies observed a dominance of transport related proteins. *Prochlorococcus* and *Synechococcus* phosphate transport-related proteins were identified in the Sargasso Sea (Sowell et al. 2009), but not in the South Atlantic (Morris et al. 2010), suggestive of the fact that lower phosphate concentrations in the Sargasso Sea are influencing the physiology of cyanobacteria in this system. Moving forward, these types of studies are likely to be expanded to other systems and the eukaryotic populations of algae.

6.2.4 Other Molecular Approaches

There are of course many molecular level approaches for tracking algal phosphorus metabolism in powerful ways. Targeted approaches will continue to be valuable for robust quantification of the transcripts and proteins that drive the cycling of phosphorus both inside and outside the cell. For example, Scanlan and Wilson (1999) purified a *Synechococcus* protein identified as the phosphate-binding protein PstS, assayed its regulation pattern using antibody probes, which then could also be used to trace the abundance of the protein across gradients in phosphorus concentration in the field. Additional examples are plentiful from both studies with the cyanobacteria (Orchard et al. 2009) and the eukaryotic algae (Dyhrman and Palenik 2001; Wurch et al. 2014).

Two additional global approaches that are gaining prominence are studies tracking global lipid composition (the lipidome) and global metabolite abundances (the metabolome) (Table 1). The lipidome is receiving intense scrutiny in the context of everything from viral susceptibility to phosphorus metabolism (Van Mooy et al. 2009; Martin et al. 2011; Fulton et al. 2014) and lipid screening methods have been instrumental in understanding how eukaryotic algae and cyanobacteria are able to modulate their lipid pool to reduce phosphorus demand (see Sect. 5.2.3). For example, lipid screening in both groups identified that betaine lipids, which are nitrogen containing can substitute for phospholipids in the eukaryotic algae (Van Mooy et al. 2009). As the authors point out, this is significant because there is then an increased demand for nitrogen, the so called nitrogen penalty (Van Mooy et al. 2009). In this way, the cycling and metabolism of nitrogen and phosphorus are linked.

Metabolomics is still in the early stages of being applied to the study of algae in culture or the environment (Kujawinski 2011). Although this tool is not as yet frequently employed to the study of phosphorus metabolism it is increasingly common. For example, studies have used metabolomic approaches to examine the induction of a carbon-concentrating mechanism in *Chlamydomonas reinhardtii* (Renberg et al. 2010) and questions related to algal-based biofuel development (Ito et al. 2013). In the end, there is an ever growing suite of molecular approaches that will continue to transform understanding of phosphorus metabolism.

7 Emerging Themes and Ongoing Challenges

New discoveries in algal phosphorus metabolism are pointing to several themes that likely will be the focus of future work and new developments in our understanding of phosphorus in both algae and aquatic systems. These themes revolve around (1) metabolism of reduced phosphorus, and (2) the importance of cell-cell interactions. There are also ongoing challenges associated with advancing research on algal phosphorus metabolism, including but not limited to (1) a dearth of methods for assaying utilization of alternative phosphorus forms and (2) a lack of tools for quantifying specific phosphorus bonds and biochemicals, among others.

7.1 Emerging Themes

7.1.1 Metabolism of Reduced Phosphorus

One of the most striking features of recent phosphorus research in algae are the diversity of phosphorus forms and

reduced valence states that appear to be utilized by the cyanobacteria in particular. In addition to serving as a phosphorus source, the oxidation of reduced phosphorus compounds could serve as an energy source – a phosphorus redox cycle in nature. Phosphorus is found in chemical forms with a range of oxidation states. Compounds with a valence of +5 include phosphate, and phosphoesters like phosphoethanolamine. Compounds with a valence of +3 include phosphite, and phosphonates like 2-AEP, as discussed in previous sections these are all potentially bioavailable to cyanobacteria. The bioavailability of compounds with a valence of +1 like hypophosphite, and phosphinates like the antibiotic phosphinothricin are less well understood, but hypophosphite is known to be bioavailable to some microbes and can be metabolized by the *htxA* operon in *Pseudomonas stutzeri* (White and Metcalf 2004a, 2007). In short, there is increasing evidence of cyanobacterial metabolism of compounds with a range of oxidation states. In *P. stutzeri*, a phosphite dehydrogenase is coupled to NADH formation suggestive of the potential for phosphorus metabolism to be linked to the energy released during oxidation (Costas et al. 2001). The long standing view that phosphorus occurs in a fully oxidized state (phosphate, Pi = +5), and that there is no redox cycle for phosphorus in the environment is increasingly being questioned as the diversity of oxidation states and compounds that cyanobacteria can metabolize is expanded and reduced phosphorus compounds are detected in nature (Pasek et al. 2014). A direct link to energy dynamics has not been established for the cyanobacteria, but the bioavailability of such a range of valence states is suggestive of a phosphorus redox cycle in nature (Karl 2014). If this is borne out with further investigation, this theme would truly reshape our understanding of algal phosphorus metabolism and their role in cycling phosphorus in the environment.

7.1.2 Cell-Cell Interactions

The central paradigm in algal phosphorus stress responses, and how algae modulate their phosphorus metabolism, is that this is controlled by cellular responses to phosphorus concentration or supply. This central tenet is what drives our conceptual modeling of how algae interact with the phosphorus cycle. This paradigm is broadly supported by observations. For example, on broad scales patterns in phosphorus-sparing lipid substitutions and alkaline phosphatase activities typically inversely correlate with inorganic phosphate concentration (Van Mooy et al. 2009; Martin et al. 2014). Despite these broad patterns there are a number of studies where there is clear variability within a species, even within the same system (Dyhrman and Palenik 1999). What drives this variability and what it means for understanding and tracking algal phosphorus metabolism is a new important theme in algal phosphorus research. One of the factors that may circumvent the known patterns and responses to phosphorus geochemistry is interactions between cells. Studies on this theme are sparse, but work by Van Mooy et al. (2012) showed that cell-cell communication via quorum sensing molecules had a major effect on *Trichodesmium* colony alkaline phosphatase activities. This study added the quorum sensing molecule to *Trichodesmium* colonies isolated from the low phosphorus Sargasso Sea and from the comparatively high phosphorus North Pacific subtropical gyre and found that the addition caused a doubling of alkaline phosphatase activity (Van Mooy et al. 2012). In short, causing cells to communicate modulated the activity of a critical enzyme, in a manner independent of the phosphorus chemistry. This is just one example, but it highlights the nuanced factors that can drive aspects of algal phosphorus metabolism, and will likely be an area of increased research in the future.

7.2 Ongoing Challenges

7.2.1 Assaying Metabolism of Alternative Phosphorus Forms

Despite many years of study, and renewed interest in aquatic phosphorus cycling, a number of ongoing challenges still impinge on our understanding of fundamental aspects of phosphorus acquisition and metabolism in algae. One such challenge involves the very isotopic composition of phosphorus. Phosphorus has at least 23 isotopes, but only one stable isotope ^{31}P. This inherently limits studies on phosphorus relative to nitrogen or carbon where there are more than one stable isotope for fractionation studies. Some progress has been made on circumventing this issue by using the oxygen isotopes of phosphate (Blake et al. 2005; Colman et al. 2005; McLaughlin et al. 2006, 2013). However the fractionation by different enzymes is not yet well understood, complicating interpretation of these signals (Liang and Blake 2009).

Of the radioactive isotopes, only ^{32}P and ^{33}P have half-lives appropriate for their use as tracers in uptake or bioavailability studies. Typically phosphate and nucleotides like ATP are the only radiolabeled phosphorus compounds commercially available. With the increasingly recognized importance of organic phosphorus metabolism, and the metabolism of phosphite, an ongoing challenge is the lack of suitable substrates for tracer studies. This is particularly critical for assaying the kinetic values that could be used for better modeling of algal community dynamics, which largely neglect utilization of phosphate alternatives. Prioritizing synthesis of radiolabeled phosphonate substrates in particular would greatly facilitate research in this area.

Just as radiolabeled substrates are lacking, so too are colormetric or fluorometric substrates for studying enzymes other than the esters and diesters. If alkaline phosphatase is taken as an example, substantial insight can result as the

number of assay tools increases. Imagine if an enzyme labeled fluorescence substrates could be developed for a phosphonate – this would dramatically expand understanding of algal phosphonate metabolism and phosphonate cycling in the environment.

7.2.2 Quantifying Phosphorus Biochemicals and Bond Classes

Analytical issues in the detection of specific phosphorus molecules, biochemical pools (polyphosphate), and bond forms (phosphonate), make tracing phosphorus mass balance in cells challenging. For example, the cellular compounds containing phosphonate bonds are present (as determined with ^{31}P NMR) but largely unknown in many systems, and phosphonate does not appear to extract well in the few studies that have tried to study this bond class in algal samples (Cade-Menun et al. 2005). In the case of polyphosphate, there are chain length biases, among other issues like signal interference from DNA, in the detection of this critical pool (Diaz and Ingall 2010; Martin and Van Mooy 2013; Martin et al. 2014). This can result in over estimation of polyphosphate, and it is not uncommon to detect polyphosphate in amounts greater than the total cellular phosphorus as a result. Improved methodologies for the characterization of phosphorus containing biochemicals are needed to more fully ascertain cellular phosphorus dynamics.

7.2.3 Heterogeneity in Capabilities and Activities

For every example highlighted herein, there seems to be a counter and our understanding of this heterogeneity in capability and activity is only increasing. Even at the strain level there are vast differences in metabolic capacity. This is observed in the variable presence of phosphorus metabolic genes in single genomes of *Prochlorococcus* (Kashtan et al. 2014). It is also seen in the genomes of multiple *E. huxleyi* strains where the number of phosphate transporter genes per genome varied by up to a factor of 5 between strains (Read et al. 2013). This level of heterogeneity may confer substantial variability in the rates and kinetics of phosphate uptake in this species, presenting an ongoing challenge in extrapolating culture findings to the field among other issues. Another example related to apparent heterogeneity is the variability in the distribution of phosphorus into different bond forms, detected with ^{31}P NMR. Here, even different species of the same genus can have very different profiles. For example, even when grown under the same conditions, *T. erythraeum* strains have a chemical shift at 18 ppm in the region of phosphonate, whereas *T. theibautii* does not (Dyhrman et al. 2009). There is no easy answer for how to deal with this heterogeneity, but one step forward would be the further development of the cell or taxon specific approaches outlined in earlier sections. Future work will need to contextualize this heterogeneity so that variability can appropriately be accounted for in modeling studies and those aimed at predictive understanding of phosphorus algae interactions.

8 Conclusions

Phosphorus plays a critical role in the metabolism, growth, ecophysiology and evolution of eukaryotic algae and cyanobacteria. Knowledge about cellular phosphorus dynamics in microalgae has been rapidly advancing with new methods and more sensitive approaches derived from flow cytometry, genomics, and mass-spectrometry among others. The chapter focused on how these advances have expanded understanding on fundamental aspects of phosphorus metabolism, and how these advances increasingly point to the molecular level underpinnings driving that metabolism. As these methods continue to evolve, new discoveries will build upon those highlighted herein.

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Sulphur and Algae: Metabolism, Ecology and Evolution

Mario Giordano and Laura Prioretti

1 Introduction

Sulphur is one of the main components of algal cells, with a cell quota typically very similar to that of phosphorus (Ho et al. 2003; Giordano 2013). The importance of S is not simply quantitative; it is also associated with its presence in numerous pivotal structural and functional compounds such as the amino acids cysteine and methionine, non-proteic thiols (glutathione), sulpholipids, vitamins and cofactors, cell wall constituents (Leustek and Saito 1999; Takahashi et al. 2011). Sulphur is also a constituent of dimethylsulphoniopropionate (DMSP), which in some algae can represent a very large portion of cell S and is involved in algal responses to a variety of abiotic and biotic stresses, in addition to being indicted of an important role in climate control (Charlson et al. 1987; Giordano et al. 2005; Ratti and Giordano 2008).

Algae acquire S as sulphate (SO_4^{2-}), the most abundant form of inorganic S in nature, in which S appears with its highest oxidation number (+VI). Sulphur is however assimilated in the organic matter as sulphide (S^{2-}), where S appears with its lowest oxidation number (−II) (Fig. 1). A non trivial amount of reducing power is thus required for S assimilation. In vascular plants, this reducing power can be generated both from the photosynthetic and the respiratory electron transfer chain; in algae, the dependence of S assimilation from photosynthesis seems to be tighter (Schmidt 1979). In both algae and plants, S assimilation mostly takes place in the chloroplast; the only known exception is *Euglena gracilis*,[1] which reduces sulphate into the mitochondrion (Brunold and Schiff 1976).

[1]Wherever possible the currently accepted names for species are used. The name used in the paper cited is also indicated. For details of names see chapter "Systematics, Taxonomy and Species Names: Do They Matter?" of this book (Borowitzka 2016).

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2 Sulphur Availability in Time and Space

Sulphate abundance in ancient seawater has been inferred by a number of proxies (Ratti et al. 2011; Giordano and Raven 2014) that collectively provide a consistent picture of oceanic sulphate concentration through time (Fig. 2).

In the Archean oceans, when the first cyanobacteria appeared, sulphate concentration was probably below 200 μmol L^{-1} (Habicht et al. 2002). In the Proterozoic, with increasing oxygenation, sulphate became more abundant, but its concentration in seawater is believed to have been no more than 1–5 mmol L^{-1} (Shen et al. 2002; Canfield 2004; Kah et al. 2004). It was in this period that green algae became the main primary producers (together with cyanobacteria) in world's oceans. Only in the later Ediacaran did the abundance of sulphate reach values as high as 15 mmol L^{-1}, to decline again in the Cambrian (Horita et al. 2002; Petrychenko et al. 2005), when, according to data obtained from the fluid inclusions in salt crystals and C and S isotopic variations, it was in the range of 3–12 mmol L^{-1} (possibly at the lower end of this range; Gill et al. 2011). Sulphate remained lower than 10 mmol L^{-1} until the Carboniferous, when it increased to values higher than 15 mmol L^{-1} (Gill et al. 2007). Sulphate levels may have declined transiently after the Carboniferous (Luo et al. 2010; Newton et al. 2011), but data at hand suggest that sulphate concentrations in Paleozoic to early Mesozoic oceans were in the range of 13–27 mmol L^{-1}. It is at this time that a transition from a phytoplankton primarily constituted of cyanobacteria and green algae (chlorophyll *a*+*b*) to one in which chlorophyll *a*+*c* algae (i.e. the algae that acquired their plastid through a secondary endosymbiotic event with a red alga, such as diatoms, dinoflagellates, coccolithophorids) became prominent occurred. The fact that chlorophyll *a*+*c* algae were better suited to take advantage of increased sulphate availability probably concurred to this transition (Sulphate Facilitation Hypothesis; Ratti et al. 2011). Interestingly, a sulphate increase in the order of what

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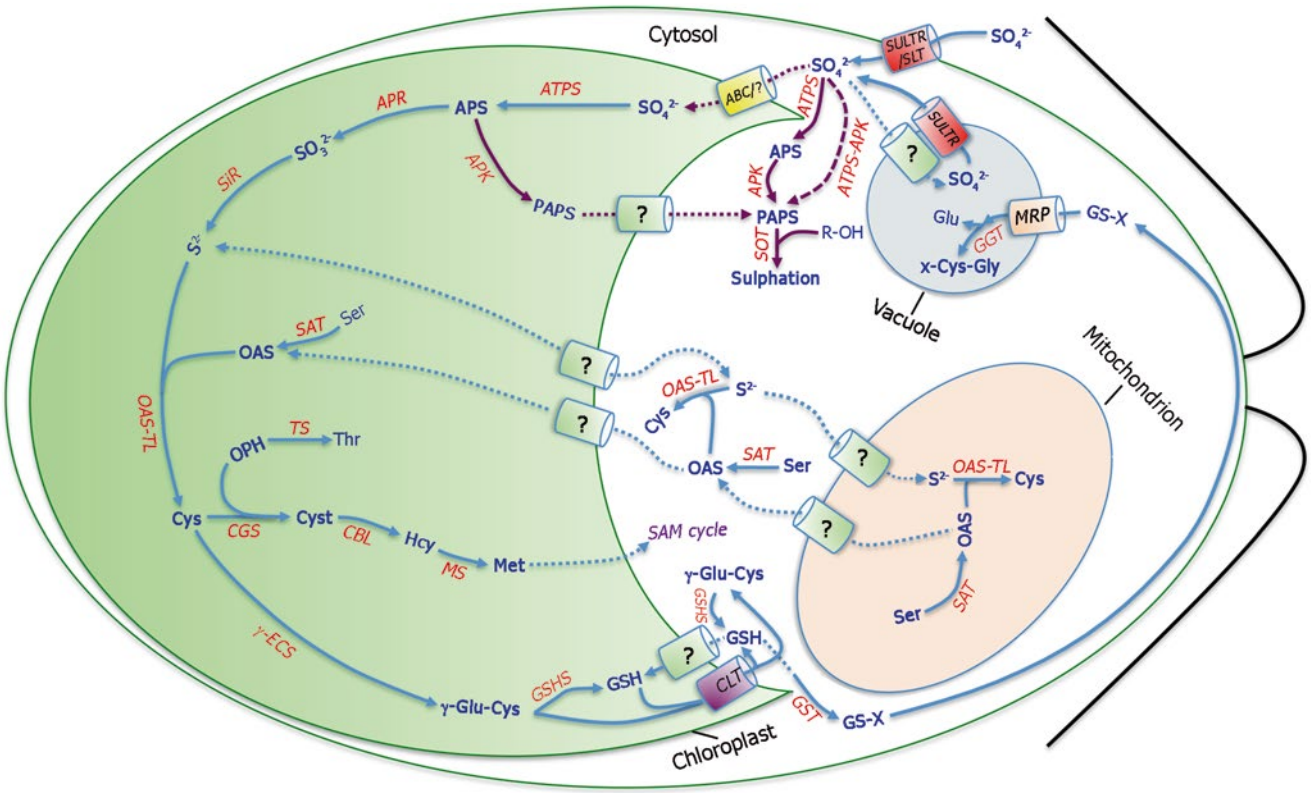

Fig. 1 Sulphate metabolism in algal cells. An overall view of sulphur assimilation and of sulphation is depicted here: the enzymes are indicated in *red*, metabolites in *blue* and transporters in *black*. The *light blue lines* indicate the reactions of the reductive sulphate assimilation pathway; the *dark purple lines* indicate reactions of the sulphation pathway; *dashed lines* indicate putative pathways for metabolite transport that are not been incontrovertibly demonstrated. *ABC* ATP-binding cassette SO_4^{2-} transporter, *APK* APS kinase, *APR* APS reductase, APS adenosine 5′-phosphosulphate, *ATPS* ATP sulphurylase, *CBL* cystathionine β-lyase, *CGS* cystathionine γ-synthase, *CLT* thiol transporter (chloroquine resistance transporter-like transporter, *Cys* cysteine, *Cyst* cystathionine, *γ-ECS* γ-glutamylcysteine synthetase, *GGT* γ-glutamyltransferase, Glu glutamate, *γ-Glu-Cys* γ-glutamylcysteine, *GSH* glutathione, *GSHS* glutathione synthetase, *GST* glutathione-S-transferase, GS-X glutathione conjugate, Hcy homocysteine, *Met* methionine, *MRP* multidrug resistance-associated protein, *MS* methionine synthase, *OAS* O-acetylserine, *OAS-TL* OAS (*thiol*)lyase, OPH O-phosphohomoserine, *PAPS* 3′-phosphoadenosine 5′-phosphosulphate, *R-OH* hydroxylated precursor, *SAM* S-adenosylmethionine (the specific reactions of the SAM cycle are depicted in Fig. 12), *SAT* serine acetyltransferase, *Ser* serine, *SiR* sulphite reductase, *SLT* Na^+/SO_4^{2-} transporter, *SOT* sulphotransferase, *SULTR* H^+/SO_4^{2-} transporter, *Thr* threonine, *TS* threonine synthase, *X-Cys-Gly* cysteinylglycine conjugate

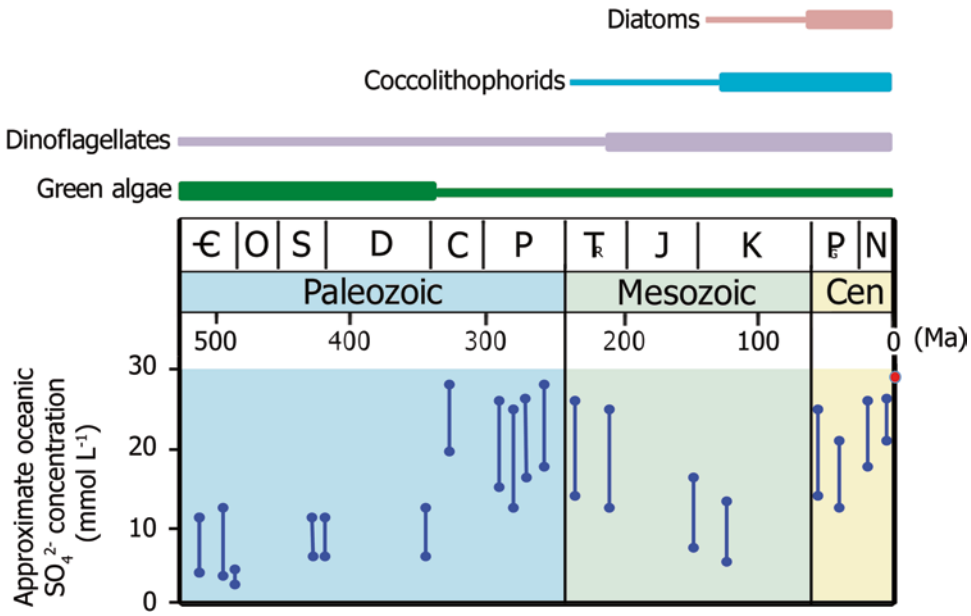

Fig. 2 Changes in secular sulphate concentrations in the oceans. In this figures the approximate variations in oceanic sulphate concentration are shown in parallel with the appearance and ecological relevance of the main algal groups (i.e. the thinner portion of the bar indicates when a given group was present but not especially important in terms of abundance, biodiversity, ecological role; the thicker bars indicates the period during which a group rose to prominence)

occurred at the Paleozoic-Mesozoic boundary induces, at least in extant taxa, compositional changes in the cells that affect their nutritional value and possibly the interaction of phytoplankters with grazers (Ratti et al. 2011, 2013).

Sulphate concentration in today's oceans is around 28 mmol L^{-1} and it likely represents the historic maximum.

Sulphate abundance varies in space as well as in time, and not all aquatic environments on Earth have sulphate concentrations as high as those in the oceans. Freshwater sulphate concentrations are commonly between 0.01 and 1 mmol L^{-1} and are strongly dependent on the catchment and on atmospheric deposition (Holmer and Storkholm 2001; Giordano et al. 2005; Giordano and Raven 2014). An increase in S concentrations in European and North American lakes has been recorded since the Industrial Revolution, as a consequence of the higher anthropogenic emissions of SO_2 and the subsequent deposition of this gas as (mostly) sulphate (Stoddard et al. 1999; Singh and Agrawal 2007). From the early 1970s, however, when legislations were enforced to limit SO_2 emissions (UNECE 1994), the input of anthropogenic S to aquatic environment decreased markedly, in European and North American lakes (Stoddard et al. 1999), and sulphate became nearly limiting in some oligotrophic lakes (Giordano et al. 2005; Giordano and Raven 2014).

In some freshwater basins (Giordano et al. 2005) and in soil (Kertesz 2000), a large proportion of S can be contained in organic molecules. Although, there is no evidence for the direct use of such molecules as a source of S for algae, it is a fact that microalgae produce arylsulphatases (ARS; mostly eukaryotic), which cleave organic SO_4^{2-} and release the residual phenol (Gonzalez-Ballester and Grossman 2009 and references therein), and alkylsulphatases, whose catalysis generates SO_4^{2-} and an aldehyde (Kahnert and Kertesz 2000; Hagelueken et al. 2006). Arylsulphatases are the most common and best studied of these enzymes and their production is a typical response to S deprivation in organisms such as the freshwater green alga *Chlamydomonas reinhardtii* (de Hostos et al. 1989). In *C. reinhardtii*, 18 putative ARS genes were identified (Gonzalez-Ballester and Grossman 2009), the transcription of two of which (*ARS1* and *ARS2*) is strongly regulated by S availability (de Hostos et al. 1989; Ravina et al. 2002).

3 S Acquisition by Algae

Algae acquire S as sulphate. The kinetics of sulphate uptake as a function of sulphate concentration shows, in most cases, a multiphasic pattern; this is due to the presence of a variety of different transporters with different affinities for the substrate (e.g. Matsuda and Colman 1995; Takahashi et al. 2012; Fig. 3).

Transporters conducting an H^+/SO_4^{2-} transport (*SULTR* gene family) have been identified in all photosynthetic organisms studied so far (Gonzalez-Ballester and Grossman 2009; Takahashi et al. 2012; Bromke et al. 2013). The driv-

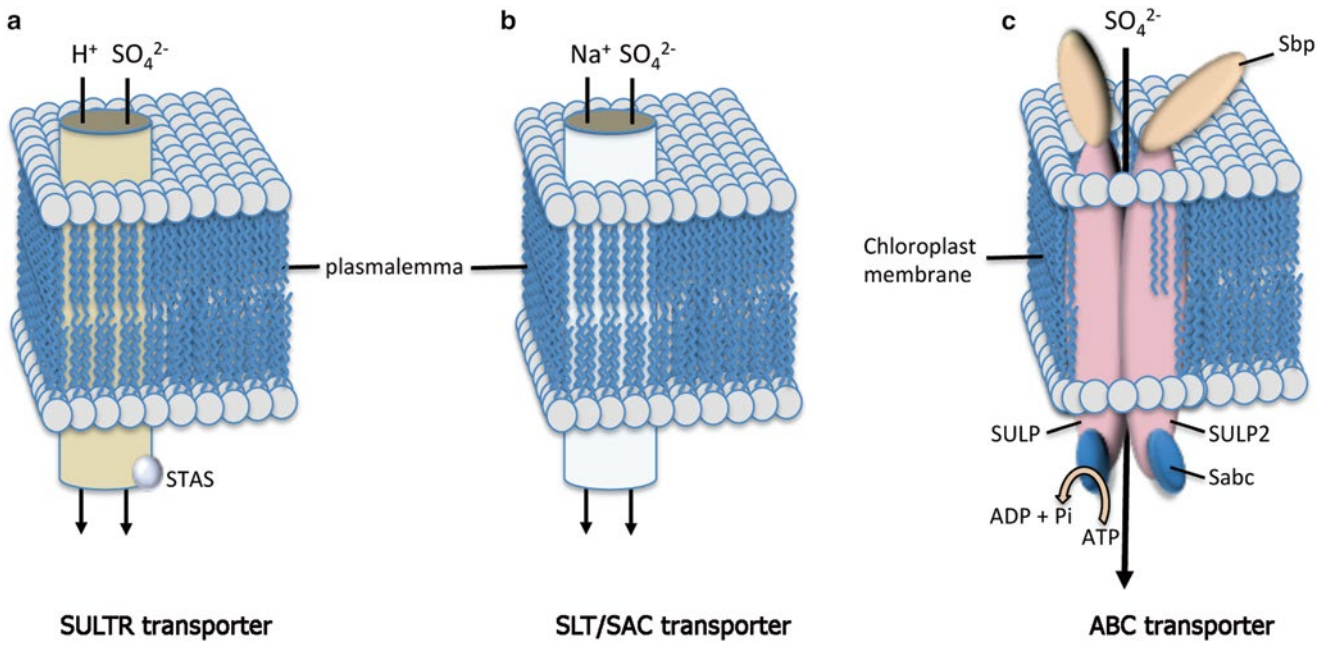

Fig. 3 Sulphate transporters. Three main types of sulphate transporters have been identified in algae: plasmalemma sulphate transporters conducting **(a)** H^+/SO_4^{2-} (SULTR) or **(b)** Na^+/SO_4^{2-} (SLT) cotransports; **(c)** ATP-binding-cassette transporter (ABC) in green algae chloroplast inner membrane and in the plasmalemma of cyanobacteria

ing force for this transport is the proton gradient across membranes; the high affinity phase of the transporters is activated when S is limiting (Yildiz et al. 1994; Bochenek et al. 2013 and references therein). The SULTR transporters possess from 10 to 14 predicted transmembrane domains and often contain a domain, at the cytosolic carboxy-terminal end, the STAS (sulphate transporters and anti-sigma factor antagonist) domain, which is common in anion transporters and is believed to have regulatory functions (Aravind and Koonin 2000). A variety of functions have been attributed to STAS domains of different proteins: some STAS are nucleotide-binding phosphoproteins or nucleotidases, some play the role of histidine and serine kinases or of transcription factors, others are involved in the sensing of light and various signalling molecules (e.g. G proteins, cyclic nucleotides, inositol phosphates and various gasotransmitters) (Sharma et al. 2011). SULTR-like sequences have been found in the diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*, in the pelagophyte *Aureococcus anophagefferens*, in the rhodophyte *Cyanidioschyzon merolae*, in the prasinophyte *Ostreococcus* sp. and in the chlorophycean *Chlamydomonas reinhardtii*; some of these sequences contain STAS domains. The actual function of the products of these genes needs however to be confirmed.

In green microalgae *Chlamydomonas reinhardtii* and *Volvox carteri*, in the bryophyte *Physcomitrella patens* and the lycophyte *Selaginella moellendorffii* (but not in vascular plants), transporters of the SLT family, induced by sulphate deficiency, also have been identified (Pootakham et al. 2010; Takahashi et al. 2012). These transporters have 10–12 predicted transmembrane domains (Smith et al. 1995; Pootakham et al. 2010; Takahashi et al. 2012 and references therein). Differently from the SULTR transporters, SLT transporters catalyze a Na^+/SO_4^{2-} antiport and have high similarity with animal sulphate transporters (Gonzalez-Ballester and Grossman 2009 and references therein). Also the regulatory element SAC1, which appears to control *Chlamydomonas* responses to S deficiency (Davies et al. 1996), belongs to this gene family (in fact, SLT stands for SAC1-like transporter). It has been suggested that the SAC1 protein is located on the plasmalemma and senses the S status of the environment; it then interacts through a threonine kinase domain with SLT proteins, initiating a signalling cascade that, when S availability is low, enhances the expression of S-starvation induced genes (Davies et al. 1994, 1996; Gonzalez-Ballester and Grossman 2009). The product of the *SAC3* gene, instead, has a negative effect on some genes induced by S starvation, possibly through the inactivation of a kinase (SNRK2.1) on the SAC1 phosphorylation pathway (Davies et al. 1994; Gonzalez-Ballester and Grossman 2009).

Takahashi et al. (2012) suggested that SLT genes were present in the ancestor of vascular plants, which then lost these genes. Sequences of the SLT type have been identified in the genome of the marine haptophyte *Emiliania huxleyi*, although these genes have a rather low degree of similarity with the *Chlamydomonas* SLT sequences and it is not sure that these proteins are indeed sulphate transporters (Bochenek et al. 2013). *E. huxleyi* was not included in the phylogenetic analysis by Takahashi et al. (2012); the evolutionary history of these transporters/regulatory genes thus remains to be clarified.

Based on Takahashi et al. (2012) analysis of eukaryotic sulphate transporters, SULTR and SAC1/SLT transporters have rather distinct phylogenies. SULTR genes constitute a sister group of animal (SLC26), yeast (SUL) and their fungal homologous genes; genes within this "tribe" are believed to share a common ancestors. The SAC1/SLT transporters would instead belong to a separate "tribe" (see Takahashi et al. 2012 for a more detailed discussion of this matter).

Interestingly, no transporters that catalyze an anion exchange with Cl^-, HCO_3^- or I^- have been found in algae, although they exist in yeasts, fungi, and animals (Takahashi et al. 2012). Takahashi et al. (2012) hypothesize that the presence of H^+ or Na^+ co-transporters in algae may depend on environmental conditions, specifically on pH and Na^+ availability. Unfortunately, the information for marine algal sulphate transporters is too scant to hypothesize what the functions of these two different types of transporters are in the oceans (at least in *E. huxleyi* they are both present in the genome).

A completely different type of sulphate transporters exists in cyanobacteria and in the chloroplast of green algae. In these membranes, sulphate transport is catalyzed by ATP-binding cassette (ABC) transporters (Melis and Chen 2005; Takahashi 2010; Takahashi et al. 2012). ABC transporters are transmembrane permease holocomplexes formed by two heterodimers; the transmembrane channel comprises the proteins SulP and SulP2 (SULP stands for sulphate permeases), each of which is bound to a sulphate-binding protein (Sbp) on the cytosolic side and to an ATP-binding protein (Sabc) on the stromal side (Melis and Chen 2005). ATP hydrolysis energizes this transport (Sirko et al. 1990; Laudenbach and Grossman 1991). As cyanobacterial sulphate permeases and other plant sulphate transporters, also the *Chlamydomonas* Sbp-SulP-SulP2-Sabc holocompex genes are regulated by S starvation (Lindberg and Melis 2008). No such transporters have been identified in other organisms (Takahashi et al. 2012) and the mechanism of plastidial sulphate transport in vascular plants is still not known (Lindberg and Melis 2008).

4 S Assimilation by Algae

Although algae mostly use sulphate as the S source, they assimilate S (into cysteine) in its most reduced form (S^{2-}), with sulphite as an intermediate step. Sulphur assimilation

Table 1 Standard reduction potential (E_0', 25 °C, 1 atm, pH 7, in aqueous solution, expressed in volts)

Oxidant	Reductant	N	E_0'
Sulphate	Sulphite	2	–0.454
Nitrate	Nitrite	2	+0.421
Adenosine 5′-phosphosulphate	Sulphite	2	–0.060
Glycerate-3-P	Glyceraldehyde-3-P	2	–0.550
1,3-biphosphoglycerate+2H$^+$	Glyceraldehyde-3-P+Pi	2	–0.290
NADH	NAD$^+$	2	–0.320
NADPH	NADP$^+$	2	–0.324
Cystine	Cysteine	2	–0.340
2GSH	GSSG	2	–0.340
Ferredoxin (ox)	Ferredoxin (red)	1	–0.430
FMN	$FMNH_2$	2	–0.300
FAD (free)	$FADH_2$	2	–0.220
UQ+2H$^+$	UQH_2	2	–0.060

N is the number of electrons transferred
The E_0' values were obtained from Rauen (1964) and Segel (1976)

is thus primarily a reductive process. In all photosynthetic organisms except *Euglena gracilis*, sulphate reduction to sulphide occurs in the chloroplast (Takahashi et al. 2011); in *E. gracilis*, the enzymes of sulphate reduction are located in the mitochondrion (Brunold and Schiff 1976; Patron et al. 2008).

The direct reduction of sulphate to sulphite requires an amount of redox energy that is hard to obtain in the cell. Table 1 shows the standard reduction potentials of sulphate/sulphite and the main electron donors in the cell. It emerges clearly that the production of sulphite from sulphate is essentially impossible unless the redox potential of sulphate is lowered. This is achieved by the activation of sulphate to adenosine 5′-phosphosulphate (APS). In APS, the mixed anhydride bond between phosphate and sulphate has a standard redox potential of about −60 mV, which is compatible with the use of thiols or pyrimidine as electron transporter (Rauen 1964; Segel 1976) (Table 1).

The activation of sulphate to APS is catalyzed by ATP sulphurylase (ATPS; EC: 2.7.7.4; Takahashi et al. 2011), which catalyses the conversion of sulphate to adenosine-phosphosulphate (Fig. 4).

As it is common for nucleotidyl transferases, ATPS requires Mg^{2+} for its catalysis. MgATP is the first substrate to enter the active site, followed by sulphate; the presence of Mg^{2+} is required for sulphate binding to the active site (Farley et al. 1976); subsequently, the terminal two phosphate groups of ATP are cleaved as pyrophosphate, and the residual AMP is transferred onto the sulphate; finally, pyrophosphate is released with Mg (Shaw and Anderson 1974). In diatoms, dinoflagellates and haptophytes, the reaction of ATPS is coupled with the hydrolysis of pyrophosphate catalyzed by a pyrophosphatase; this exergonic reaction contributes to drive APS production in vivo (Patron et al. 2008; Bradley et al. 2009).

In plants, two to four distinct ATPSes are located in the plastid and, at least in some species, in the cytosol (Lunn et al. 1990; Klonus et al. 1994; Rotte and Leustek 2000); since APS reduction appears to occur exclusively in the plastid, the plastidial ATPSes are probably the main ones in the pathway of S assimilation; the role of the cytosolic ATPSes still has to be fully elucidated (Rotte and Leustek 2000). Plant ATPS is typically a homotetramer with no allosteric regulation (Kopriva et al. 2009). In bacteria, instead, ATPS is a heterotetramer with a GTPase subunit with regulatory functions (Leyh 1993; Liu et al. 1994). Fungi and yeasts ATPSes are homoexamers with C-terminal APS kinase (APK; see the paragraph on sulphation) domains in each subunit (MacRae et al. 2001; Ullrich et al. 2001); the APK domain apparently has no catalytic function, but plays a structural role in the interaction among the catalytic portions of the holoenzyme (Lalor et al. 2003). Also the mammalian enzyme has an APK domain, but it is located at the N-terminus (see Ravilious et al. 2013 for details and further references).

Until recently, the ATP sulphurylase of algae has been assumed to be similar to that of higher plants, in terms of regulation and catalysis. The actual story is possibly more complicated (Table 2). All algae have a plastidial ATPS very similar to the bacterial enzyme. In most species studied so far, a single gene encodes for this ATPS. *Chlamydomonas reinhardtii* represents an exception to this, since it has two putatively plastidial ATPS (Patron et al. 2008). The algae with chlorophyll *a*+*c* possess two ATPS, one in the chloroplast and the other in the cytosol; the plastidial enzyme is responsible for the production of APS for primary S assimilation. The cytosolic ATPS is fused to an APK at the N-terminus and is rather similar to the metazoan enzyme

Table 2 Algal ATP sulphurylase (ATPS) and their cysteine content

Phylum	Species	Environment	Number and type of genes	Localization	Total number of Cys	Number of conserved Cys
Cyanobacteria	*Acaryochloris* sp.	M	1 ATPS	–	4	4 A
	Anabaena sp.	F	1 ATPS	–	4	4 A
	Arthrospira platensis	F	1 ATPS	–	4	4 A
	Crocosphaera sp.	M	1 ATPS	–	4	4 A
	Cyanobacterium sp.	M	1 ATPS	–	4	4 A
	Cyanothece sp.	M	1 ATPS	–	4	4 A
	Cylindrospermopsis sp.	F	1 ATPS	–	5	4 A
	Fischerella sp.	F	1 ATPS	–	4	4 A
	Gloeobacter sp.	F	1 ATPS	–	4	4 A
	Lyngbia sp.	M	1 ATPS	–	4	4 A
	Microcoleus vaginatus	F	1 ATPS	–	4	4 A
	Microcystis aeruginosa	F	1 ATPS	–	4	4 A
	Nodularia sp.	M	1 ATPS	–	4	4 A
	Nostoc sp.	F	1 ATPS	–	4	4 A
	Oscillatoria sp.	F	1 ATPS	–	4	4 A
	Prochlorococcus marinus	M	1 ATPS	–	9–11	5 B
	Raphidiopsis sp.	F	1 ATPS	–	4	4 A
	Spirulina subsalsa	M	1 ATPS	–	4	4 A
	Synechococcus elongatus	F	1 ATPS	–	4	4 A
	Synechococcus sp. CB0101	M	1 ATPS	–	8	5 B
	Synechococcus sp. CC9902	M	1 ATPS	–	10	5 B
	Synechococcus sp. JA-3-3Ab	F	1 ATPS	–	5	4 A
	Synechococcus sp. JA-2-3B′a	F	1 ATPS	–	6	4 A
	Synechococcus sp. RCC307	M	1 ATPS	–	8	5 B
	Synechococcus sp. RS9916	M	1 ATPS	–	9	5 B
	Synechococcus sp. WH7803	M	1 ATPS	–	10	5 B
	Synechococcus sp. WH8102	M	1 ATPS	–	9	5 B
	Synechocystis sp. PCC6803	F	1 ATPS	–	4	4 A
	Thermosynechococcus sp.	F	1 ATPS	–	6	4 A
	Trichodesmium sp.	M	1 ATPS	–	4	4 A
Chlorophyta	*Chlamydomonas reinhardtii*	F	2 ATPS	P	9	5 B
					10	5 B
	Chlorella variabilis	F	1 ATPS	P	12	5 B
	Coccomyxa subellipsoidea	F	1 ATPS	P	10	5 B
	Micromonas pusilla	M	1 ATPS	P	8	5 B
	Ostreococcus lucimarinus	M	1 ATPS	P	9	5 B
	Ostreococcus tauri	M	1 ATPS	P	8	5 B
	Tetraselmis suecica	M	1 ATPS		8	5 B
	Volvox carteri	F	1 ATPS	P	9	5 B
Rhodophyta	*Porphyra purpurea*	M	1 ATPS	P	2	1 C
	Pyropia yezoensis	M	1 ATPS	P	1	1 C
Cryptophyta	*Guillardia theta*	M	2 ATPS	P	9	5 B
					7	2 B
Dinophyta	*Amphidinium klebsii*	M	1 ATPS	?	6	2 B
			1 APR-ATPS		7	3 (1 A; 2 B)
	Heterocapsa triquetra	M	1 APR-ATPS	P	7	3 (1 A; 2 B)
Haptophyta	*Emiliania huxleyi*	M	1 ATPS	P	9	5 B
			1 APK-ATPS	C	5	2 D

(continued)

Table 2 (continued)

Phylum	Species	Environment	Number and type of genes	Localization	Total number of Cys	Number of conserved Cys
Heterokontophyta	*Aureococcus anophagefferens*	M	1 ATPS	P	17	5 B
			1 APK-ATPS	C	7	2 D
	Ectocarpus siliculosus	M	1 ATPS	P	10	5 B
	Fragilariopsis cylindrus	M	1 ATPS	P	8	5 B
			1 APK-ATPS	C	6	2 D
	Phaeodactylum tricornutum	M	1 ATPS	P	9	5 B
			1 APK-ATPS	C	8	2 D
	Pseudo-nitzschia multiseries	M	1 ATPS	P	7	5 B
			1 APK-ATPS	C	5	2 D
	Thalassiosira pseudonana	M	1 ATPS	C	8	5 B
			1 APK-ATPS	P	7	2 D

All species for which the ATPS sequence is known are included in the table, except for Cyanobacteria, for which only representative species or strains are shown. The column "Environ." indicates whether the species is marine (M) or freshwater (F). The column "Number and type of genes" indicates how many ATPS are present and of what type (i.e. if the protein contains the sole ATPS domain or contains domains with potentially other catalytic activity). The column "Localization" shows whether the protein is plastidial (P) or cytosolic (C); in the case of prokaryotes, no compartmentalization is indicated. The column "Total number of Cys" indicates how many cysteine residues are contained in the ATPS domain of the enzyme. The column "Number of conserved Cys" refers to the number of Cys residues that are retained in the same position in the sequences identified by the same letter (A, B, C or D). All sequences, except those of *Heterocapsa triquetra*, *Tetraselmis suecica* and *Amphidinium klebsii* were obtained from either the NCBI protein database or the JGI genome database; *H. triquetra* ATPS sequence was kindly provided by Stanislav Kopriva (University of Cologne). *T. suecica* and *A. klebsii* sequences were produced by the authors in collaboration with Charles F. Delwich (University of Maryland)

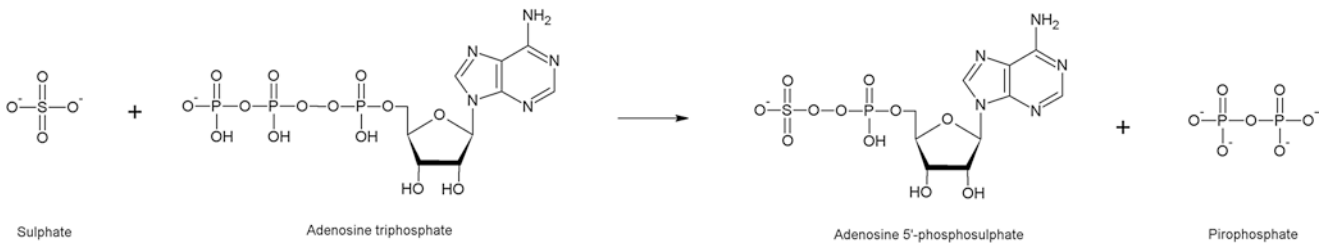

Fig. 4 Reaction catalyzed by ATP sulfurylase (ATPS)

(Patron et al. 2008). This enzyme is believed to be involved in sulphation (see below). In the diatom *Thalassiosira pseudonana*, however, based on the presence/absence of transit sequences, the location of the two ATPS is believed to be reversed, with the enzyme with the sole ATPS moiety in the cytosol and the APK-ATPS in the plastid; it is still to be unambiguously ascertained where assimilatory APS reduction takes place in this diatom. Another exception is represented by the dinoflagellate *Heterocapsa triquetra*, in which a plastidial ATPS is fused at the N-terminus to an APS reductase (APR; see below); it is possible that this arrangement facilitates APS reduction during S assimilation (Patron et al. 2008). Also in *Amphidinium klebsii* an APR-ATPS sequence was identified. In addition to the APR-ATPS, this dinoflagellate possesses an ATPS sequence with the sole ATPS domain (Prioretti et al 2014).

It has been hypothesized that ATPS originated in an ancestral eukaryote as a bifunctional enzyme with ATPS and APK activities. Bacteria and cyanobacteria acquired a descendent of this enzyme by lateral gene transfer (Patron et al. 2008). The evolutionary processes that led to the ATPS of Chlorophyta and of algae with secondary plastids is still a matter of debate.

Very little is known about ATPS regulation in algae. In vascular plants, all steps of S assimilation are up-regulated by sulphate deprivation (Leustek et al. 2000; Kopriva et al. 2009; Davidian and Kopriva 2010). The modulation of S metabolism in algae has unfortunately been studied only in a small number of organisms and generalization is difficult; a certain degree of taxon-specificity appears to exist in the regulation of ATPS expression and activity (Giordano et al. 2000; Bochenek et al. 2013; Prioretti 2014; Prioretti et al 2014). Recent findings suggest that a number of algal ATPS might be susceptible to redox regulation (Prioretti 2014; Prioretti et al 2014). Whether this is a common trait of algal ATPS is presently unsure, although preliminary experimental data point to that direction (Prioretti 2014; Prioretti et al 2014). The suggestion that algae ATPS is

redox regulated (possibly via thioredoxins) originates from two lines of evidence:

1. Most algal ATPSes (except red algae – see below) contain a large number (five to ten) of cysteine residues in their sequences (Table 2); this contrasts with the case of the ATPSes of vascular plants, fungi and yeasts, which at the most contain two cysteine residues in positions different from those in algal ATPSes. Among algae, five main groups of ATPS can be identified: (A) the ATPSes of freshwater cyanobacteria and of marine cyanobacteria that do not belong to the genera *Synechococcus* and *Prochlorococcus*; these enzymes contain four conserved cysteines. (B) The plastidial ATPSes of Chlorophyta, Cryptophyta, Haptophyta and Heterokontophyta (notice the exception of *Thalassiosira pseudonana* cytosolic ATPS mentioned above) and the enzyme of marine cyanobacteria of the genera *Synechococcus* and *Prochlorococcus* (possibly the most abundant marine cyanobacteria; Flombaum et al. 2013); these ATPSes constitute a consistent phylogenetic group (Patron et al. 2008) and contain seven to ten cysteines, five of which are highly conserved (Prioretti et al. 2014), although in positions different from those of the ATPSes of the former group. (C) ATPSes from red algae; only one or two cysteine residues are present in these sequences, one of which is conserved. (D) The cytosolic bifunctional APK-ATPSes enzyme of algae with red secondary plastid; in this case, five to eight cysteine residues are present in the ATPS domain of the protein, two of which are conserved, but in positions different from those of the cysteines of the ATPSes of the other groups. (E) Dinoflagellate ATPSes; these ATPSes are different from all those mentioned above; at this stage, information is available only for two species *Heterocapsa triquetra* (Patron et al. 2008) and *Amphidinium klebsii*; it is therefore difficult to be sure that what follows is true for all dinoflagellates. Both *Heterocapsa triquetra* and *Amphidinium klebsii* possess an enzyme with fused APR and ATPS domains; these APR-ATPSes contain seven cysteines residues in the ATPS domain, one of which is in the same position as one of the cysteines of the enzymes from group A and two have the same position as two of the cysteines of group B ATPSes. In *Amphidinium klebsii*, also a protein with the sole ATPS domain is present; it contains six cysteine residues, two of which are in the same position as the cysteines of the bifunctional APR-ATPS enzyme (Prioretti 2014; Prioretti et al. 2014; Table 2).

   More work is needed to ascertain the exact function of the various cysteines in ATPS.
2. Interestingly, the activity of the ATPS of group A can be stimulated by thiol reducing agents and this stimulation can be reversed by the addition of thiol oxidizing agents. At this stage, it cannot be excluded that also ATPS of group D have a similar regulation. The enzymes of group B, instead, are not affected by thiol reducing agents and are weakly stimulated by oxidizing agents; dinoflagellates ATPS are not affected by neither thiol reducing or oxidizing agents.

The fact that, with rare exceptions, a clear difference exists between the ATPSes of marine and freshwater cyanobacteria poses the question of whether these differences are related to the ecology of the algae. The fact that eukaryotic algae have the same type of ATPS regardless of whether they inhabit freshwaters or the sea is indicative of the fact that the number of cysteines is probably not a decisive feature for life in the oceans. Yet, it cannot be excluded that the ATPS of extant eukaryotic algae represent a frozen accident with which the descendents of the ancestral organisms with group a) ATPS have coped.

The APS generated by ATPS can be used in both the reductive assimilation of S into cysteine and in sulphation reactions (i.e. the catalyzed addition of sulphate to a variety of organic molecules).

We shall first describe the pathway of S assimilation.

APS reduction – Two electrons are required to reduce the sulphate of APS, where S has an oxidation number of +VI, to the S of sulphite, with an oxidation number of +IV. As we mentioned above, in most algae APS reduction occurs in the chloroplast. The sulphate moiety of APS is reduced to sulphite by APS reductase (APR; EC 1.8.4.9; Fig. 5), using reduced glutathione as the electron donor; AMP is released.

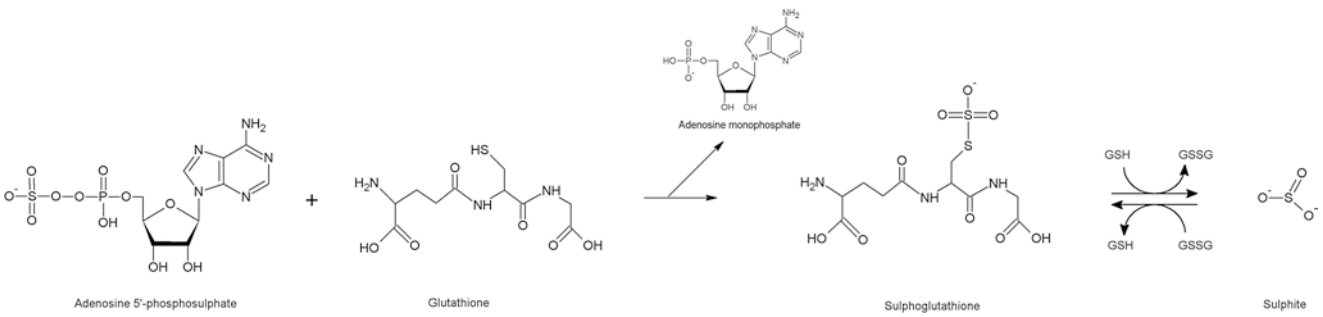

Fig. 5 Reaction catalyzed by adenosine 5′-phosphosulphate reductase (APR)

APR plays a crucial regulatory role in vascular plants (Takahashi et al. 2011): in *Arabidopsis*, it has been shown that the redox regulation of a disulphide bond of APR decreases its activity (Bick et al. 2001). Gene expression and enzyme activity are also negatively affected by the availability of reduced S in cysteine and glutathione: high abundance of cysteine and glutathione inhibit APR activity (Vauclare et al. 2002). Nutrient deprivation (especially S deprivation), instead, stimulates APR activity (Phartiyal et al. 2008). In green algae, as in plants, APR is characterised by a C-terminal thioredoxin/glutharedoxin-like domain and an N-terminal reductase domain (Takahashi et al. 2011 and references therein). The reductase domain contains a Fe_4S_4 cluster (Kopriva et al. 2001, 2002) that is presumably involved in the electron transfer to sulphate, although the actual mechanism has not been elucidated. In basal vascular plants, in the moss *Physcomitrella patens* and in marine chromalveolates and chlorarachniophytes, an APR-B form is present; in this enzyme, the Fe-S cluster is missing. This has been interpreted as an adaptation to an environment where Fe is a scarce resource; phylogenetic analyses confirmed that APR-B is derived from the APR containing the Fe-S cluster (Patron et al. 2008).

Sulphite reduction – The sulphite (+IV) produced by APS is reduced to sulphide (−II) in a reductive step requiring six electrons. This reaction is catalyzed by a Ferrodoxin-dependent sulphite reductase (SiR; EC 1.8.7.1; Fig. 6), a plastidial enzyme with a high affinity for its substrate (Schmidt 1973; Krueger and Siegel 1982; Nakayama et al. 2000). In bacterial SiR (EC 1.8.1.2), NADPH donates the electrons (Yonekura-Sakakibara et al. 2000).

SiR is a very old enzyme, possibly already present before the split between Bacteria and Archea or exchanged via lateral transfer soon after these two domains diverged (Wagner et al. 1998). The ancestral enzyme probably functioned in the dissimilatory direction to produce sulphate as a terminal acceptor of respiratory electrons (little sulphate was present before the great oxygenation event). An early gene duplication is believed to have given origin to modern SiR and its paralogue nitrite reductase (NiR) (Patron et al. 2008). It is thus not by chance that SiR can usually also reduce nitrite, although with lower affinity (Nakayama et al. 2000). The interchangeability of the two enzymes is such that, in the thermophilic red alga *Cyanidioschyzon merolae*, sequences putatively annotated as SiR appear to act as nitrite reductase (Imamura et al. 2010). It has been shown that substrate preference can be switched by a single amino acid mutation (Nakayama et al. 2000). It should be noted however, that some NiR appear unable to reduce sulphite (e.g. Hattori and Uesugi 1968). SiR and NiR share the same prosthetic groups: siroheme (with a mid-point redox potential, E_m', of about −400 mV, in plants) and a Fe_4S_4 cluster (E_m'=−285 mV, in plants) (Krueger and Siegel 1982; Crane et al. 1995; Swamy et al. 2005; the E_m' were obtained from Hirasawa et al. 2004). Siroheme is a porphyrin with Fe in its center and eight carboxylate side chains; it appears to be necessary for the "multi-electron" reductions that SiR and NiR catalyze. Siroheme has been interpreted as an ancestral type of heme and may have played a key role in the evolution of redox metabolism (Murphy et al. 1974). The N-terminal part of assimilatory SiR, in cyanobacteria (Gisselmann et al. 1993) as in algae (e.g. Yonekura-Sakakibara et al. 2000) and in plants (Krueger and Siegel 1982), binds a plant-type (2Fe-s2S) ferredoxin, which thus acts as the *trait-de-union* between the thylakoidal electron transport chain and nitrite reduction. Ferredoxin apparently interacts with SiR acidic amino acid residues, whose mutation greatly reduces the ability of ferredoxin to bind to SiR and transfer electrons (Akashi et al. 1999; Saitoh et al. 2006). The transfer of electrons follows the gradient of redox potential and proceeds from ferredoxin to siroheme, from there to Fe_4S_4 cluster, and finally to sulphite. In plants SiR, the electron transfer from ferredoxin to siroheme appears to occur with a very small redox potential difference (Hirasawa et al. 2004); interestingly, in algae the redox potential of ferredoxin is appreciably lower than in vascular plants (vascular plants E_m' > green algae E_m' > red algae E_m' > cyanobacteria E_m'; Cammack et al. 1977). This may imply that the electron transfer in algal and cyanobacterial SiR occurs in an appreciably less reducing environment. This must be especially true for *Euglena gracilis* (Brunold and Schiff 1976; Rotte et al. 2001), in which sulphite reduction occurs in the much more oxidative environment of the mitochondrion, with the catalysis of a bacterial type SiR that receives the electrons from r NADH (whose redox potential is substantially less negative than that of ferredoxin).

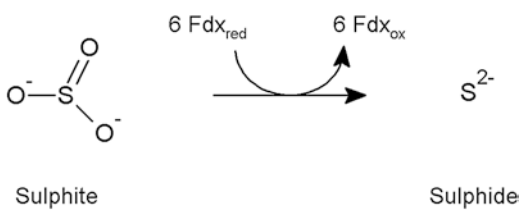

Fig. 6 Reaction catalyzed by sulphite reductase (SiR)

The knowledge of algal sulphite reductase structure and catalysis is mostly based on papers from the 1960s to 1970s (e.g. Ohmori and Hattori 1970; Ho et al. 1976; Hattori ad Uesugi 1968). From these publications and more recent molecular analyses (e.g. Patron et al. 2008), no substantial differences emerge between the ferredoxin-SiR of algae and that of vascular plants (e.g. Patron et al. 2008). In most algae, only one gene encodes for SiR, with the exceptions of *Cyanidioschizon merolae* (but see above) and *Chlamydomonas reinhardtii* (Patron et al. 2008), which have

two plant-type SiRs. In C*hlamydomonas*, the expression of the two genes that encode for ferrodoxin-SiR, *SIR1* and *SIR2*, is stimulated by S deprivation. Very little is known on the regulation of *SIR* in other algae. *Chlamydomonas* also possess a bacterial-type SiR (*SIR3*) whose expression is unresponsive to a decrease of S availability (Zhang et al. 2004). Bacterial-type SiRs have also been found in the nuclear genomes of the diatom *Thalassiosira pseudonana* and of the red alga *Cyanidioschizon merolae* (Shibagaki and Grossman 2008); the functions of the products of these genes remain elusive. As we mentioned above, an active bacterial-type SiR is present in the mitochondrion of *Euglena gracilis*; a nuclear gene presumably encodes this protein (Brunold and Schiff 1976; Rotte et al. 2001; Patron et al. 2008).

Sulphide incorporation into cysteine – The fixation of reduced S into organic matter occurs by insertion of sulphide into the amino acid O-acetylserine (OAS) to produce cysteine; this reaction is catalyzed by OAS (*thiol*)lyase (OAS-TL; EC 2.5.1.47). Serine acetyltransferase (SAT; EC 2.3.1.30) catalyzes OAS production from serine and acetyl-coenzyme A (Fig. 7; Takahashi et al. 2011).

OAS-TL and SAT are encoded by a number of genes: in *C. reinhardtii*, *P. tricornutum*, *T. pseudonana*, three genes encode OAS-TL; in *Emiliania huxleyi*, 11 tentative OAS-TL sequences have been identified. Three genes have been annotated as SAT in *P. tricornutum*, *T. pseudonana* and *E. huxleyi*, and two genes in *C. reinhardtii* (Kopriva et al. 2009). In the plant *Arabidopsis thaliana*, SAT and OAS-TL are localized in the plastid, mitochondrion and cytosol. In this plant, cysteine synthesis enzymes appear to be redundant and the analysis of mutants defective in the various SAT isoforms show that both the main cytosolic isoform (SERAT1;1) and the mitochondrial isoform (SERAT2;2) are capable of producing enough OAS to support normal growth; this has been interpreted as an indication that cysteine can be moved across compartments and used where it is needed (Watanabe et al. 2008; Kopriva et al. 2009). In vivo, most of *A. thaliana* cysteine production takes place in the cytosol (Birke et al. 2012). In the moss *Physcomitrella patens*, although most of cysteine synthesis occurs in the chloroplast, OAS-TL was detected in both the chloroplast and in the cytosol (but not in the mitochondrion); in fact, it should be said that what was detected was the fluorescence emitted by green fluorescent proteins-OAS-TL fusion constructs that had been transfected into *Physcomitrella* (Birke et al. 2012). In *C. reinhardtii*, cysteine synthesis appears to be restricted to the chloroplast (Giordano et al. 2008; Patron et al. 2008; Shibagaki and Grossman 2008; Birke et al. 2012). Based on these evidences, Birke et al. (2012) suggested that redundancy/multiple compartmentalization of cysteine synthesis may have developed along an evolutionary trajectory proceeding from green algae to vascular plants. Given the fact that this conclusion is only based on a very small number of species and that *Physcomitrella* OAS-TL localization requires further confirmation, these results must be taken solely as a possibility, at this stage. Further investigations are required to more confidently address the matter of the evolution of cysteine synthesis compartmentalization.

Unfortunately, OAS-TL and SAT have not been studied in algae and no direct information on their mode of action, isoform location or regulation of expression is available (Kopriva et al. 2009). However, some peculiarities emerge from the OAS-TL sequences of the dinoflagellate *Karlodinium micrum* and the chlorarachniophyte *Bigelowiella natans*: in both cases, the C- terminal domain is characterized by a glutaredoxin-like sequence (Kopriva et al.

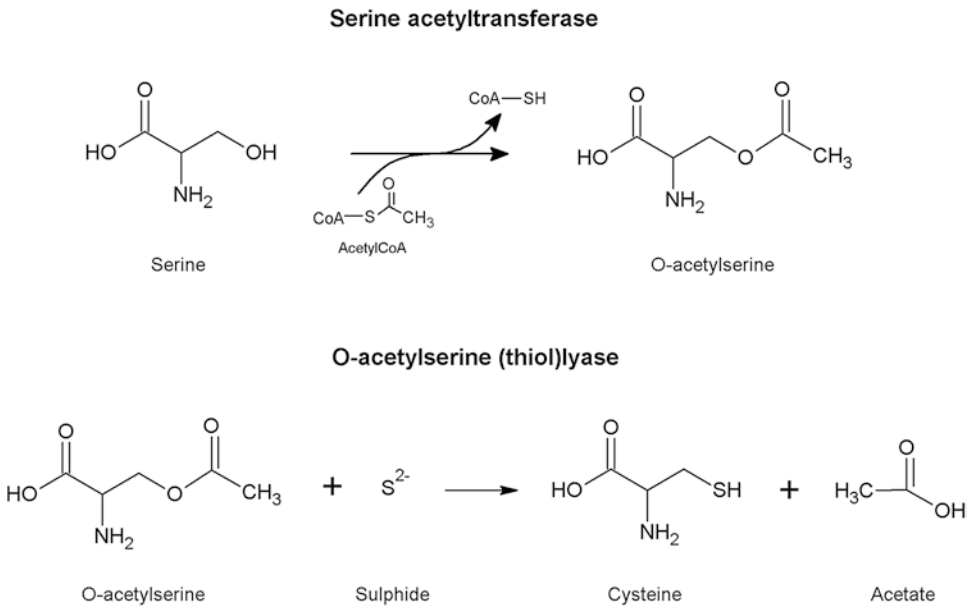

Fig. 7 Reactions catalyzed by O-acetylserine (*thiol*)lyase (OAS-TL) and serine acetyltransferase (SAT)

2008). Gluaredoxin are nearly ubiquitary glutathione- or thioredoxin-dependent oxidoreductase (only some bacteria and archea do not have them; Couturier et al. 2009), which are commonly involved in the redox regulation of enzymes (Meyer et al. 2012). This would therefore point towards a direct redox regulation of cysteine synthesis, in these organisms. Again, this points toward a higher tendency to redox regulation of sulphur assimilation in algae than in vascular plants (Giordano and Raven 2014).

In the absence of information on the mode of action and regulation of algal OAS-TL and SAT, we shall assume that they operate in algae as in vascular plants. In plants (and in bacteria) OAS-TL and a SAT associate to constitute the Cysteine Synthase Complex (Takahashi et al. 2011). According to Wirtz et al. (2010), *Arabidopsis* cysteine synthase complex is constituted by one SAT homohexamer and two OAS-TL homodimers (Fig. 8).

When the two enzymes form the complex, OAS-TL is catalytically inactive and operates solely as a regulatory subunit; thus no substrate channeling takes place in the complex (Droux et al. 1998). Free OAS-TL homodimers are however always present and active; their presence is necessary to attain maximum SAT activity and for cysteine synthesis. SAT, instead, is most likely inactive when is free. The presence of OAS causes the complex to disassemble due to the fact that OAS competes with OAS-TL for the C-terminus of SAT (Huang et al. 2005; Francois et al. 2006). On the contrary, the complex is stabilized by sulphide, so that a metabolic regulation of complex assembling/disassembling is enacted to control the assimilation of reduced S (Wirtz and Hell 2006; Fig. 8). In plants, OAS is mostly produced in the mitochondrion, where SAT is highly active, but appears to limit cysteine synthesis in the chloroplast, where SAT expression is enhanced by oxidative stress. The overall picture suggests that, in vascular plants, the chloroplast is mostly in charge of sulphide production, the mitochondrion of OAS synthesis, whereas cysteine mostly originates in the cytosol (Takahashi et al. 2011 and references therein). It is not possible, at the present state of knowledge, to say that a similar repartition takes place in algae.

5 Glutathione and Phytochleatins Synthesis

In photosynthetic organisms, cysteine is also the substrate from which glutathione synthesis begins (Noctor et al. 2012). Glutathione is the main non-protein thiol of photosynthetic cells and it is usually present at concentrations that are orders of magnitudes higher than those of cysteine (which is typically maintained in the micromolar range). It is an oligopeptide that is synthesized enzymatically, not on ribosomes (Rennenberg 1995; Noctor et al. 2012). Glutathione, in algae as in plants (e.g. Rennenberg and Brunold 1994; Nagalakshmi

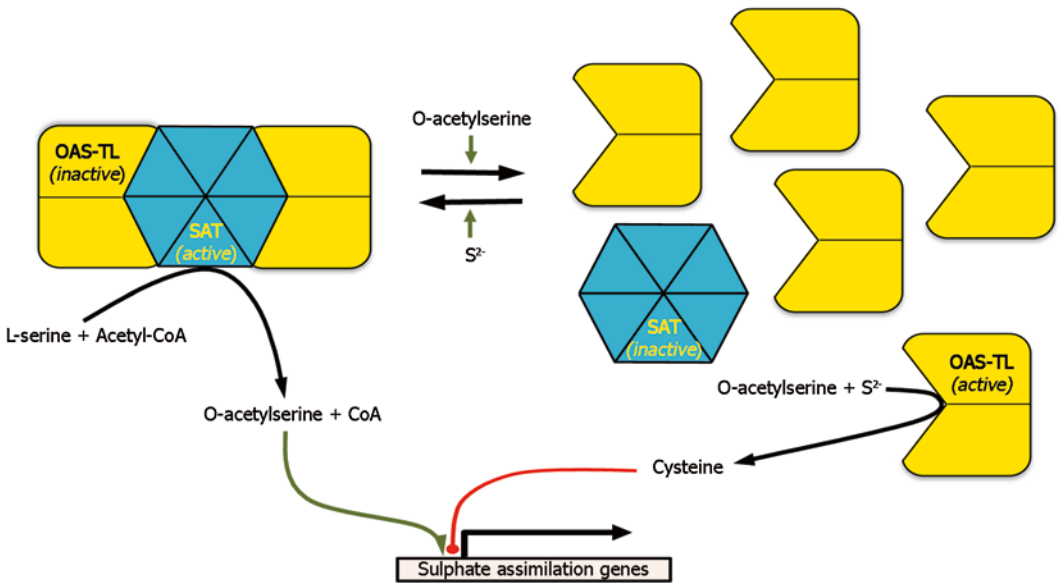

Fig. 8 Mechanism of assembling/disassembling and regulation of the cysteine synthase complex (OAS-TL/SAT complex). Six SAT and two OAS-TL subunits are necessary to form a complex. In the presence of S^{2-}, the complex assembles. In the complex, serine acetyltransferase (SAT) is active and catalyzes O-acetylserine (OAS) formation, whereas OAS (*thiol*)yase (OAS-TL) probably only plays a regulatory function. The OAS produced by the SAT in the complex is released because of its low affinity to the inactive OAS-TL. OAS triggers the transcription of the sulphate assimilation genes. When OAS accumulates in the cell, the cysteine synthase complex dissociates in SAT hexamers and OAS-TL dimers. In the free form, SAT is inactive (or little active), while OAS-TL dimers become active and use OAS and S^{2-} to produce cysteine. When cysteine is abundant in the cell, the transcription of sulphate assimilation genes is repressed

and Prasad 2001), thanks to its thiol groups, is profoundly involved in redox regulation, in the detoxification of the cell from reactive oxygen species (ROS) and xenobiotics, it is a pivotal signaling molecule and contributes to buffering and monitoring of reduced S within the cell; glutathione possibly also represents a S storage pool, although in a S rich environment such as the ocean this function may be of little relevance. The thiol groups of glutathione are reduced and oxidated by the flavoprotein glutathione reductase (EC 1.6.4.2), which uses NADPH/$NADP^+$ as electron donor/acceptor and plays a fundamental role in controlling the redox state of the glutathione pool (Leisinger et al. 1999; Vitova et al. 2011; Ding et al. 2012; Lu et al. 2013; Zhang et al. 2013). The continuous interconversion of reduced glutathione and its oxidized dimeric form (generated by the formation of a disulphide bond between the thiols of two glutathione molecules) is crucial to the various processes in which glutathione is involved (see Noctor et al. 2012 for a more detailed description of such processes).

Two enzymes are involved in glutathione biosynthesis: γ-glutamylcysteine synthetase (EC 6.3.2.2) and glutathione synthetase (EC 6.3.2.3) (Fig. 9).

The first enzyme catalyzes the formation of a gamma peptide bond between a cysteine and a glutamate (i.e. a bond between the γ-carboxyl of glutamate and the α-amino group of cysteine). Subsequently, glutathione synthetase generates glutathione by linking γ-glutamylcysteine and glycine by the formation of an alpha peptide bond. Both enzymes hydrolyze ATP to generate the energy required for the catalysis. These enzymes have been located in the chloroplasts and in the cytosol, but not in the mitochondria (Preuss et al. 2013). Regulation of γ-glutamilcysteine synthetase occurs via feedback inhibition by glutathione (Hell and Bergmann 1990). This avoids the accumulation of excessive amounts of glutathione in case of a surplus of its constitutive amino acids. Glutathione synthesis also depends on the supply of cysteine and glycine (Noctor et al. 1996; Hartmann et al. 2004); this is due to the fact that γ-glutamilcysteine synthetase has a Km for cysteine close to the rather low basal cysteine concentration in the cell (Noctor et al. 1996). In other words, the activity of this enzyme often operates well below substrate saturation and is thus limited by cysteine availability (and sulphate assimilation). When cells are subject to oxidative stresses associated with the presence of ROS and xenobiotics, sulphate assimilation ad glutathione synthesis are stimulated (Mallick and Mohn 2000; Dupont et al. 2004).

In plants and algae, the conjugation of glutathione, through its thiol group, to xenobiotics or to endogenous molecules that must be inactivated is catalyzed by the enzyme glutathione S-transferase (e.g. Tang et al. 1998). The resulting conjugate is then transferred into the vacuoles via a transporter of the ATP Binding Cassette (ABC) type (Martinoia et al. 1993; Foyer et al. 2001). At least in plants, these complexes are then hydrolyzed to cysteine-xenobiotic conjugates (Noctor et al. 2012 and references therein).

When cells are exposed to toxic concentrations of heavy metals, glutathione is used to produce phytochelatins (Ahner et al. 1995, 2002; Scheidegger et al. 2011).

$$\gamma-(\mathrm{Glu}-\mathrm{Cys})_n\,\mathrm{Gly}(n=2-11)$$

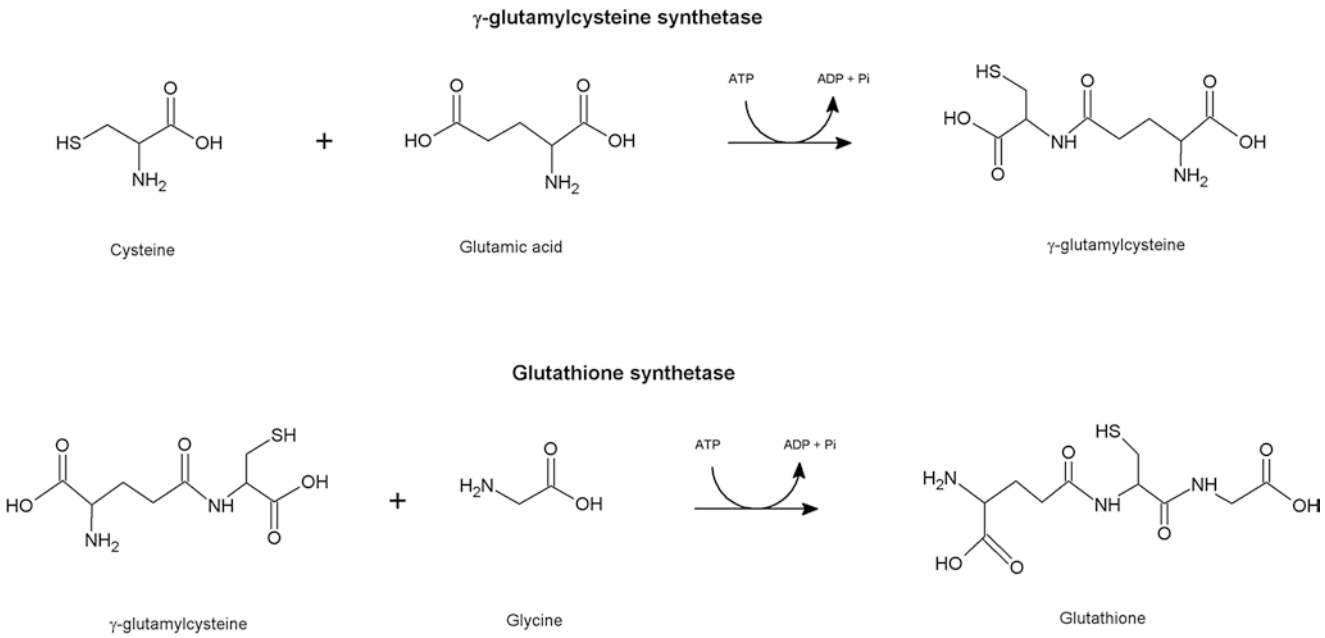

Fig. 9 Glutathione synthesis. The upper panel depicts the reaction catalyzed by γ-glutamilcysteine synthetase; the lower panel depicts glutathione synthetase reaction

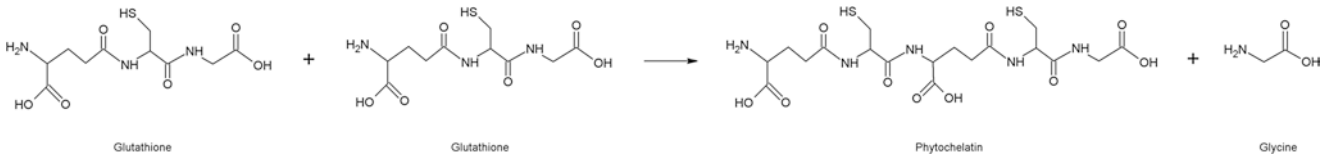

Fig. 10 Phytochelatins biosynthetic pathway

Phytochelatin synthesis is catalyzed by γ-glutamilcysteine dipeptidyl transpeptidase or phtochelatin synthase (Fig. 10). Much of what is known about this enzyme and its regulation is derived from studies on higher plants, especially *Arabidopsis thaliana*, but some information on algal enzymes is beginning to emerge (Gupton-Campolongo et al. 2013 and references therein). Phytochelatin synthase binds the carboxyl group of a cysteine in a glutathione molecule to the amino group of a glutamate in another glutathione molecule. The glycine originally linked to the cysteine involved in the bond is released. The same mechanism is used to elongate existing phytochelatins (up to 11 Gly-Cys units).

Phytochelatins bind heavy metals (Vatamaniuk et al. 2000; Le Faucheur et al. 2006); the phytochelatin-heavy metal complexes are transported into the vacuole through an ABC transporter. Within the vacuole these complexes bind additional heavy metals, especially cadmium (transported independently through Cd/H^+ antiporters); also sulphide participates to the construction of these heavier phytochelatin complexes, which tend to crystallize and precipitate (Noctor et al. 2012).

6 Methionine Metabolism

In photosynthetic organisms cysteine is the entryway for S into the organic matter. Methionine, the other S amino acid, is synthesized in a pathway that uses cysteine as a donor of reduced S and aspartate (via homoserine and O-phosphohomoserine) as the donor of C skeletons and N (Fig. 11).

In plants and, for the little that is known, in algae, methionine synthesis comprises three reactions; (1) cytathionine γ-synthase (CGS; EC 2.5.1.48) catalyzes the reaction between cysteine and O-phosphohomoserine (which is also a precursor of threonine synthesis) to produce cystathionine; (2) cytathionine is cleaved to homocysteine and pyruvate, with the release of the amino group originally contained in cysteine; the enzyme that catalyzes this reaction is cystathionine β-lyase (CBL; EC 4.4.1.8); (3) finally, methionine synthase transfers a methyl group from methyltetrahydrofolate to homocysteine to produce methionine. In plants, reactions 1 and 2, take place in the chloroplast, whereas methionine synthase has been found in both the chloroplast and the cytosol (Ravanel et al. 2004; Takahashi et al. 2011). In cyanobacteria, the reduced sulphur of cysteine can also be directly employed to synthesize homocysteine in a reaction with either succinyl-homoserine or acetyl-homoserine; the enzymes that catalyze these reactions (O-succinyl homoserine sulphydrylase and O-acetyl homoserine sulfhydrylase) are collectively named homocysteine synthase (HS). This pathway is most likely absent in eukaryotic photosynthetic organisms (Hesse and Hoefgen 2008; Vallon and Spalding 2009). It has been proposed that where HS is not present, CGS may also catalyze the direct production of homocysteine from cysteine, although no hard evidence exist for this collateral pathway (Hesse and Hoefgen 2008 and references therein).

Two main forms of methionine synthase exist in nature: METH (EC 2.1.1.13), which transfer the methyl group from tetrahydrofolate via cobalamine (vitamin B_{12}), and METE (EC 2.1.1.14), which does not use cobalamine in its catalysis (Helliwell et al. 2011). In vascular plants, only METE is present. Among algae, organisms such as the red alga *Cyanidioschyzon merolae* and the green alga *Coccomyxa* sp. C-169 only have METE; the green algae *Chlamydomonas reinhardtii*, *Chlorella* sp. NC64A and possibly also the oleaginous *Botryococcus braunii*, the diatom *Phaeodactylum tricornutum*, the brown macroalga *Ectocarpus siliculosus* posses both types of methionine synthase; the chlorophyte *Micromonas pusilla*, various *Ostreococcus* species, the diatom *Thalassiosira pseudonana*, the haptophyte *Emiliania huxleyi*, the toxic pelagophyte *Aureococcus anophagefferens* only have METH (Helliwell et al. 2011; Koch et al. 2013; Tanabe et al. 2014). Croft et al. (2005) found that 171 algal species out of the 326 they investigated require exogenous cobalamine for growth. The exclusive presence of METH determines the need for mixotrophic acquisition of cobalamine (Helliwell et al. 2011). The requirement for cobalamine thus has no phylogenetic consistency and is the result of the loss of METE genes. No alga appears to be able to synthesize cobalamine. This poses a number of ecological questions, whose implications are various, complex and unclear (Croft et al. 2005; Helliwell et al. 2011; Bertrand et al. 2013); further studies are required to attain a better understanding of vitamin mixotrophy in algae. The suggestion by Croft et al. (2005) that cobalamine is fed to algae by symbiotic bacteria is extremely stimulating but this symbiotic associations need to be more firmly demonstrated, before this suggestion can be fully accepted.

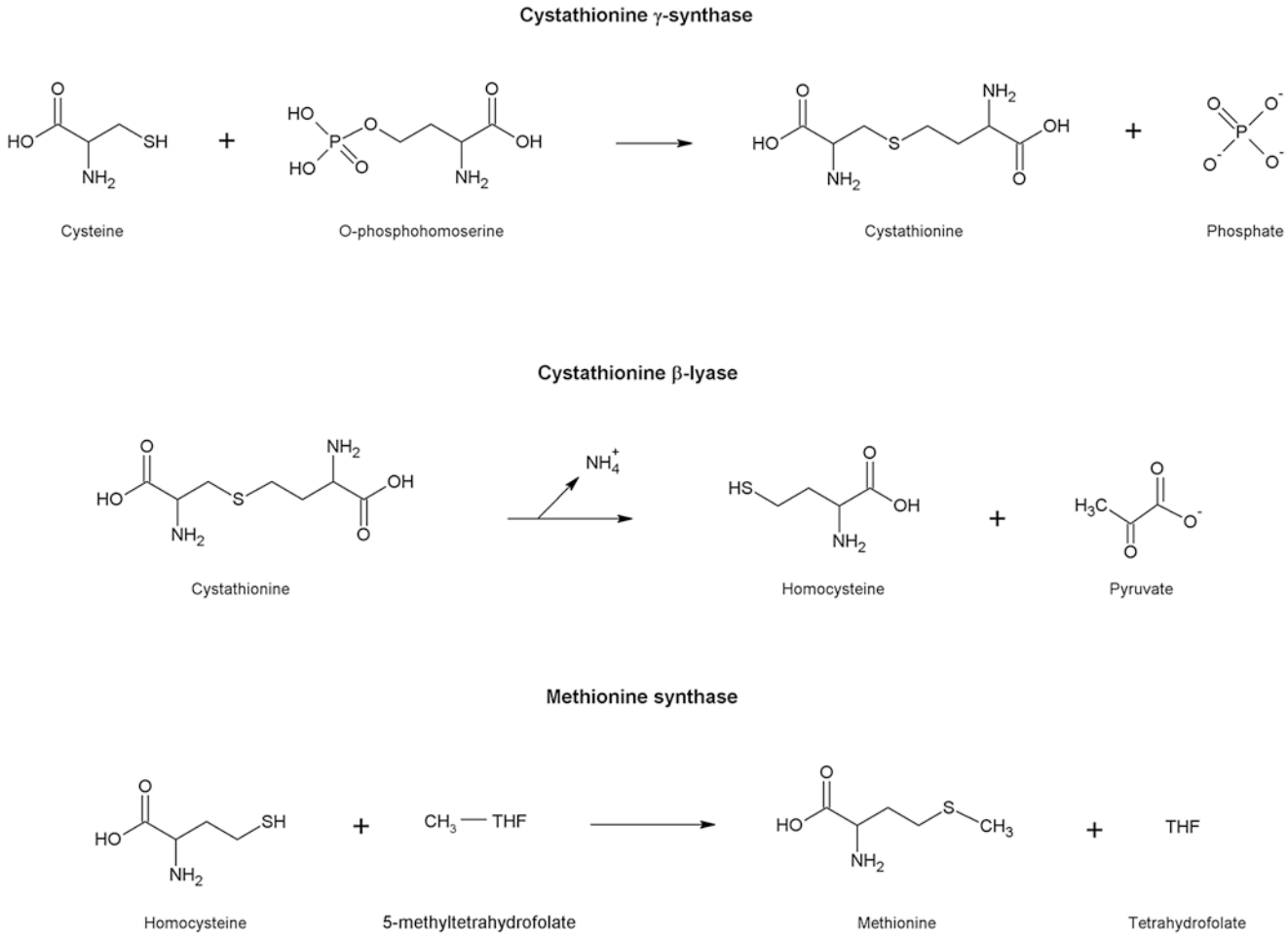

Fig. 11 Methionine synthesis. The three reactions are catalyzed, respectively, by cystathionine γ-synthase, cystathionine β-lyase and methionine synthase

At least in plants, the largest proportion of methionine is converted to S-adenosyl-L-methionine (SAM), whereas only a smaller proportion is incorporated into proteins. SAM synthesis occurs in the cytosol thanks to the catalysis of SAM synthetase, which generates SAM from methionine and ATP (Harlow et al. 2007; Fig. 12). The catalysis of this enzyme requires the formation of a membrane bound tripoliphosphate that is then cleaved to orthophosphate and pyrophosphate by the tripolyphosphatase activity of the enzyme (Ravanel et al. 1998). The available information suggests that SAM synthesis occurs in algae through a pathway similar to that of plants. In dinoflagellates, due to the unusual codon usage of dinoflagellate, SAM synthetase genes show rather low similarity with sequences from other organisms, although the corresponding protein are instead homologous to SAM from various sources (Harlow et al. 2007). In the cytosol, SAM serves has the donor of a methyl group to L-methionine, with the catalysis of cytosolic SAM:methionine S-methyltransferase (EC 2.1.1.12); this reaction generates S-methyl-L-methionine (SMM) and S-adenosylhomocysteine (SAH). The enzyme S-adenosylhomocysteine hydrolase (EC 3.3.1.1) then converts SAH to homocysteine and homocysteine can regenerate methionine in a reaction catalyzed by cytosolic MS. The ensemble of these cytosolic reactions constitutes the so-called SAM cycle (Fig. 12). SMM, SAH and methionine are transferred across the chloroplast membrane to satisfy the requirements for these compounds of both the chloroplast stroma and the cytosol (Ravanel et al. 2004; Takahashi et al. 2011).

SAM plays a central role in the regulation of methionine synthesis. The control of methionine synthesis, in plants, is mostly exerted at the intersection between methionine and threonine synthesis, and is based on the different affinities of CGS and threonine synthetase for the common intermediate O-phosphohomoserine and on the stimulation by SAM of the activity of threonine synthetase and of its affinity for O-phosphohomoserine (Takahashi et al. 2011 and references therein). Bochenek et al. (2013) showed that, in *Emiliania huxleyi*, SAM synthetase and S-adenosylhomocysteine hydrolase, two enzymes of the cytosolic SAM cycle, are

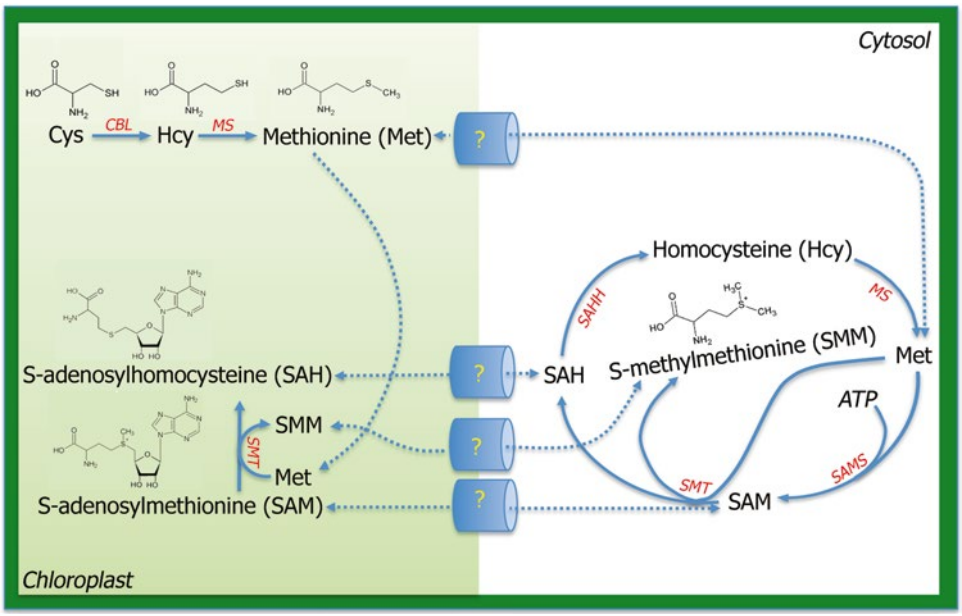

Fig. 12 S-adenosylmetionine (SAM) cycle. The enzymes are in *red. CBL* cystathionine β-lyase, *Cys* cysteine, *Hcy* homocysteine, *Met* methionine, *MS* methionine synthase, *SAH* S-adenosylhomocysteine, *SAHH* S-adenosylhomocysteine hydrolase, *SAM* S-adenosylmethionine, *SAMS* S-adenosylmethionine synthetase, *SMM* S-methylmethionine, *SMT* SAM:methionine S-methyl transferase. *Dashed lines* indicate putative pathways for metabolite transport

down-regulated when the algae were deprived of sulphate, whereas, under the same conditions, the expression of methionine synthetase is induced. In *Chlamydomonas*, the genes of the SAM cycle and in general of methionine metabolism are consistently down-regulated when the algae are S starved (González-Ballester et al. 2010), while the opposite is true for the vascular plant *Arabidopsis thaliana* (Nikiforova et al. 2003).

SAM is involved in polyamine ad ethylene synthesis (Ratti and Giordano 2008 and references therein), but its primary metabolic role is as methyl donor in a variety of fundamental processes, including DNA and phospholipid methylation (Hesse and Hoefgen 2008). Halides are among the possible target for SAM-dependent methylation. This conspicuously contributes to biogenic halide production and methyl halides are the main carriers of natural halogens from the oceans (Toda and Itoh 2011). The activity of the SAM-dependent halide/thiol methyltransferases is present in many algae, although the amount of activity and methyl halide productivity can vary greatly (Toda and Itoh 2011). Since halides are also connected with ozone layer disruption, this fate of methionine may also play an ecological role (Toda and Itoh 2011). SAM is also believed to be a precursor of some toxins causing paralytic shellfish poisoning (Harlow et al. 2007).

7 Methionine Is the Precursors of Algal Sulphonium Compounds

In plants, the SMM produced from the methylation of L-methionine by SAM, is the precursor of dimethylsufide (DMS). SMM hydrolases (EC 3.3.1.2) catalyzes this reaction, which also leads to the production of L-homoserine (Giordano et al. 2005 and references therein). Interestingly, in algae (at least in those species in which this pathway has been elucidated), DMS is generated only through the action of DMSP lyases after cell lysis (Giordano et al. 2005; Norici et al. 2005; Caruana and Malin 2014). DMSP is also produced by few vascular plants (Hanson et al. 1994; Trossat et al. 1998; Husband et al. 2012). Although both algae and plants use methionine as the precursor of DMSP and, possibly, both synthesize it in the chloroplast, they use very different biosynthetic pathways (Giordano et al. 2005; Ratti and Giordano 2008; Oduro et al. 2012; Bochenek et al. 2013). In vascular plants, DMSP synthesis is a major sink for SMM (Hanson et al. 1994; Bürstenbinder and Sauter 2012). In the green alga *Ulva intestinalis* and possibly in other algal taxa (Gage et al. 1997; Summers et al. 1998; Fig. 13), the instable chetoacid 4-methylthio-2-oxobutyrate (MTOB) is generated by transamination (possibly the amino group is transferred to 2-oxoglutarate; see Giordano et al. 2005 and references therein). Subsequently, MTOB reductase converts MTOB to 4-methylthio-2-hydroxybutyrate (MTHB) using NADPH. These two reactions have been found also in non-DMSP producers. The following step in the pathway, i.e. the S-methylation of MTHB to 4-dimethylsufonio-2-hydroxybutyrate (DMSHB), instead seems to be exclusive of DMSP producers; this reaction is catalyzed by the SAM-dependent enzyme MTHB S-methyltransferase. DMSHB has osmoprotectant properties and has been proposed to be the evolutionary precursor of DMSP. In *Ulva intestinalis*, DMSP is finally produced by the oxydative decarboxylation of DMSHB.

Although a large number of algal species produce DMSP, the amount that a cell can contain is extremely variable (for instance see Caruana and Malin 2014, who showed the very

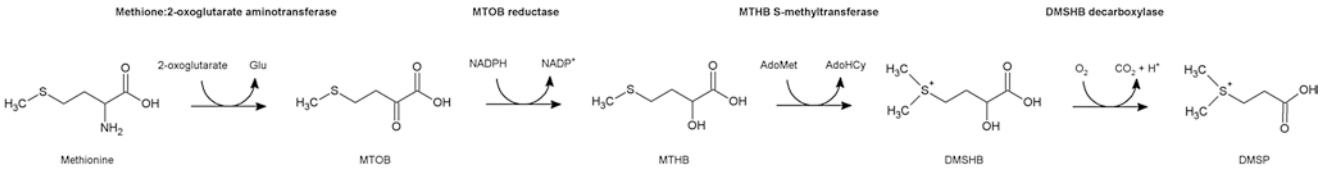

Fig. 13 DMSP biosynthetic pathway in algae. *DMSHB* 4-dimethylsufonio-2-hydroxybutyrate, *DMSP* dimethylsulphoniopropionate, *MTHB* 4-methylthio-2-hydroxybutyrate, *MTOB* 4-methylthio-2-oxobutyrate

large variability of DMSP production within dinoflagellates). High DMSP producers often have relatively low C:S ratios (Norici et al. 2005). The interest on DMSP metabolism largely originated from the hypothesis that the release in the atmosphere of the product of its enzymatic degradation, dimethyl sulphide (DMS), represents a major planetary feedback mechanism to control global temperature (Charlson et al. 1987). In other words, the stimulation of phytoplankton growth caused by an increase in oceanic surface temperature would lead to a higher production of DMS, whose oxidation in the atmosphere by hydroxyl radicals would cause the formation of sulphate and methanesulphate that greatly contribute to the formation of aerosol with a strong sunlight-scattering power and to cloud condensation (Andreae and Crutzen 1997; Giordano et al. 2005; Orellana et al. 2011). Indeed, the DMS production catalyzed by DMSP lyase in the ocean represents the largest natural source of S to the atmosphere (Bates et al. 1992; Giordano et al. 2005; Norici et al. 2005). DMSP lyases have been detected in fungi, bacteria and a variety of algae (Stefels et al. 2007; Franklin et al. 2010; Curson et al. 2011; Caruana et al. 2012; Caruana and Malin 2014; Mohapatra et al. 2014). It should be noted, however, that a rather small fraction of the DMSP produced by healthy algal cells is converted to DMS (Giordano et al. 2005; Norici et al. 2005); what is thus the function of DMSP in algal cells? The literature on this matter is vast (Giordano et al. 2005; Norici et al. 2005); here we shall just provide succinct information on the main hypotheses on DMSP functions:

1. *DMSP is an osmolyte*. The tertiary sulphonium compound DMSP bears an obvious resemblance with a common osmolyte in phytoplankters, the quaternary ammonium compound glycine betaine (Fig. 14). In some organisms, but not all, DMSP is produced instead of glycine betaine when N is in short supply (Keller et al. 1999a, b).

   Rather early in the history of the planet, N became limiting for primary production in the ocean (Anbar and Knoll 2002). This may have represented a strong selective force in favor of those organisms that were able to produce an alternative osmolyte that contained the much more abundant S in the place of N. It is possible that this original function of DMSP later diversified, thanks to the metabolic versatility of tertiary sulphonium compounds (Ratti and Giordano 2008). The acquisition of additional functions besides osmoregulation and the persistence of high S abundance in the oceans (see above) may have then altered the modulation of DMSP synthesis in response to N availability, which may explain the inconsistent results obtained by Keller et al. (1999a, b).
2. *DMSP is a cryoprotectant*. The observation that, in contrast to the hypothesis of Charlson et al. (1987), lower temperature elicits an increase of DMSP concentration in some algal cells (Sheets and Rhodes 1996), that some polar microalgae contain more DMSP than their temperate and tropical relatives (Karsten et al. 1996 and references therein), and that DMS protects enzymes and in some cases even stimulates their activity (Karsten et al. 1996) led to the hypothesis that DMSP acts as an osmoprotectant.
3. *DMSP has an anti-grazing action*. DMSP and the products of its cleavage by DMSP lyase, DMS and acrylate, have been shown to deter grazers (Freidig et al. 1999; Giordano et al. 2005; Van Alstyne and Puglisi 2007; Lyons et al. 2010). Very little (if any) DMSP, DMS and acrylate are released by healthy cells. Most DMSP cleavage occurs after cells break (healthy cells do not usually produce large amounts of DMS). DMSP release and DMS and acrylate production are thus high when algae are subjected to grazing (especially if the grazers break the cells rather than swallowing them). High DMSP producers may therefore be more able to deter these kinds of grazers. If this function of DMSP and the products of its cleavage is indeed of ecological relevance (and more data are needed to ascertain it), it may provide the causative link (or one of the causative links; see Ratti et al. 2011) between DMSP production and the evolutionary success of the algae with plastids containing chlorophyll a+c,

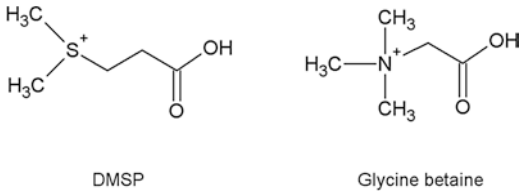

Fig. 14 DMSP and glycine betaine: two osmolytes with similar structure

which on average have lower C:S ratios and, with exceptions (e.g. most diatoms), are better DMSP producers than chlorophyll a+b algae (Giordano et al. 2005; Norici et al. 2005).

Function of DMSP as an antioxidant, methyl group donor and S storage have also been proposed (Karsten et al. 1996). Furthermore, DMS may convey chemical information that contributes to interspecific communication within the food web (Steinke et al. 2002; Seymour et al. 2010). All these functions are entirely compatible with the chemical properties of the molecule. It is thus possible that DMSP, whatever the selective pressure that caused the initial affirmation of its biosynthetic pathway, has acquired a variety of different functions.

8 Sulphation

The APS produced by the catalysis of ATPS, besides being funnelled into the reductive S assimilation pathway, can also be used in the non-reductive modification of proteins and metabolites by sulphate addition. This process is generically indicated as sulphation (Shibagaki and Grossman 2008). Prior to its utilization in sulphation, APS must be further phosphotylated to 3′-phosphoadenosine-5′-phosphosulphate (PAPS) (Takahashi et al. 2011). This phosphorylation is catalyzed by ATP:adenylyl-sulphate 3′-phosphotransferase, or APS kinase (APK; EC 2.7.1.25). APK transfers the γ-phosphate from ATP to the 3′-hydroxyl group of APS (Leipe et al. 2003); both in bacteria and eukaryotes, similarly to what happens in ATPS, APK binds MgATP before APS; if the opposite occurs, the enzyme is unable to perform the catalysis (Satishchandran and Markham 1989; MacRae and Segel 1999; Lillig et al. 2001). In plants, APK is located both in the cytosol and in the chloroplast (Kopriva et al. 2012). The location of this enzyme in algae has not been thoroughly investigated. In metazoa, fungi and in stramenopile algae such as diatoms, brown algae and Cryptophyceae, APK is bound to ATPS (Mueller and Shafqat 2013). It has been demonstrated that, at least in *Arabidopsis thaliana*, a precise coordination of sulphate reduction and sulphation pathways, i.e. of the destiny of APS, occurs (Mugford et al. 2011). The finding that *Arabidopsis* APK is redox regulated (Ravilious et al. 2012) suggests that the destiny of APS may be mediated by the plastid redox state and by oxidative stress (Kopriva et al. 2012).

In yeasts and fungi, PAPS is utilized in S assimilation and is reduced to sulphite by a PAPS reductase (EC 1.8.4.8), which lacks the FeS cluster of APR and contains instead a thioredoxin-like domain (Kopriva et al. 2008; Shibagaki and Grossman 2008; Bromke et al. 2013). Genes similar to those encoding PAPS reductase have been found in the genome of the diatom *Thalassiosira pseudonana*; unfortunately, no information is yet available on the proteins that these genes produce and on the function they play (Bromke et al. 2013). Kopriva et al. (2002) suggested that the PAPS reductive pathway evolved after APR in response to periods of low Fe availability (Kopriva et al. 2002). If this is true, it is noteworthy that no marine alga, for what is known, uses this pathway for sulphur assimilation, in spite of the frequent severe shortage of Fe in seawater. The consensus, in spite of the PAPS reductase-like sequences in *Thalassiosira*, is that algae use PAPS exclusively for sulphation.

In plants and mammals, sulphotransferases (SOTs; EC 2.8.2) are responsible for sulphation (Klein and Papenbrock 2004; Hernández-Sebastià et al. 2008), with PAPS as the sulphate donor. The information on algal SOTs is rather scarce and in-depth studies are lacking (Hernández-Sebastià et al. 2008). Putative SOTs have been identified in cyanobacteria (Soto-Liebe et al. 2010), red algae (Arad et al. 2013) and brown algae (Dittami et al. 2012). In the toxic freshwater cyanobacterium *Cylindrospermopsis raciborskii*, for example, two putative SOTs are believed to be involved in the biosynthesis of a paralytic shellfish poisoning toxin (Soto-Liebe et al. 2010). The brown alga *Ectocarpus siliculosus* possesses at least 15 genes encoding SOTs (Michel et al. 2010); some of these are related with animal arylsulphatases and plant SOTs involved in the biosynthesis of secondary metabolites like glucosinolates, a second group of SOTs are more similar to bacterial genes which, nevertheless, have not been characterized, the remaining *Ectocarpus* SOT genes are homologous with animal SOTs responsible of the synthesis of sulphate carbohydrates and are thought to be involved in the sulphation of cell wall polysaccharides (Michel et al. 2010). In macroalgae, conspicuous amounts of sulphated polysaccharides are produced; it is therefore expected that high levels of SOT activity are present in these organisms. The interest in sulphated polysaccharides also derives from their utilization by the food, pharmaceutical and chemical industries (Itoh et al. 1993; Witvrouw and De Clercq 1997). Fucans are among the most common sulphated carbohydrates in macroalgae; they comprise fairly heterogeneous compounds and are typical of brown algae. Fucans function in vivo is mostly associated with protection against desiccation and with structural support (Percival 1979). Chemically, they are polymers of L-fucose bonded by alternating α(1→3) and α(1→4) glycosidic bonds; sulphate is usually attached to the position 4 of the fucose ring (Berteau and Mulloy 2003).

Galactans are class of sulphated polysaccharides; they include carrageenan and agarans. Although it was shown that green algae and marine angiosperms are able to produce these compounds (Aquino et al. 2005; Bilan et al. 2007; Farias et al. 2008), these polysaccharides are especially abundant in Rhodophyta. Galactans have structural functions in vegetative thalli and contribute to the formation of

mucilaginous layers around reproductive spores (Percival 1979). Although some species-specific differences exist in the structure of galactans, they typically have a linear backbone with alternated β-D-galactopyranose and α-D/L--galactopyranose, bound with a α(1→4) link; the position of sulphate in the molecule is variable (Pomin and Mourao 2008). The main difference between carrageenan and agaran is that the former contains α-D-galactopyranose whereas the latter contains α-L-galactopyranose; this simple modification is responsible for the different physical properties of these molecules. For further information about the properties and uses of carrageenans and agarans see Campo et al. (2009) and Pomin (2010).

Among the various molecules that are produced in sulphation reaction, sulpholipids are especially important in algae. Sulpholipids are a class of glycerolipids present in all photosynthetic organisms; they are typical and abundant components of the thylakoid membranes (Goss and Wilhelm 2010; Shimojima 2011). The most common sulpholipid is sulphoquinovosyldiacylglycerol (SQDG); its derivative 2-O-acyl-sulphoquinovosyldiacylglycerol (ASQD) is also frequently found in algae (Riekhof et al. 2003). Algae contain substantially larger amounts of SQDG than plants, and stramenopile algae possess higher levels of SQDG than green algae (Vieler et al. 2007; Lepetit et al. 2012). SQDG is also abundant in cyanobacteria where it often represents the 10–20 % of the total membrane lipid pool (Sato and Wada 2010) and in species of the genera *Synechococcus* and *Prochlorococcus* it can sum up to 66 % of total membrane lipids (Van Mooy et al. 2006). The first step in the biosynthesis of SQDG is the donation of the sulphate group by PAPS to UDP-glucose; this reaction leads to the synthesis of UDP-sulphoquinovose (UDP-SQ) and is catalyzed by UDP-SQ synthase (SQD1; EC 3.13.1.1); subsequently, SQDG synthase (SQD2) catalyzes the transfer of the sulphoquinovose group to a molecule of diacylglycerol (see Benning et al. 2008 for further details). Sulphoquinovosyldiacylglycerol seems to be the involved in the promotion of protein-pigment complexes formation and in their stabilization. Loll et al. (2007) found that SQDG, together with other membrane lipids (e.g. monogalactosyldiacylglycerol and digalactosyldiacylglycerol), forms a lipid envelope around the D1 and D2 proteins of photosystem II that provides a suitable environment for the turnover and repair of these proteins. In cyanobacterial membranes, SQDG is also believed to facilitate the dimerization of PSII and the binding of Q_B to PSII, although the mechanism by which this occurs has not yet been clarified. Pick et al. (1985) found an association between SQDG and the plastidial ATP synthase in the green alga *Dunaliella salina* (Pick et al. 1985); the same authors showed that, in spinach chloroplasts, SQDG might contribute to ATP synthase activity down-regulation by reducing the permeability of the plastidial membranes to protons (Pick et al. 1987).

There are indications of the fact that sulpholipid biosynthesis is stimulated by N and P limitation (Goss and Wilhelm 2010). This may explain why very high levels of SQDG were found in cyanobacteria of the genera *Prochlorococcus* and *Synechococcus* (Van Mooy et al. 2006), which typically dominate phytoplankton in the oligotrophic and phosphate poor open ocean gyres; these organisms may have acquired a competitive advantage over other species by substituting phospholipids with sulpholipids and thus decreasing P demand (Van Mooy et al. 2006, 2009). Sulphate limitation, instead, leads to a decrease in the size of the SQDG pool (Sato et al. 2000); when this happens, the thylakoid membrane lipid structure and ionic composition is altered; the lack of SQDG is compensated by an increase in the phosphatidylglycerol (PG) pool. In *Chlamydomonas reinhardtii*, the sulpholipid pool is broken down in response to S starvation in order to provide S for protein biosynthesis (Sugimoto et al. 2010).

9 Interaction of S Metabolism with C, N and P Metabolisms

The stoichiometry of organisms is reciprocally connected to the chemistry of the environment (Redfield 1934; Falkowksi and Raven 2007). The changes in elemental stoichiometry are however rather tightly constrained by the basal metabolic requirement of the organisms. These constraints clearly emerge from the similarity between the stoichiometry of organisms grown in "standard conditions" (i.e. mostly resource-replete conditions; C:N:P:S = 124:16:1:1.3) and the expected oceanic Redfield Ratio (C:N:P:S = 106:16:1:1.7; Giordano 2013 and references therein). In the case of C, N and S, their co-existence in numerous molecules, among which proteins, requires that their assimilation is, at least to some extent, co-regulated and that the limitation of any of these nutrients leads to a stimulation in the uptake of the limiting nutrient and/or concomitant adjustments in the uptake and assimilation capacity and fluxes and of the intermediate storage of the non-limiting nutrients (Giordano et al. 2005; Giordano 2013). In the specific case of low S availability, the reduction in photosynthetic activity is a rather typical consequence, associated with alteration of both light and dark reactions (Wykoff et al. 1998; Giordano et al. 2000). This is accompanied by a major reorganization of the protein complement, which leads to an overall size reduction of the protein pool and appears to differentially affect specific proteins (especially Rubisco amount decreases substantially; protein such as periplasmic carbonic anhydrases are much less affected; Giordano et al. 2000). The mobilization of sulphur from proteins such as Rubisco may also allow the reallocation of S to proteins directly involved in S-starvation (Giordano et al. 2000, 2005). Sulphur and nutrient limitation

in general affects photosynthetic electron transfer in various ways. Primarily, the decreased demand of ATP and reducing power for the assimilation of the nutrients in short supply may generate a negative feedback to the rate of electron transport; the down-regulation of pigment-protein complexes synthesis, the lower functionality of photosystem II associated with a reduction of D1 ex novo synthesis (as observed in *Chlamydomonas reinhardtii*; Wykoff et al. 1998; also see references in Maneeruttanarungroj et al. 2012) or a higher rate of PSII degradation (as detected in the cyanobacterium *Synechococcus* sp.; Collier et al. 1994) may decrease the number of active reaction centers. The down-regulation of photosynthesis in S-deprived *C. reinhardtii* is believed to be a specific response necessary to cope with such conditions, which is under the control of SAC1 (see above) (Davies et al. 1996; Wykoff et al. 1998).

In the green alga *Dunaliella salina*, S limitation profoundly alters C ad N metabolism: when S supply decreases, the main anaplerotic enzyme, phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31), is down-regulated. This hampers the export of C-skeletons from the tricarboxylic acid cycle (TCAC) for ammonium assimilation; the accumulation of intracellular NH_4^+ inhibits nitrate reduction (Fernandez et al. 2009). The fact that the TCA cycle is forced to slow down also affects the upstream metabolic pathways; this is probably the reason why, under S starvation, *D. salina* shows a rather dramatic change in the amino acid profile; as compared to the S-replete cells, S-starved cells show a much larger contribution of C3 amino acids such as alanine (especially) and serine to the total amino acid pools (Giordano et al. 2000). Transcriptomic data are suggestive of different modes of regulation in different algal taxa; the metabolic changes in *D. salina* subject to S starvation are thus not necessarily generalizable.

10 Summary

- Sulphur is one of the most important macronutrients for photosynthetic organisms; it is mostly available in aquatic environments as sulphate (SO_4^{2-}).
- Sulphate concentrations have changed substantially in the course of Earth history. This has likely affected the evolutionary trajectories of phytoplankton.
- Today, sulphate is very abundant in the oceans (around 28 mmol L^{-1}), but much less concentrated in most freshwater systems.
- Sulphate is the form in which sulphur is acquired by algae, but sulphide is the form in which it is assimilated (in cysteine).
- Sulphate reduction occurs in the chloroplast, in most algae. *Euglena gracilis* is the only known exception: in this alga, sulphate is reduced in the mitochondrion.
- Sulphate reduction requires activation of sulphate to adenosine 5′-phosphosulphate (APS). Subsequently, APS is reduced by a glutathione-dependent reductase.
- In vascular plants, APS reductase is believed to be the main control point in the pathway of sulphate reduction. In algae, there are strong indications of redox regulation at the first step of the pathway, that of sulphate activation catalyzed by ATP sulphurylase.
- Cysteine is the entryway of sulphur in the organic matter in algae.
- Cysteine is also the point of origin of the biosynthetic pathways that lead to the production of glutathione and phytochelatins.
- Methionine is derived from cysteine. Most of the methionine produced is used to synthesize S-adenosyl-L-methionine (SAM), from which S-methyl-L-methionine (SMM) is generated. SMM is a precursor of dimethylsuylphoniopropionate (DMSP).
- DMSP possibly originated as an N-free osmolite in environments (such as the ocean) where N is often limiting. It has then acquired a number of functions that may today have great relevance in algal ecology. When DMSP is cleaved, DMS and acrylate are produced; DMS is believed to play an important role in climate control; DMSP, DMS and acrylate seem to have antigrazing activity.
- The APS produced by ATP sulphurylase can also be used in non-reductive reactions leading to the sulphation of numerous secondary metabolites such as sulpholipids, sulphated galactans (carrageenan and agaran), and sulphated fucans.

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Micronutrients

Antonietta Quigg

1 Introduction

In the 40 years since the chapter 'Inorganic nutrients' was written by O'Kelley in the book '*Algal Physiology and Biochemistry*' (Stewart 1974), our understanding of the types, amounts, and roles of micronutrients in microalgae has expanded enormously, as has our ability to measure and decipher their activities, fate and behavior in cells and the surrounding environment. This chapter aims to provide a state-of-the art account of micronutrients in microalgae. Unlike the original chapter by O'Kelley, which included the macronutrient elements Sulfur, Potassium, Calcium and Magnesium, the reader is referred to other chapters in this book for an update on those elements. Given the extensive literature towards our understanding of some micronutrients, the reader is also referred to chapters "Iron" (Fe) (Marchetti and Maldonado 2016), "Selenium in Algae" (Se) (Araie and Shiraiwa 2016), and "Silicification in the Microalgae" (Si) (Finkel 2016) which are dedicated exclusively to each of these micronutrient elements.

Although each micronutrient is considered on an element-by-element basis briefly below, following O'Kelley, it is recognized that each functions in the presence of others and is affected by them, such that these interactions, as we know them, will also be examined. The reader is referred to excellent reviews and/or treatises published over the decades since O'Kelley's chapter, particularly the book by Frausto da Silva and Williams (2001) and papers by Raven (1988, 1990, Raven et al. (1999), Whitfield (2001), Morel et al. (2003), Worms et al. (2006), Glass et al. (2009), and Sunda (1994, 2012). More recent papers have begun to reveal the nature of yet-to-be-discovered metalloproteins involved in biochemistry and physiology (e.g. Cvetkovic et al. 2010) and these understudied and unknown roles and activities of micronutrients will be the subject of future research efforts.

Unlike other groups of organisms, microalgae have polyphyletic origins (e.g., Delwiche 1999; Falkowski et al. 2004). They are not only morphologically but also physiologically and biochemically heterogeneous, making generalizations about their micronutrient requirements challenging. As O'Kelley pointed out in 1974, whereas higher plants are thought to have essentially the same elemental requirements, there appear to be differences in elemental requirements between algal species. This includes the obvious fundamental requirements for Si in diatoms and some chrysophytes (see chapter "Silicification in the Microalgae", Finkel 2016) and Ca in coccolithophores (see chapter "Calcification", Taylor and Brownlee 2016), but also the lesser known and understood differences in micronutrient requirements between prokaryotes and eukaryotes as well as between eukaryotes.

In addition, micronutrient requirements are known to differ between oceanic, coastal (neritic) and freshwater microalgae. The majority of examples in this Chapter will be microalgae that have a coastal and oceanic origin; this is in no way intended to discount the importance of freshwater systems. Anthropogenic inputs of micronutrients to the environment exceed inputs from natural sources by 10- to 100-fold, particularly to lakes, rivers, and the coastal ocean. There has been a concurrent steady increase in their concentrations in the biota, altering ecological stoichiometries, food webs and trophic movement of these elements. We also raise concerns of a new emergent pollutant (engineered nanoparticles), which will likely also be the focus of future studies of micronutrient effects on microalgae.

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2 Evolution of Micronutrient Requirements in Microalgae

Throughout Earth's history, it can be (and has been) argued that the environment and microalgae have influenced each other's composition (e.g., Redfield 1934; Falkowski and Raven 1997; Falkowski 1997; Frausto da Silva and Williams 2001; Anbar and Knoll 2002; Morel and Price 2003; Quigg et al. 2003a, 2011; Falkowski et al. 2004; Martin et al. 2008; Sunda 2012; Martin and Quigg 2013). In order to do so, microalgae appear to have evolved simultaneously mechanisms that maximize use of available micronutrient fluxes (e.g., by releasing strong complexing agents) and strategies to promote more efficient recycling of some elements compared to others (e.g., by catalyzing redox reactions that modify the bioavailability of micronutrients). Microalgal uptake of some essential elements results in their extraordinarily low concentrations in surface seawater (except for Mo). This in turn, controls the rate of photosynthetic fixation of carbon (primary production) and the transformation and uptake of major nutrients, particularly nitrogen (see e.g. Falkowski 1997; Anbar and Knoll 2002; Martin et al. 2008). In this way, the extremely low concentrations of essential micronutrients results in ultra-efficient uptake systems in microalgae. It may also explain the widespread replacement of micronutrients in metallo-centers of enzymes by one another in important biochemical reactions (e.g., nitrogenase, superoxide dismutase, carbonic anhydrase). Recent studies have shown that the ability to acquire (uptake) and eliminate (efflux) micronutrients is a physiological trait that varies between taxa and can be linked to evolutionary histories and changes in ocean chemistry. Such species-specific traits play an important role in determining the micronutrient quota's (intracellular concentrations), their response to different environmental perturbations including upwelling and pollution, and consequently successional patterns, community composition and/or competition.

The growth of marine microalgae in the environment was thought for a very long time to be primarily limited by the availability of the major or macro-nutrients (nitrogen and phosphorus, and to a lesser extent silicate in diatoms) (see chapters "Combined Nitrogen", "Nutrients and Their Acquisition: Phosphorus Physiology in Microalgae", and "Sulphur and Algae: Metabolism, Ecology and Evolution"; Raven and Giordano 2016; Dyhrman 2016; Finkel 2016). Although some early laboratory studies suggested that Fe may limit microalgal growth in the oceans (see chapter "Iron", Marchetti and Maldonado 2016), it was not until Martin and Fitzwater (1988) and Martin et al. (1991) provided the first evidence of a micronutrient (Fe) playing a major role in microalgal growth and primary productivity that scientists more seriously considered the role of micronutrients. Despite being the fourth most abundant element in the earth's crust, Fe is relatively scarce (in terms of bioavailability) in today's oxygenated oceans (0.02–1 nmol L^{-1}) (Bruland et al. 1991). We now know that Fe limits microalgae in as much as 30–40 % of the world oceans, particularly in high nitrate-low chlorophyll regions (see chapter "Iron"; Marchetti and Maldonado 2016). Studies have also found that Fe limits cyanobacterial N_2 fixation and thereby controls oceanic inventories of biologically available fixed nitrogen over long (geological) time scales (see e.g., Falkowski 1997; Anbar and Knoll 2002; Sunda 2012). The interaction between N and Fe and microalgae is now so well recognized and studied that these are discussed in greater detail in other parts of this book (see Raven and Giordano 2016; Marchetti and Maldonado 2016).

By contrast, during the Archean, some 3.8–2.5 billions of years ago, as a result of widespread ocean anoxia Fe is thought to have been relatively abundant (50 μmol L^{-1}) in the surface and deep oceans (Anbar and Knoll 2002). This may explain why it is used in so many important proteins associated with the photosynthetic pathways (Raven 1988, 1990; Raven et al. 1999; Larkum 2016). The opposite is thought to be true for Mo with prebiotic concentrations ten-times less than present values of ~100 nmol L^{-1} in surface oceans (Collier 1985; Anbar and Knoll 2002; Scott et al. 2008). Anbar and Knoll (2002) hypothesized that Mo–N co-limitation likely influenced the early evolution of cyanobacteria. Heterocystous cyanobacteria, which diversified over 2 billion years ago (Tomitani et al. 2006), evolved during a period when bioavailable Mo was low in the oceans (Anbar and Knoll 2002; Scott et al. 2008). This would have created strong selection pressures for Mo acquisition and storage, especially for the N_2 fixation enzyme, nitrogenase, which has both high Fe and high Mo requirements (in a Fe_7MoS_9 cluster). As Mo levels rose in the sea, ca. 500 million years ago (Scott et al. 2008), Mo no longer limited nitrogen assimilation pathways (nitrate reduction) thereby allowing eukaryotes to diversify (Anbar and Knoll 2002). Frausto da Silva and Williams (2001) reported that there was a proliferation of Zn enzymes in microalgae that evolved only after the establishment of an oxidizing environment; however, biological utilization of Co shows the opposite trend. The significance of this will be discussed below in the section describing enzymes which use a variety of metallo-centers and in particular can use Zn or Co for the same catalysis reaction. Consistent with this is the observation of generally lower Zn quotas in prokaryotes because they produce fewer Zn finger proteins and are less likely to use Zn as a metallo-center in the enzyme superoxide dismutase relative to eukaryotes (Saito et al. 2002; Dupont et al. 2010; Quigg et al. 2011). Comparable shifts in micronutrient utilization are thought to have occurred for other micronutrients with redox-sensitive environmental chemis-

tries but supporting evidence for these less abundant elements is not easy to gain from the geologic record.

Laboratory studies have suggested that there is an evolutionary inheritance hypothesis which can be used to predict similarities in elemental composition within related taxonomic lineages of microalgae; driven at least in part by changing ocean chemistry at the time of their evolution and radiation (Quigg et al. 2003a, 2011; Falkowski et al. 2004). Twenty-nine species of microalgae from ten taxa were grown under identical conditions (light, temperature, nutrient-replete media) to examine macro- and micronutrient composition. Initially it was found that eukaryotic algae of the green (chlorophyll $a+b$ plastids) and red (chlorophyll $a+c$ plastids) lineages (see definitions of lineage in Delwiche 1999; Falkowski et al. 2004) had stoichiometrically distinct elemental signatures. Diatoms, dinoflagellates and coccolithophores of the red lineage generally had lower Fe:P, Zn:P and Mn:P but higher Co:P, Mo:P and Cd:P relative to chlorophytes and prasinophytes in the green lineage (Quigg et al. 2003a). Dinoflagellates have plastids (chloroplasts) derived from tertiary endosymbiotic events such that both green and red types are present in extant species. Quigg et al. (2003a) found that the dinoflagellate with the green plastid (*Gymnodinium chlorophorum* (= *Lepidodinium chlorophorum*))[1] clustered with the green lineage in terms of micronutrient utilization while those with red plastids (*Prorocentrum minimum* (= *Prorocentrum cordatum*), *Amphidinium carterae* and *Thoracosphaera heimii*) clustered with the red lineage, suggesting the micronutrient requirements of the plastids plays a significant role in determining micronutrient requirements of microalgae. In a later study, cyanobacteria, Glaucocystophyta, and additional members from both of the green and red lineages were included in a further analysis (Falkowski et al. 2004; Quigg et al. 2011). The largest differences in the elemental profiles distinguished prokaryotic cyanobacteria and primary endosymbiotic events that resulted in the green and red plastid lineages (Quigg et al. 2011). Smaller differences in micronutrient stoichiometry within the red and green plastid lineages were consistent with changes in micronutrient stoichiometry owing to the processes associated with secondary endosymbioses and inheritance by descent with modification. The authors hypothesized that differences in elemental composition among species primarily represents phylogenetic differences in biochemical requirements and the ability of the organisms to take up and store these elements, not environmental or culture conditions. Hence, phenotypic variation in micronutrient stoichiometry is much smaller than genetic differences between phylogenetic groups. Studies by Ho et al. (2003) and Finkel et al. (2006, 2007) have supported these contentions, finding that phenotypic differences in elemental composition due to environmental conditions rarely result in more than two to fivefold variability in element to phosphorus ratios in response to an order-of-magnitude variation in irradiance or macronutrient concentration, or an order-of magnitude variability for over three orders-of-magnitude variation in trace metal concentrations in the external media.

3 Essential, Required, Replaceable?

The following inorganic elements were reported by O'Kelley (1974) as being found in microalgae: N, P, K, Mg, Ca, S, Fe, Cu, Mn, Zn, Mo, Na, Co, V, Si, Se, Cr, Cd, Cl, B, I. Of these, N, P, Mg, Fe, Cu, Mn, Zn and Mo were thought to be required by all microalgae. Early studies on micronutrients focused on the roles of essential versus required elements. The presence of a particular element in an organism is not to be mistaken with that element being essential, as some elements, particularly micronutrients, may be absorbed at levels in excess of requirements or may be absorbed but not required by microalgae (see Quigg 2008). The essential transition metal ions (referred to as micronutrients in this chapter) used in enzymes include vanadium to zinc (first-row transition metal series) and molybdenum (second-row series). Because these elements exist in nature in multiple oxidation states, they are ubiquitously utilized as micronutrients.

The accepted criteria for confirming a micronutrient as essential were: (1) growth ceases in its absence or is optimal (or close to) in its presence (Arnon 1953) and (2) the demonstration by in vitro experiments that an element has a non-replaceable role in a fundamental life process (O'Kelley 1974). According to Liebig's law of the minimum, when all other factors are favorable (e.g., light, temperature), the nutrient available in the smallest quantity will limit the growth of an organism. Given that such studies require axenic cultures and the ability to exclude the micronutrient of interest; they are very difficult to perform (e.g., presence of contaminants in 'pure' chemicals, non-selective binding or complexation which in turn alters bioavailability). Thirdly it was thought that a comparison of the composition of microalgae and that of seawater may provide insights into which micronutrients may be most limiting for growth (O'Kelley 1974). This approach however was challenged once we had a greater understanding of the differences in micronutrient concentrations in coastal versus open ocean environments and the corresponding microalgae present in these distinct ecosystems. Further, a deeper understanding of the complex nature of micronutrient chemistry has challenged this latter approach for studying micronutrient requirements by microalgae (more below), specifically the

[1]Wherever possible the currently accepted names for species are used. The name used in the paper cited is also indicated. For details of names see chapter "Systematics, Taxonomy and Species Names: Do They Matter?" of this book (Borowitzka 2016).

ability of some micronutrients to replace one another in times of limitation or as a result of synergistic/antagonistic behaviors when one element is present in excess of another.

4 Micronutrient Elements Considered Essential to All Microalgae

Arguably, the most important micronutrients in microalgae are those used in redox reactions (e.g. Fe, Mn, Cu), acid-base catalysis (e.g. Zn, Ni), transmission and storage of information and energy (K, Ca), and in structural cross-links (e.g. S, Si) (Table 1). While this selection may have been biased from life's beginnings by abundance, later in evolution energy costs and functional advantages would have become more important. The bioavailability of these micronutrients influences the flow of energy and nutrients in ecosystems impacting both biogeochemical processes and community structure. Various biotic and abiotic factors affect accumulation of micronutrients in microalgae, including their size, water quality and environmental contamination. Further, the organic ligands (chelators) produced by microalgae play an important role in mediating the positive (and negative) impacts of micronutrients. Intracellularly, micronutrient quotas in microalgae are driven largely by the biochemical demands of the cells as they respond to various external forces.

In oceans, macro- and micronutrients essentially have the same profiles: surface waters have depleted concentrations (close to or below detection limits) which then increase with depth (e.g., Bruland 1980; Bruland et al. 1991) with one exception, Mo. Similarities between vertical profiles of micronutrients with those of macronutrients have been taken to suggest that similar biological uptake and regeneration processes are occurring. Examples of these will be given below and are discussed in more detail in reviews such as Whitfield (2001), Morel et al. (2003), Sunda (2012) and Twining and Baines (2013). Similar profiles are not found in coastal and freshwater environments; this is a result of the complex chemistries and physical interactions at play in these systems. Mo concentration is roughly proportional to salinity, hence the same concentration (~105 nmol L^{-1}) has been measured at all depths and ocean basins (Collier 1985; Bruland et al. 1991). The lack of a nutrient-like profile

Table 1 Roles of micronutrients in microalgal cells

Element	Functions	Examples of compounds
Fe	Photosynthetic pathways, active groups in porphyrin molecules and enzymes; component of cytochromes and certain nonheme iron-proteins and a cofactor for some enzymatic reactions, reactions with oxygen and nitrogen (e.g. N_2 fixation, N uptake, photosystems), cytochrome oxidase, cytochrome P-450, chlororespiration and cyclic electron flow around PS I, Mehler reaction (production of superoxide),	PS II complex (cytochrome *b*559 and one additional Fe which is not in haem or part of a FeS complex), the cytochrome *b*6-*f* complex (cytochrome *b*563, cytochrome *f*, Rieske non-haem iron-sulfur complex containing 2 Fe), cytochrome *c*6 (an alternate to Cu containing plastocyanin), PS I complex (3 non-haem iron-sulfur complexes FX, FB and FA, each containing 4 Fe) and ferredoxin (a protein with one non-haem iron-sulfur complex containing 2 Fe, an alternate to flavodoxin which contains no metals), nitrate reductase, nitrite reductase, catalase, cytochromes (e.g. *b* and *c* for electron transport in photosynthesis and respiration; *f* for photosynthetic electron transport), superoxide dismutase (alternatively with Mn or Zn), NAD(P)H dehydrogenase,
Mn	Electron transport in PSII, maintenance of chloroplast membrane structure, breakdown reactions and those involving halogens, Mehler reaction (production of superoxide)	O_2 evolving complex, catalases, peroxidases (alternatively with Fe, Se, or V), manganin
Zn	Enzymes (ca. 150 known), ribosome structure, nucleic acid replication and polymerization, hydration and dehydration of CO_2, hydrolysis of phosphate esters, Mehler reaction (production of superoxide)	DNA and RNA polymerases, carbonic anhydrase
Cu	Electron transport (photosynthesis and respiration) enzymes, disproportionation of O_2 radicals to O_2 and H_2O_2 in reaction	Plastocyanin, cytochrome oxidases, superoxide dismutase (alternatively with Fe or Mn), at trace element levels, has a role in the thylakoid lumen in facilitating H_2O dehydrogenation and O_2 evolution
Mo	Nitrogen reduction (nitrate and nitrite reduction to ammonium), ion absorption	Nitrate and nitrite reductase, nitrogenase enzyme
Co	Component of Vitamin B_{12}, C4 photosynthesis pathway, C and H transfer reactions with glycols and ribose	Vitamin B_{12}, carbonic anhydrase
V	May be a component of nitrogenase	Nitrogenase enzyme
Ni	Hydrolysis of urea; co-factor in enzymes	Urease
Cd	May substitute into enzymes that typically use Zn	Carbonic anhydrase in diatoms

suggests minimal removal of Mo by microalgae relative to its seawater concentration (Sunda 2012).

As mentioned earlier, the reader is referred to other Chapters in the book for details on Fe (Marchetti and Maldonado 2016). Below the focus is on a small sub-set of important micronutrients following O'Kelley's original list: Mn, Zn, Cu, Mo, Co, V and Ni. The level of detail reflects, perhaps coincidentally, our current interest in understanding of the roles and activities of these micronutrients.

4.1 Manganese

Hopkins (1930) was the first report a Mn requirement for microalgae. Like higher plants, microalgae become chlorotic when Mn is deficient as it alters chlorophyll formation, galactosyldiglyceride content and/or glycolate synthesis (see O'Kelley 1974). More recent studies have found Mn (II) forms strong nitrogen (N)- and S-ligands and is a cofactor in several Krebs-cycle enzymes. Mn (III) is used in superoxide dismutases, acid phosphatases, and ribonucleotide reductases and in transfers across membranes (Table 1; Raven et al. 1999; Frausto da Silva and Williams 2001). While earliest micronutrient studies revealed Mn was associated with PSII but not PSI (Cheniae and Martin 1969; Teicheler-Zallen 1969), today the most studied role of Mn in algal metabolism is its function in the oxygen-evolving complex of photosynthesis. Mn is second only to iron in its quantitative role in the thylakoids reactions of all microalgae; the reader is referred to the excellent reviews of Raven (1990), Raven et al. (1999) and Morel et al. (2003) for very specific details.

Evolutionarily, Mn was thought to be selected for the oxygen-evolving complex (and some other functions) because of its greater overall availability (reduced and oxidized) relative to Fe, rather than because of its unique chemistry (Raven et al. 1999; Frausto da Silva and Williams 2001). While it is recognized that four atoms of Mn (in oxidation states ranging from +3 to +5) and one Ca^{2+} are required to split water in the protein heterodimer, there is still a great deal of attention directed at understanding the exact process by which this occurs at a molecular level (e.g. see Ferreira et al. 2004). The Mn complex provides the redox system for the accumulation of the four oxidizing equivalents required for water oxidation, providing a gating mechanism between the single electron turnover of the reaction centers and the oxidation of water. Three of the five oxidation states of the water oxidizing complex are known to involve different Mn redox states. There are some inefficiencies thought to be associated with the process such as backward transitions (double misses) which lead to the formation of super reduced states (Messinger et al. 1997; Quigg et al. 2003b); current evidence however suggests that these may have a more photoprotective role allowing the cells to dissipate excess photon energy.

4.2 Zinc

Zn is an essential element (Table 1) with a long list of important metabolic functions (over 150 enzymes are known) as a result of its ability to function as a Lewis acid and its bioavailability in aquatic habitats (Frausto da Silva and Williams 2001; Morel et al. 2003). But it can also inhibit the growth of microalgae if present at elevated concentrations or as a result of antagonistic behaviors in the presence of some other micronutrients (Anderson et al. 1978; Sunda and Huntsman 1992, 1995a, 2000; Sunda 1994, 2012). The earliest demonstration of a Zn requirement in microalgae was in 1926 in the green alga *Stichococcus bacillaris* (Eilers 1926). Early studies focused on its role in the nucleic apparatus (such as DNA and RNA polymerases), finding the RNA content was low in Zn deficient algal cells (e.g., Wacker 1962; Altman et al. 1968; Prask and Plocke 1971; Frausto da Silva and Williams 2001). Because of Zn's essential role in DNA metabolism, zinc-limited *Euglena* cells were found to be incapable of completing their mitotic cycle and were unable to undergo cell division (Falchuk et al. 1975). Since then studies have shown its requirement in important proteins and enzymes such as Zn finger transcription factors, reverse transcriptase, alkaline phosphatases, superoxide dismutase, lactate dehydrogenase and in phycobilin production (Table 1; Frausto da Silva and Williams 2001). Zn may also be involved in silica uptake and frustule deposition in diatoms, as evidenced by decreased silica uptake rates and Si:P ratios at low dissolved inorganic zinc concentrations (see Sunda and Huntsman 2005 for references).

Because of its importance in cellular process, Zn internalization can be up regulated to the point where Zn diffusion becomes limiting and thermodynamically strong Zn complexes become bioavailable (Hassler and Wilkinson 2003; Hassler et al. 2005). In natural waters, Zn has a nutrient type profile and is present as the free ion or in relatively weak complexes (Bruland 1980; references in Sunda 1994, 2012). Further, Zn uptake is tightly regulated such that Zn internalization and intracellular Zn contents can be maintained relatively constant despite external variations of several orders of magnitude: $10^{-8.5}$ to 10^{-11} M (Sunda and Huntsman 1992; Hassler and Wilkinson 2003). There is evidence both that Zn limits productivity in some regions of the ocean (e.g., sub-Arctic Pacific) and coastal waters (e.g., California and Costa Rica) (see references in Sunda and Huntsman 2005) and that this limitation affects the composition and structure of marine microalgal communities because of differences in Zn requirements among microalgal species (Brand et al. 1983; Sunda and Huntsman 1995a, 2000; Quigg et al. 2003a, 2011). Support for this also comes from Sunda and Huntsman (1992) who found that the ability of the oceanic species (*Thalassiosira oceanica* and *Emiliania huxleyi*) to outgrow coastal ones (*Thalassiosira pseudonana* and *Conticribra* (*Thalassiosira*) *weissflogii*) at low Zn ion concentrations was

due almost entirely to a reduced growth requirement for cellular Zn rather than to an increased capability for uptake.

In terms of photosynthesis in microalgae, Zn plays a critical role in the enzyme carbonic anhydrase, involved in CO_2 supply to RUBISCO for C fixation (see reviews such as Raven et al. 1999; Morel et al. 2003; Beardall et al. 2009; Sunda 2012), one of the most catalytically active enzymes known across plant and animal kingdoms. In marine microalgae, carbonic anhydrase catalyzes the following reversible reaction:

$$HCO_3^- + H^+ \leftrightarrow CO_2 + H_2O$$

In the last decade, the activity, function and micronutrient requirements of this enzyme have been the focus of many studies including those addressing questions of the carbon concentrating mechanism, C4 versus C3 photosynthesis pathways, C acquisition (see chapter "Carbon Acquisition by Microalgae", Beardall and Raven 2016), and partially in order to understand how microalgae will respond to climate change factors such as ocean acidification. In terms of micronutrients, predications of a ~40 % increase in CO_2 in the atmosphere and surface ocean waters suggests that co-limitation by Zn and CO_2 are unlikely as lower quantities of this enzyme are needed at high CO_2 concentrations (more below). In eukaryotes, there is evidence from both laboratory and field measurements that Zn limitation may at least be partially alleviated by the use of Co as a metallo-center in the enzyme carbonic anhydrase (Morel et al. 1994; Sunda and Huntsman 1995a, 2000), and in diatoms Cd has been found to be replaceable for Zn or Co in this important enzyme (Lee and Morel 1995; Yee and Morel 1996; Lane et al. 2005; Xu and Morel 2013).

4.3 Copper

The importance of Cu as a micronutrient was first established by Guseva (1940). Its activity may be related to the three accessible oxidation states (I, II, III) of Cu in biological systems resulting in this micronutrient having a redox potential range from +0.2 to 0.8 V (Falkowski and Raven 1997). Cu is known to play a role in the photosynthetic electron transport chain of microalgae that use the metallo-protein plastocyanin (Katoh et al. 1961; Hill et al. 1996), in PSI activity (Bishop 1964) and the respiratory electron transport chain, as well as in the Cu-Zn form of superoxide dismutase, and in the transmembrane uptake of Fe (La Fontaine et al. 2002; Maldonado et al. 2006) (Table 1).

Cu-plastocyanin it is not used ubiquitously in all microalgae (Raven et al. 1999). In some species, in times of differential Fe/Cu availabilities, cells can switch from using the Fe-containing enzyme cytochrome c_6 to plastocyanin in the photosynthetic electron transport pathways without compromising photosynthetic activity (Hill et al. 1996; Raven et al. 1999; Peers and Price 2006). In the respiratory electron transport pathway, cytochrome oxidase reduces oxygen to water (Frausto da Silva and Williams 2001).

A variety of adverse effects in the presence of elevated Cu have been reported, including reductions in growth, photosynthesis and respiration. The severity of the effect is dependent on pH, ambient light, the presence of other ions (e.g., Fe) and whether cells are grown under light or dark, aerobic or anaerobic conditions (see O'Kelley 1974; Brand et al. 1986; Frausto da Silva and Williams 2001). Not surprisingly, the internal concentration (homeostasis) of copper is tightly regulated by a range of cellular processes. These can quickly internalize Cu to vesicles or externalize it; high Cu efflux rates have been measured within seconds of elevated Cu exposures in microalgae (Croot et al. 2000, 2003; Quigg et al. 2006). Unlike the response to other micronutrients, the response to Cu seems to vary with microalgal species such that there is an evolutionary imprint of ocean chemistries and physiologies (more below).

Cu is present in total concentrations of 0.5–4.5 nmol L^{-1} in open ocean surface waters (Bruland 1980) whereas coastal Cu concentrations can reach 50 nmol L^{-1} (references in Glass et al. 2009; Sunda 2012). The free metal of Cu is more toxic than the complexed form of the metal because the former is considered more available (Brand et al. 1986; Moffett and Brand 1996; Croot et al. 2000, 2003). Cu is heavily complexed in the euphotic zone by a group of organic ligands (weak and strong) (Moffett et al. 1990; Moffett and Dupont 2007); the ligand concentration generally covaries with that of Cu, resulting in a relatively constant free cupric ion concentration of 10^{-13} M in coastal and near-surface oceanic waters. It is not known whether ligands bind Cu ions intracellularly and then are released as a detoxification method or if the ligands are first released and then bind Cu ions extracellularly. In both scenarios, complexation to Cu decreases the amount of biologically available Cu around the cell (Moffett and Brand 1996). It is however known that chemical speciation in the environment is dominated by complexed Cu, which may elevate or reduce Cu uptake in regions with low and high Cu concentrations respectively (Moffett and Dupont 2007).

4.4 Molybdenum

Bortels (1940) was the first to demonstrate the importance of Mo in N_2 fixation by heterocystous cyanobacteria. Wolfe (1954) then showed that the N content of N_2 fixing *Anabaena cylindrica* was positively correlated with the Mo concentration of the growth media. Shortly thereafter, studies found Mo essential for the biological assimilation of N either as dinitrogen (N_2) gas by nitrogenase (Hallenbeck et al. 1979)

or nitrate (e.g., Arnon et al. 1955; Arnon and Ichioka 1955; Rao 1963) because of its role in the nicotinamide adenine dinucleotide nitrate-reducing complex (Vega et al. 1971). Since then, the role of Mo in the nitrogen fixation and reduction processes has been the subject of many investigations (see Raven 1988; Glass et al. 2010; Hoffman et al. 2014) including those focused on evolutionary aspects of micronutrients and microalgal evolution (Anbar and Knoll 2002; Martin et al. 2008; Martin and Quigg 2013).

Nitrogenase is the oxygen-sensitive protein in cyanobacteria, which reduces N_2 to ammonia. It is composed of two subunits: a Mo-Fe containing multi-subunit dinitrogenase reductase protein (NifDK) (contained in a cofactor called MoFe-cofactor or MoFe-co, believed to be the substrate binding site) and a Fe-containing dinitrogenase protein (NifH) (see reviews such by Raven 1988; Raven et al. 1999; Hoffman et al. 2014). The process is both energetically and nutrient expensive. The Mo content of purified nitrogenase from *Anabaena cylindrica* is 2 atoms per enzyme complex (and per 20 Fe atoms) with a specific activity of 1.2 mmol C_2H_2 mg $NifDK^{-1}$ min^{-1} (Hallenbeck et al. 1979). The specific activity of MoFe-nitrogenase for N_2 reduction is 1.5 times that of VFe-nitrogenase at 30 °C and it is at least this much more efficient than Fe-nitrogenase. This difference in specificity of the known nitrogenase metalloenzyme systems has been used to explain the prevalence of MoFe-nitrogenase relative to other forms (see references in Anbar and Knoll 2002; Hoffman et al. 2014).

Nitrate reductase, used ubiquitously by all organisms in N metabolism for reducing nitrate to nitrite, also has a Mo metallo-center (Mikami and Ida 1984). Cyanobacterial nitrate reductase (NArB) contains only one Mo atom and has a specific activity of ~300 μmol NO_2^- mg^{-1} NarB protein min^{-1} in *Plectonema boryanum* (= *Leptolyngbya boryana*) (Mikami and Ida 1984). In terms of N assimilation, NarB is 37 times more efficient per mol N than NifD (nitrogenase in *Nostoc* sp.). This indicates that significantly less cellular Mo is required to support NO_3^- assimilation than N_2 fixation, consistent with calculations by Raven (1988).

Early studies showed that pigment content and nitrogenase activity was found to decline after 7–10 days of growth in Mo-deficient media for *Anabaena cylindrica* (Fay and Vasconcelos 1974; Jacob and Lind 1977) but these physiological changes could be reversed by Mo resupply to the growth media of *Anabaena oscillarioides* (Ter Steeg et al. 1986). Intracellular Mo and Fe concentrations concurrently increase with nitrogenase activity (Tuit et al. 2004), reflecting the greater need for these micronutrients when N_2 fixation is taking place in cyanobacteria. In general, N_2-fixation requires 10 nmol Mo L^{-1} in the medium for growth of heterocystous cyanobacteria such as *Anabaena cylindrica* (Jacob and Lind 1977), *Trichormus* (*Anabaena*) *variabilis* (Zerkle et al. 2006), and both freshwater and coastal strains of *Nostoc* sp. (Glass et al. 2010). Similar findings were found by Quigg et al. (2011) for the growth of a variety of both marine and freshwater cyanobacteria.

Concentrations of Mo vary widely from <20 nmol L^{-1} in freshwater, to 30–160 nmol L^{-1} in coastal areas, to ~105 nmol L^{-1} in the open ocean were its profile is dictated by salinity concentrations (see Collier 1985; Bruland et al. 1991; Glass et al. 2010; Sunda 2012). Howarth et al. (1988) hypothesized that the difference in Mo bioavailability in aquatic environments influences the expression and the activity of Mo-containing enzymes involved in N assimilation. These authors found low Mo concentrations associated with freshwaters could limit the function of Mo-based nitrogenase and nitrate reductase, slowing cyanobacterial growth due N-limitation. Sulfate has been found to inhibit Mo assimilation by microalgae (Raven 1988; Frausto da Silva and Williams 2001), making Mo less available in seawater than in freshwater despite its higher concentration because of analogue differences in sulfate distributions in these water masses. As a result, N assimilation may require greater energy expenditure in seawater than freshwater.

In a comparison of Mo requirements for N_2 fixation between freshwater and coastal strains of the heterocystous cyanobacterium *Nostoc* sp., Glass et al. (2010) set out to test the hypothesis that the aquatic environment plays a role in Mo requirements. In the coastal strain, 3 days were required for chlorophyll *a* concentrations to fall below those of corresponding Mo replete cultures but it was not until 12 days had elapsed that N_2 fixation rates began to decline. By comparison, 7 and 11 days, respectively, were required for N_2 fixation rates and chlorophyll levels to decline in Mo limited freshwater cultures. Contrary to their hypothesis, at <1 nmol Mo L^{-1}, N-limitation was induced (increased C:N ratios, decreased nitrogenase expression and activity). Further, when Mo in the medium was >1 μmol L^{-1}, the freshwater strain stored Mo (>100 μmol mol^{-1} Mo:C) but this did not occur the coastal strain. The high Mo content and extended time required for N_2 fixation to decrease in the freshwater strain was thought to be due to expression of the gene *mop*, which encodes a putative molybdate-storage protein, coded just upstream of the *nif* operon in freshwater heterocystous cyanobacteria (Markowitz et al. 2008). Studies have also investigated the role of Fe requirements for N_2 fixation (Berman-Frank et al. 2007). These fundamentally different biochemical and physiological strategies exhibited by strains of the same species found in different aquatic environments reflects both acclimation (phenotypic) and adaptation (genotypic) in response to micronutrient acquisition and utilization. Similar studies are required to understand such strategies for other micronutrients and in other microalgae.

5 Microelements Required by Some Microalgae

5.1 Cobalt

Co is known to be required in vitamin B_{12} (metallo-centre) in flagellates such as *Euglena* (Hutner et al. 1949) or in the inorganic form by some cyanobacteria (Holm-Hansen et al. 1954) and in a variety of other microalgae (see references in O'Kelley 1974). These early studies revealed that in DNA synthesis, vitamin B_{12} plays an important role in the reduction of ribotides to deoxyribotides, and in the synthesis of thymine, through the conversion of glutamate to beta-methyl aspartate (see summary of Provasoli and Carlucci 1974). Cyanobacteria such as *Synechococcus bacillaris* (= *Cyanobium bacillare*) have an absolute Co requirement (Sunda and Huntsman 1995a; Saito et al. 2002). Eukaryotic cells however do not, this maybe because they cannot make their own vitamin B_{12} but are dependent on bacteria for this and other vitamins (Frausto da Silva and Williams 2001).

The coccolithophore *Emiliania huxleyi* has a Co requirement that can be partly met by Zn while the diatoms *Thalassiosira pseudonana* and *Thalassiosira oceanica* have Zn requirements that can be largely met by Co and/or Cd (Sunda and Huntsman 1995a). Eukaryotes, particularly diatoms, are able to switch between these micronutrients in the enzyme carbonic anhydrase. Associated with this replacement, there was a 700-fold increase in cellular Co uptake rates with decreasing Zn ion concentration, indicating that Zn will have a major influence on biological scavenging of Co.

While it has long been known that Zn is typically the metallo-center for alkaline phosphatase, recent studies suggest Co may substitute under a range of conditions (Wojciechowski et al. 2002; Gong et al. 2005; Jakuba et al. 2008). Further, the Zn, Co, and Cd contents of cells appear to increase under low inorganic phosphorus availability (Ji and Sherrell 2008; Twining et al. 2010), implying a potential for Cd substitution in alkaline phosphatase, but there is as yet no evidence for this taking place.

5.2 Vanadium

Early studies revealed a requirement for vanadium (V) in a green alga (*Scenedesmus obliquus*; Arnon and Wessel 1953) and the N_2 fixing cyanobacterium, *Anabaena cylindrica* (Allen and Arnon 1955). *Anabaena cylindrica* was also found to require Ca^{2+} in both the presence and absence of a nitrogen source, strontium could not replace Ca^{2+} and Mo could not replace V (Allen and Arnon 1955). Some freshwater diazotrophs have one or two nitrogenase isoforms with V or Fe taking the place of Mo (e.g., Raven 1988; Thiel 1993; Raven et al. 1999; Boison et al. 2006). V is thought to stimulate CO_2 uptake in photosynthesis by serving as a catalyst for CO_2 reduction (Warburg et al. 1955). The requirement for V was ×1,000 higher than for Mo in the green alga (*Acutodesmus* (*Scenedesmus*) *obliquus*; Arnon and Wessel 1953).

Vanadium enzymes which catalyze peroxidative halogenation reactions (haloperoxidases) are found in virtually all classes of marine algae (Butler 1998); these are thought to be responsible for the vast array of halogenated marine natural products (Lee and Morel 1995; Yee and Morel 1996). On a global level, algal blooms produce massive quantities of volatile chlorinated and brominated hydrocarbons; the significance of these halogenated compounds is not yet entirely understood and requires further investigation.

5.3 Nickel

Ni is present in total concentrations of 2–12 nmol L^{-1} in open ocean surface waters (Bruland 1980) and amol (10^{-18}) or lower concentrations in microalgae (see references in Quigg 2008; Quigg et al. 2011). It is often below detection limits in microalgal biomass and requires concentrating cells before analysis, especially in cells grown on nitrate. Little is known about the precise catalytic activity of Ni in biology. Its redox functions are replaceable by Fe, Cu or Mn. An example of this would be in the enzyme superoxide dismutase which has a Ni metallo-center in cyanobacteria but typically Fe or Mn in eukaryotes (Table 1; Frausto da Silva and Williams 2001; Morel et al. 2003). Most work has focused on investigating the role of Ni as the cofactor for the enzyme urease which catalyzes the hydrolysis of urea to ammonium (Frausto da Silva and Williams 2001; Dyhrman and Anderson 2003). Microalgae which can take advantage of this N-source are thought to have a competitive advantage over those which cannot. More recently, it has been speculated that Ni scarcity limits urea utilization in the marine environment as a result of organic complexation and slow uptake kinetics (see references in Dupont et al. 2007).

6 Microelements Which Are Known to Be Replaceable in Some but Not All Microalgae

Given that photosynthetic pathways are key to survival, micronutrient stress in microalgae (limitations as well as excess, synergistic as well as antagonistic behaviors) to meet the demands for proteins with metallo-centers has been addressed using a variety biochemically and physiologically sophisticated strategies. Here we will focus on the enzyme carbonic anhydrase as is studied in this respect. For observations related to the roles and functions of Fe in the photosyn-

thetic process and other activities, see details in Larkum (2016). One example which considers Fe/Cu is: in low Fe waters, microalgae may substitute the Cu-containing enzyme plastocyanin for the Fe-containing enzyme cytochrome c_6 (Hill et al. 1996; Raven et al. 1999; Peers and Price 2006). As a result, low-Fe conditions may increase Cu requirements in microalgae. Another example is the strategy which involves substitution of the metal-free protein flavodoxin under conditions of low Fe for the Fe-S protein ferredoxin in photosystem I (LaRoche et al. 1996; McKay et al. 1999). Further, some microalgae are able to reduce the abundance of Fe-rich photosystem I relative to photosystem II under conditions of Fe limitation (Strzepek and Harrison 2004).

Co, Cd and Zn are known to functionally substitute for each other in the enzyme carbonic anhydrase of the marine diatom *Conticribra* (*Thalassiosira*) *weissflogii* to maintain optimal growth under Zn limitation (e.g., Lane et al. 2005; Xu and Morel 2013). In *Conticribra weissflogii*, Cd is exported as a phytochelatin complex (Lee et al. 1996) suggesting cells recognize this metal ion as a nutrient and/or as potentially toxic. Lee and Morel (1995) and Finkel et al. (2006, 2007) found biological uptake of Cd (as measured by Cd quotas in cells) in a majority of species tested. This is thought possible as Zn and Co have similar size/charge ratios and that Zn and Cd have similar chemical natures such that Zn and Co transporters will also mobilize Cd (Xu and Morel 2013). Many studies have also linked biogeochemical cycles of Cd and P through microalgae in surface waters, export into the deep sea and remineralization at depth (see Sunda and Huntsman 2000; Morel et al. 2003; Finkel et al. 2007). Sunda and Huntsman, (2005) determined from measured relationships between cellular Zn:C ratios and Zn concentrations and depth profiles for Zn and PO_4^{3-}, that Zn chelation in the nutricline of the North Pacific provide evidence that concentrations of this micronutrient are controlled by biological uptake and regeneration. A similar relationship to Zn but for Cu was reported in Sunda and Huntsman (1995b). Despite Cd having a nutrient-like distribution profile in the ocean, that is, depleted at the surface and remineralized at depth, and being complexed by organic ligands (Bruland 1980; Xu and Morel 2013), present findings do not prove there is ubiquitous biological utilization of Cd.

7 Uptake, Homeostasis, Toxicity and Efflux of Micronutrients

The acquisition and retention of micronutrients in microalgae is under the influence of internal (physiological) and external (physio-chemical) factors as well as by the size and nature of the cell (Hudson and Morel 1993; Hudson 1998; Raven et al. 1999; Pinheiro and van Leeuwen 2001; Franklin et al. 2002; Morel and Price 2003; Wilkinson and Buffle 2004; Sunda 1994; Finkel et al. 2006, 2007). If available, microalgae may concentrate micronutrients up to 10^3–10^5 higher than in their environment. This process, known as *bioaccumulation*, concerns essential micronutrients such as Fe, Cu, Se, and Zn, but also toxic elements such as Cd or Hg (see also Quigg 2008). Physico-chemical factors (e.g., pH, salinity, temperature, light, and particulate and organic matter concentrations) influence their accumulation inside cells (*bioconcentration*).

Major pathways for the uptake of micronutrients by microalgae are described in excellent reviews such as those by Raven et al. (1999), Worms et al. (2006), Glass et al. (2009), and Sunda (1994, 2012). Most recent studies have focused attention on the biological (transport across membrane), physical (diffusion) and chemical (dissociation kinetics of metal complexes) reactions occurring immediately on biological surfaces. Major questions still to be addressed were summarized by Worms et al. (2006) as: what is the behavior of micronutrients species during their transport from the bulk solution (i.e. >few microns from the biological surface) to the biological interface? How does transfer of the chemical occur across the biological membrane? And, what is the role of the organism in modifying the chemistry and biology of the uptake process? These questions are not entirely new (see e.g. Hudson and Morel 1993; Hudson 1998; and others) but in some cases reflects that a lack of understanding of some of these process still requires concurrent technological developments which are only now emerging.

7.1 Uptake of Micronutrients

Briefly, unlike macronutrients, the key to determining the concentrations of micronutrients which are available to microalgae for growth and protein function, and the uptake mechanisms themselves, is determining the *bioavailable* concentration of a specific micronutrient, denoted commonly as M′ and not simply its total concentration in the medium/environment (Sunda and Guillard 1976; Anderson et al. 1978; Morel et al. 2003; Worms et al. 2006). Rates of micronutrient uptake from solution have subsequently been found to be directly proportional to M′, which in turn, is highly dependent on the redox potential of the element in the environment (directly or indirectly powered in part by photochemistry) and the presence and type of chelator and/or complexation process(es) (Morel and Hudson 1985; Hudson and Morel 1993; Sunda and Huntsman 1992, 1995a, b, 2000; Hudson 1998). It is now known that chemical speciation must be taken into account when predicting bioavailability of micronutrients to microalgae.

Over the past 25 years, equilibrium models including the free ion activity model (FIAM) and the biotic ligand model

(BLM) have used extensively to describe micronutrient bioavailability in environmental systems (e.g., Sunda and Guillard 1976; Sunda 1994; Campbell 1995; Campbell et al. 2002). Synthetic chelators such as EDTA have been used extensively to control M′ in laboratory experiments. With knowledge of complexation constants, the free metal ion concentration in a media can be determined (Hudson and Morel 1993; Morel and Hudson 1985; Hudson 1998). FIAM and BLM predict the role of chemical speciation on micronutrient bioavailability. In this respect, they are often (qualitatively) successful in predicting a reduction in micronutrient effects due to complexation (inorganic and organic ligands), competition or other reactions which may reduce micronutrient bioaccumulation; these processes result in a decreased interaction of the micronutrient with uptake sites on the surface of the cells. Yet environmental systems are rarely at equilibrium. These models and the challenges have been recently reviewed by Morel et al. (2003) and Worms et al. (2006) and the reader is referred to these for details.

Arguably, it has been more challenging, and ongoing efforts have been directed towards understanding the types, roles and activities of the naturally occurring complexing agents. As with the synthetic chelators, the availability and uptake of micronutrients is controlled by complexation with organic ligands. These phenomena are currently best studied for Fe (see chapter "Iron", Marchetti and Maldonado 2016) but less so for other micronutrients. Organic ligands acting as chelators can release micronutrient ions into the medium while internalizing others into cells. Siderophores (low molecular weight compounds) are a type of natural chelator produced by bacteria and cyanobacteria that are thought to aid in the assimilation of otherwise difficult to obtain micronutrients, especially Fe (see e.g., Butler 1998).

In response to recently upwelled waters, bacteria and microalgae produce specific chelators to reduce concentrations of Cu ions in their immediate surroundings, thereby diminishing their immediate potential toxicity (Moffett and Brand 1996; Croot et al. 2000, 2003; Quigg et al. 2006). For more on the important role of ligands in controlling micronutrients in the natural environment, see reviews by Vraspir and Butler (2009) and Sunda (2012).

7.2 Internalization of Micronutrients

The vast majority of micronutrients are hydrophilic and their transport through biological membranes is mediated by specific proteins. In some cases, a single transport site may be used by several micronutrients or several transport sites may be used by a single micronutrient. If micronutrient internalization occurs via anionic transporters (Campbell et al. 2002), they essentially are "piggy-backed" across transporters meant for low molecular weight ligands such as citrate, thiosulfate or phosphate (e.g., Errecalde and Campbell 2000; Fortin and Campbell 2001). Such transport is dependent on micronutrient speciation and cell nutrition. Other highly specific ligands such as siderophores may be produced by the cell specifically to facilitate the transport of essential cations that are present at low concentrations in the environment (see above). This further complicates the prediction of uptake or effects when using simple chemical models such as FIAM or BLM.

The majority of micronutrients are thought to be transported as the free ion. While transport sites often demonstrate a high affinity for required micronutrients, they are not always highly selectivity. Toxicity may occur when a potentially toxic micronutrient binds to the site of an essential micronutrient with a similar ionic radius or coordination geometry. Calcium has been shown to reduce the internalization of Cd and Zn (e.g., Kola and Wilkinson 2005; Heijerick et al. 2002) and Cd has been shown to replace Zn or Co (e.g., Lane et al. 2005) while Mn and Zn directly compete for the same transporters in some diatoms (Sunda and Huntsman 1992, 1998a, b, c, d).

If microalgae are adapted to low micronutrient concentrations, especially open ocean species, micronutrient uptake can be enhanced by increasing the production of transporters (e.g. Mn; *Thalassiosira pseudonana*; Sunda and Huntsman 1998b, c) or by increasing the affinity of the transporters (e.g. Zn; *T. pseudonana*; Sunda and Huntsman 1992). Internalization has also been linked to low and high affinity transporters (e.g. Sunda and Huntsman 1992; for Zn). As a result, maximum internalization fluxes will be sufficiently different (most often by several orders of magnitude) so that these two uptake mechanisms can be distinguished as two distinct and saturable processes (see e.g. Sunda and Huntsman 1998a; Sunda 1994, 2012). In a direct comparison of Mn′ classical saturation kinetics for an oceanic species and an estuarine species, Sunda and Huntsman (2008) found the half-saturation constant[2] K_s of Mn uptake in *Thalassiosira oceanica* was one-seventh of that for *T. pseudonana*. V_{max} appeared to be under negative feedback control in both species. Six- to 12-fold variations in this parameter allowed cells to regulate intracellular Mn at nearly constant values. While Mn′ and V_{max} varied in relation to each other, K_s did not. They found that the range in Mn′ concentrations over which regulation occurred was different for the two species, and coincided with the Mn′ concentrations of the natural habitat of each species.

For micronutrients which are both essential but can be toxic if over accumulated, microalgae have developed at set of processes to manage their utilization whilst preventing harmful consequences. Cu is such a micronutrient. Studies of Cu accumulation have revealed that Cu ions bind to

[2]See Harrison et al. (1989) for definition.

specialized transport ligands associated with the cell membrane. Cu uptake is light- and ATP-dependent (Verma and Singh 1990, 1991) as well as temperature dependent (Hill et al. 1996), and follows Michaelis–Menten kinetics for facilitated and active transport (Sunda and Huntsman 1995b; Hill et al. 1996). The Cu transport rate is also known to be determined by the activity of the free metal ion in the medium (FIAM model applies) and not the total complexed Cu concentration (Sunda 1994; Van Leeuwen 1999). Knauer et al. (1997) found that Cu uptake is mediated by two systems in the freshwater chlorophyte *Desmodesmus* (*Scenedesmus*) *subspicatus*: a high-affinity and a low affinity system operating at Cu′ of $10^{-14–12}$ M and $10^{<12}$ M, respectively.

This management of micronutrient internalization can also result in species-specific uptake rates (e.g. for Cu). When comparing short-term (2–20 min) uptake rates in the laboratory, Quigg et al. (2006) found cellular net uptake of Cu was two to three orders of magnitude higher in the cyanobacterium *Synechococcus* sp., on the basis of carbon and surface-area–normalized Cu-accumulation rates (46 μmol Cu mol^{-1} C min^{-1} and 1,100 zmol Cu μm^{-2} min^{-1}) (zmol = 10^{-21} mol) relative to those measured in diatoms, chlorophytes, a dinoflagellate, and a coccolithophore. Cu-accumulation rates for *Conticribra* (*Thalassiosira*) *weissflogii* were found to be three times faster in natural seawater than in EDTA-buffered artificial seawater containing an inorganic Cu concentration of 28 pmol L^{-1}. Calculations showing that the diffusive flux of inorganic Cu was insufficient to account for observed short-term uptake rates suggest that some of the Cu bound to naturally occurring organic ligands is released through the rapid dissociation of those complexes in the cell's boundary layer.

7.3 Homeostasis of Micronutrients

Homeostasis (Cannon 1932) of element composition is one of the central concepts of ecological stoichiometry (see below for more) but while much has been done to understand C, N, P (Redfield 1934; Sterner and Elser 2002) and even Si, less is known about the controls on homeostasis for micronutrients in microalgae. Understanding implications of physiological homeostasis, how microalgae (and other organisms) regulate and maintain their fundamental biochemical functions despite continuous internal and external changes in their environment, is certainly a challenge for future generations. Increased anthropogenic activities since the start of the industrial revolution have resulted in environmental issues such as eutrophication, acidification, carbon dioxide increase, and climate warming. The ecological consequences of these can be measured by examining the response(s) of microalgae. Hence, the ability of microalgae to maintain themselves against recognized changes in environmental conditions depends on their ability to regulate internal conditions despite changes in external elemental supplies.

A general observation has been that the cellular concentrations of micronutrients vary sigmoidally with the concentration of the unchelated micronutrient (i.e., M′) in the medium of microalgae cultured with chelators such as EDTA or NTA (see FIAM model above) (reviewed in Sunda 1994). Evidence for this comes from Sunda and Huntsman (1992) who grew the diatoms *Conticribra* (*Thalassiosira*) *weissflogii*, *Thalassiosira pseudonana* and *Thalassiosira oceanica* and the coccolithophore *Emiliana huxleyi* across a range of Zn concentrations (10^{-11} to $10^{-8.5}$ M). Sunda and Huntsman (1992) found that all isolates exhibited similar sigmoidal relationships between cellular Zn:C ratios and Zn concentrations with minimal slopes at $10^{-10.5}$ to ~$10^{-9.5}$ M and increasing slopes above and below this range. The minimal slopes at intermediate Zn concentrations were explained by negative feedback regulation of a high-affinity Zn uptake system, while increased slopes at high Zn concentrations appeared to be related to uptake by a low-affinity site. By balancing micronutrient uptake and export rates, and all other conditions being optimal, these microalgae were able to sustain maximum growth rates.

While cellular concentrations may increase in direct proportion to free metal ion concentration for Zn, cells regulate concentrations of other micronutrients (e.g., Fe, Mn, Cu) much more tightly (e.g., see Marchetti and Maldonado 2016 for Fe, see Twining and Baines 2013; for other micronutrients). Sunda and Huntsman (1995b) did find a similar sigmoidal relationship between cellular Cu:C ratios and free cupric ion concentration for *Thalassiosira pseudonana*, *Thalassiosira oceanica* and *Emiliania huxleyi* grown in trace metal ion buffered media. Only five to ninefold variations in cellular Cu:C were measured despite 10^{-15} to 10^{-12} M Cu ions in the growth medium. There is clearly a strong internal economy for the use and re-use such that microalgal cells have a *homeostatic system* to maintain appropriate cellular concentrations of micronutrients (Sunda and Huntsman 1992; Sunda 1994).

When quotas increase above what is needed to sustain maximal growth, luxury uptake appears a strategy for storage of potentially important limiting micronutrients (e.g., for Fe, see chapter "Iron"; Marchetti and Maldonado 2016) while rapid efflux is used for potentially toxic micronutrients (see above for Cu). Little is still known about micronutrient storage in microalgae (Morel et al. 2003). Bioaccumulation and bioconcentration of micronutrients affects their ecology as a consequence of the energy and costs associated with excreting and/or detoxifying. If the costs are significant, then this may reduce growth, reproduction and/or competitive ability. This also influences which and how micronutrients may be biotransferred or biomagnified to higher trophic levels (see Quigg 2008).

7.4 Toxicity Caused by Some but Not All Micronutrients

Toxic effects may be observed when proteins (enzymes) are inactivated as a result of their interaction with micronutrients or due to a competitive interference on the uptake of one micronutrient by another or by the induction of an oxidative stress with subsequent cell damage (Sunda and Huntsman 1998a; Payne and Price 1999; Worms et al. 2006). Microalgae are known to use the following protective mechanisms: intracellular binding or sequestration by metal-complexing agents; compartmentalization and transport of micronutrient to subcellular compartments; efflux; and/or extracellular sequestration. Once complexed, toxic micronutrients may be stored in internal compartments such as vacuoles (Nagel et al. 1996; Cobbett 2000). The transport of chelated metal ion from the cytosol into the vacuole is mediated by transporter proteins.

In terms of intracellular binding or sequestration, microalgal strategies to preserve low intracellular concentrations of potentially toxic micronutrients include (i) biomethylation and transport through cell membranes of metal alkyl compounds by diffusion controlled processes, (ii) the biosynthesis of intracellular polymers that serve as traps for the removal of metal ions from solution, (iii) the sequestration of metal ions to cell surfaces, (iv) the precipitation of insoluble metal complexes (e.g., metal sulfides), and (v) metal exclusion from cells (Foster 1977; Frausto da Silva and Williams 2001; Whitfield 2001). While biomethylation appears to be limited to nonessential metals (e.g., HgII), the other strategies are pertinent to our understanding of Cu accumulation and homeostasis in algal cells (see Quigg et al. 2006).

Once inside cells, intracellular sequestration is an effective mechanism used by eukaryotic microalgae to reduce the bioavailability of toxic ions. Chelators produced include glutathione, amino acids, phytochelatins, metallothioneins, organic acids and thioredoxins (e.g., Ahner and Morel 1995; Mendez-Alvarez et al. 1999; Lemaire et al. 1999; Le Faucheur et al. 2005). Induction of these compounds depends on both the concentration and nature of the micronutrients present in excess (mainly Cd, Cu, Ni). For example, thioredoxins are small but ubiquitous proteins with highly reactive exposed disulfide sites that can be reduced to thiols under stressful conditions (Lemaire et al. 1999). Cd is known to be very effective at inducing the production of phytochelatins and other thiols in microalgae; they use this both as a mechanism to control Cd homeostasis but also as a detoxification mechanism (Payne and Price 1999; Xu and Morel 2013).

Two of the most extensive studies on toxicity, in terms of number of marine microalgae and micronutrients examined, were performed by Brand et al. (1983, 1986). In Brand et al. (1983), alterations to the growth rate of 21 species by a range of Zn′, Mn′ and Fe′ concentrations were measured using an EDTA-buffered medium. Coastal species tended to be limited by Zn′ below $10^{-11.5}$ M whereas Zn′ at 10^{-13} M did not limit all oceanic species. This gradient of Zn requirements is consistent with distributions of both species and micronutrient availability. Mn and Fe were not found to be toxic but ultra-low levels reduced growth even in oceanic species. We now know that the oceanic species of eukaryotic microalgae in the study by Brand et al. (1983) and Sunda and Huntsman (1992) (and others) likely could not be limited even at these very low Zn ion concentrations as Co and Cd in the media could alleviate Zn limitation.

The sensitivity of 20 species of marine microalgae to free cadmium ion activity was measured by Brand et al. (1986) using a NTA-cadmium ion buffer system that controlled free Cd ion activity. Prokaryotic cyanobacteria were the most sensitive to Cd toxicity, diatoms were the least sensitive, and coccolithophores and dinoflagellates were intermediate in their response. Cyanobacteria were killed by Cd′ of $10^{-9.3}$ M whereas reproduction rates of most of the eukaryotic algae were not reduced significantly until $10^{-8.3}$ Payne and Price (1999), Sunda and Huntsman (1998d, 2000), and Finkel et al. (2007) found similar patterns, suggesting an apparent metabolic demand for Cd in diatoms, no functional response in Cd:P ratios of prasinophyte and dinoflagellate species, and a metabolic sensitivity to Cd by cyanobacteria. Several potential physiological mechanisms have been proposed to explain these phylogenetic differences in Cd:P regulation (see Finkel et al. 2007). For example, there are taxonomic differences in elemental transport systems and their specificity for Cd versus other elements in response to environmental conditions in microalgae. Or, it may be more energetically costly to restrict and select entry of different ions than induce efflux, which would require a direct input of ATP. Finkel et al. (2007) added a new hypothesis, that the fundamental and striking differences in the regulation of Cd:P in cyanobacteria versus eukaryotes, especially the diatoms, may reflect the active selection of different biochemical regulatory networks in response to the changing availability of elements over Earth's history (Ho et al. 2003; Quigg et al. 2003a, 2011; Falkowski et al. 2004).

Cu toxicity (measured as a decrease in growth rate) of 38 marine microalgae, measured using a NTA-cupric ion buffer system that controlled free cupric ion activity, was found to be dependent on the phylogenetic origin of the species (Brand et al. 1986). On a smaller subset of microalgae, Croot et al. (2000, 2003) and Quigg et al. (2006) found the response to Cu could be related to the phylogenetic origins of the species investigated. Cyanobacteria were typically found to be the most sensitive (with reproduction inhibited at Cu′ of 10^{-12} M), diatoms the least sensitive, with coccolithophores and dinoflagellates having an intermediate sensitivity. *Emiliania huxleyi*, *Skeletonema costatum*, *Thalassiosira pseudonana* and *Thalassiosira oceanica* were most resistant

to Cu, with growth continuing at the highest Cu′ tested ($10^{-9.5}$ M and $10^{-9.2}$ M). Brand et al. (1986) did not find differences between coastal and oceanic species in their terms of their sensitivity to Cu; more recent studies concur with this finding. It appears that Cu′ influences seasonal succession of species, particularly in regions of upwelling (Moffett and Brand 1996; Moffett and Dupont 2007), by species specific toxicity, which can be ameliorated by the production of Cu complexing ligands that reduce Cu′ extracellularly.

7.5 Efflux of Micronutrients

The effects of excess micronutrients in the cytosol may also be reduced by efflux. In addition to decreasing toxicity, efflux reduces the net micronutrient flux and may modify the chemical speciation of the micronutrient through the expulsion of complexes. Microalgae can also excrete compounds that complex with micronutrients in the extracellular medium in order to reduce their bioavailability and therefore, entry to microalgal cells (see references in Chen et al. 2011; Quigg et al. 2013). These complexing agents include polysaccharides, proteins, peptides and small organic acids that are able to decrease the concentration of bioavailable micronutrients in the immediate vicinity of the cell. In marine systems, Cu, Cd and Zn tend to be strongly complexed by exudates produced by microalgae. See reviews by Worms et al. (2006) and Quigg et al. (2013) for more details.

8 Relationships among Individual Micronutrients Used by Microalgae

There is a developing understanding of antagonistic and/or synergistic relationships among micronutrients in microalgae and in aquatic environments. How different microalgae respond to and interact with bioavailable micronutrients (M′) in various combinations is still poorly understood. What we do know is that the cellular concentration of one micronutrient can strongly influence or be influenced by others via competition for transport through transmembrane channels, coordination chemistry, or charge (Bruland et al. 1991; Hudson and Morel 1993; Sunda and Huntsman 1998a, b, c, d; Morel et al. 2003; Worms et al. 2006).

An example of this behavior comes from examining the response of microalgae to different concentrations of the micronutrients Zn, Co and Cd. This provides a good example of what is called "biochemical substitution" (Morel et al. 2003). If all three micronutrients are at bioavailable concentrations, Zn is used preferentially (Sunda and Huntsman 1995a, 2000). However, when Zn′ is depleted but Co is available, the coccolithophore *Emiliania huxleyi* is able to utilize the Co to reach their maximum growth rates (Sunda and Huntsman 1995a). If Zn′ is increased from 1 to 25 pM, then Co quotas decrease twofold in *Emiliania huxleyi* or if Zn′ is increased from 10 to 100 pM, the Cd quota of *Conticribra* (*Thalassiosira*) *weissflogii* is decreased fivefold (Sunda and Huntsman 1995a, 2000). In another study, high levels of Cu and Zn were shown to antagonize Mn nutrition by inhibiting cellular Mn uptake in the diatom *Thalassiosira pseudonana* (Sunda and Huntsman 1998c). Cu and Zn inhibition of growth rate occurs only at low Mn′ (independent of the irradiance used for growth). Studies with coastal diatoms have shown that algal Cd concentrations are not only controlled by aqueous Cd ion concentrations but are also inversely related to Mn and Zn ion concentrations (Lee and Morel 1995; Sunda and Huntsman 1996, 1998d, 2000). Hence, a second example comes from investigating the antagonistic relationships between the micronutrient Mn and Zn and Cd – while Zn can be a micronutrient, it is also known to be toxic at elevated concentrations whereas Cd is generally consider to be toxic and not a micronutrient by Sunda and Huntsman (1996). Zn and Cd where found to inhibit Mn uptake by interfering with intracellular feedback regulation of Mn uptake capacity and by competitively blocking Mn binding to membrane uptake sites. Further, Cd was found to be transported into the cell via the Mn uptake system, an effect Sunda and Huntsman (1996) found to be accentuated at low Mn free ion concentrations by negative feedback which increases uptake capacity and by lowering Mn competition for binding to the uptake sites. The net result of these two effects was higher intracellular Cd:Mn ratios at low Mn free ion concentrations which exacerbates Mn limitation due to competitive binding of Cd to Mn nutritional sites. To alleviate such problems in the natural environment, phytochelatins and efflux mechanisms are activated. If these processes do not work, Mn limitation may occur. Enhanced cellular Cd concentrations at low ionic Mn lead to reduced biodilution rates associated with Mn limitation of growth rate.

In some situations, toxicity is observed even for micronutrients which are typically consider primarily nutritive in nature. Mn can be inhibitory or toxic in excess amounts to cyanobacteria (*Microcystis* sp.; Velichko, 1968 in O'Kelley 1974) and a negative fertility factor for diatoms (*Ditylum brightwellii*; Steele 1965). In this situation, what typically occurs is that the concentration of the micronutrient becomes elevated beyond the homeostatic range required by a microalgal cell. In this situation, the microalgae will accumulate the micronutrient uncontrollably by simple diffusion (usually electrically uncharged complexes) through membranes or by leakage through the transport system of another micronutrient (Morel et al. 2003). Sunda and Huntsman (1998b) found that Zn enters diatoms cells through Mn transport systems, if present in elevated concentrations. This is an example of "competitive inhibition" (Morel et al. 2003). Zn will then become toxic to the cell and a corresponding decrease in

growth rate is observed. This in turn results in an even faster rate of accumulation (i.e., the micronutrient quota is no longer being diluted by growth) so that growth stops abruptly as M′ increases above a critical threshold in the growth medium (Morel et al. 2003). In the studies by Brand et al. (1983, 1986), authors examined this phenomenon in a range of microalgae with different phylogenetic origins and with a range of Zn′, Mn′, Fe′, Cu′ and Cd′ in buffered media. They were able to define the "tipping point", i.e., the concentration where these micronutrients switched from being nutritional to toxic; further studies are required to elucidate the molecular mechanisms in most microalgae.

9 Importance of Light

Changing the irradiance for growth changes the cellular concentration of elements required for light harvesting and photosynthesis: while N, Fe, and to a lesser extent, Mn increase with decreasing irradiance in many species (Raven 1988, 1990; Sunda and Huntsman 1997; 1998c; Raven et al. 1999; Morel et al. 2003) the opposite pattern has been observed for Zn in the coastal diatom *Thalassiosira pseudonana* (Sunda and Huntsman 2004, 2005). The increased need for cellular Fe:P and Mn:P at low irradiances is triggered by requirements for these elements in the photosynthetic reaction centers, photosynthetic electron transport, and the water splitting in the oxygen evolving complex of photosystem II. This is described in great detail in Raven (1988, 1990) and Raven et al. (1999). When microalgae are photoacclimated to extremely low irradiances, they have a larger ratio of light-harvesting pigments to thylakoid protein complexes, that is, an increase in photosynthetic unit size. This requirement for the photosynthetic pathways is so significant that when the irradiance for the growth of the dinoflagellate *Prorocentrum minimum* (= *P. cordatum*) was increased from 50 to 500 µmol quanta m^{-2} s^{-1}, the Fe quota decreased by a factor of 2 (Sunda and Huntsman 1997). In contrast, a tenfold rise in irradiance increased the Zn requirements in *Thalassiosira pseudonana*. Sunda and Huntsman (2005) associated this with a 2.8-fold increase in the maximum specific growth rate, and resultant increased demand for carbonic anhydrase and other biosynthetic Zn enzymes. Miao and Wang (2004) also observed increases in the uptake of Cd and Zn in the coastal diatom *Thalassiosira pseudonana* with increases in irradiance. Cellular micronutrient concentrations in general are directly related to the uptake rate and inversely related to the specific growth rate. Consequently, the reduced growth rate under low irradiances allow cells to accumulate the increased Fe, Mn and other elements needed for synthesis of additional photosynthetic units.

On the basis of the changing elemental composition of five species (*Cyanothece* sp., diazotroph; *Pycnococcus provasolii*, prasinophyte; *Amphidinium carterae*, dinoflagellate; *Conticribra* (*Thalassiosira*) *weissflogii*, diatom; *Chaetoceros calcitrans*, diatom) examined when growing a five light intensities from 15 to 500 µmol quanta m^{-2} s^{-1}, Finkel et al. (2006) concluded that phenotypic responses to light were responsible for less than one to about three orders of magnitude in micronutrient:P ratios within species, comparable to the phylogenetic differences observed in Quigg et al. (2003a). Finkel et al. (2006) found that many of the micronutrients were present in highest concentrations at very low irradiance (<30 µmol quanta m^{-2} s^{-1}): Fe:P, Mn:P, Zn:P, Cu:P Co:P, Mo:P, and Ni:P. However, under saturating irradiances, and in a few species, micronutrients were enriched relative to P. This included Fe, Mn, and Zn in the coastal diatom *C. weissflogii*, and Co in the diazotroph *Cyanothece* sp. and the prasinophyte, *Pycnococcus provasolii*. Further, they found evidence of storage of cellular micronutrients under 50 µmol m^{-2} s^{-1}. These authors found a facultative increase in micronutrients not related to photosynthesis. There is little known about micronutrient storage (aside from perhaps Fe). Finkel et al. (2006) hypothesized that dilution of cellular micronutrients could occur in response to the release of cells from light limitation, allowing microalgal growth to increase rapidly. This warrants further investigation as we need to better understand how microalgae modulate micronutrients for metabolic requirements.

Micronutrients interact with light within the euphotic zone, thereby controlling algal growth rates (see Sunda and Huntsman 1998a, c; Finkel et al. 2006). This occurs as a result of several processes. Complexation is known to be a highly light dependent process (see Worms et al. 2006; Sunda 1994, 2012). Given microalgae growing at very low irradiances (growth-limiting) have higher concentrations of Mn and chlorophyll *a*, needed for the synthesis of additional photosynthetic units (Raven 1990; Raven et al. 1999), there is a concurrent need for more Mn. Sunda and Huntsman (1998c) found this additional Mn was not provided by higher cellular Mn uptake rates, since these rates were found to be the same in light-saturated and light-limited cells of *Thalassiosira pseudonana*. Rather, the steady-state cell Mn was equal to the uptake rate divided by the specific growth rate such that cells accumulated the additional Mn needed for photoacclimation once light limitation caused growth rate to decline, an inherent negative feedback relationship. In addition, the fractionation of micronutrients relative to phosphorus into microalgal biomass under low light is consistent with depleted levels of Cu^{2+} and Mn^{2+} in deep chlorophyll maxima (Finkel et al. 2006), suggesting that the export of low light-acclimated microalgae is a major source of trace element flux to the deep ocean and an important factor in the biogeochemical cycles of many of the biologically limiting elements in the oceans.

Also to be considered is the diel photoperiod which regulates daily rates of carbon-fixation, growth, and cell division.

Because photosynthesis occurs only during the day, the growth and composition of cells must vary over the diel cycle. While this is well documented for cell carbon, nitrogen, carbohydrate, protein, chlorophyll *a* in cultured and natural microalgal populations, very few studies have investigated changes on micronutrients. One such study is that of Sunda and Huntsman (2004) in which they examined variations in cellular Fe and Zn in the diatom *Thalassiosira pseudonana*. Cellular concentrations of carbon, chlorophyll *a*, zinc, and iron varied during the light period in response to day–night differences in rates of C-fixation, chlorophyll *a* synthesis, growth, and metal uptake. Higher cellular Fe intracellular levels measured increased concurrently with rates of C-fixation thereby allowing the diatom cells to synthesis additional iron-rich proteins (e.g., those utilized in photosynthetic electron transport – See Raven et al. 1999). Cellular Zn concentrations decreased by 25 % during the light period because of higher daytime specific growth rates, which led to higher rates of biodilution. Mean chlorophyll *a* concentration decreased linearly with decreasing specific growth rate under Fe and Zn limitation, thereby allowing the cells to maintain a balance between light harvesting and biosynthesis. The diel cycle also influenced cellular Fe concentrations through day–night differences in iron chelation, linked to photochemical redox cycling. Differences between Fe and Zn can be accounted for by complexation, that is, for Zn this process this is unaffected by light, but for iron chelates photo-redox cycling increases the concentration of dissolved inorganic Fe(II) and Fe(III) species and thus increases algal iron uptake rates in the light (see e.g., Sunda and Huntsman 1995b).

10 Importance of CO_2

Given the current interest in global increases in atmospheric CO_2 and temperature and the associated with changes in ocean chemistry (not only acidification) and circulation, light and nutrient regimes, there are concurrent changes in the availability of macronutrients (reviewed by Finkel et al. 2010 and elsewhere in this book) and micronutrients for microalgae. The consequences include, but are not limited to, alterations in marine microalgal communities which in turn will affect primary and export production, food web dynamics and structure and biogeochemical cycling of carbon and nutrients in the oceans. While it is well known that carbonic anhydrase is required for the uptake and fixation of inorganic carbon, especially at low CO_2 concentrations at the cell surface (Morel et al. 1994; Raven et al. 1999; Beardall et al. 2009), microalgae are known to activate carbon concentrating mechanisms in order to increase CO_2 concentrations at the active site of RUBISCO (see chapter "Carbon Acquisition by Microalgae", Beardall and Raven 2016). Few studies to date have however investigated the relationships between changes in micronutrients and CO_2 concentrations.

Sunda and Huntsman (2005) found decreasing the CO_2 concentration (via an increase in pH from 8.2 to 9.0; CO_2 values not reported) in the culture medium for the diatom *Thalassiosira pseudonana* resulted in an increased cellular Zn requirement to achieve a given growth rate and also that needed to achieve maximum growth. This increase was linked to a greater demand for Zn in the enzyme carbonic anhydrase, which is needed for cellular CO_2 acquisition. The increased demand for cellular Zn in *T. pseudonana* was possibly because of a decrease in the daily specific growth rate, which increased cellular Zn concentrations by decreasing biodilution rates. The coastal diatom *Conticribra weissflogii* responds similarly to changes in CO_2 and Zn concentrations in its environment (Morel et al. 1994). Particulate Cd:P in field samples of microalgae have been shown to respond to changes in CO_2 (Cullen et al. 1999). In the study by Morel et al. (1994), a change in cellular demand for Cd (or Zn and Co) in carbonic anhydrase of diatoms was measured, the only taxonomic group with a demonstrated use for Cd, in response to the availability of inorganic carbon.

As a result of the high concentrations of carbonic anhydrase, the increased demand for cellular Zn needed for algal growth under conditions of low CO_2 availability may lead to CO_2 and zinc co-limitation in some oceanic environments (Morel et al. 1994). To test this hypothesis, Zn additions to bottle incubation experiments were performed in the different parts of the Pacific (see references in Sunda 2012). Consistently results showed a modest stimulation of algal growth (compared to Fe additions), but a correspondingly significant effect on microalgal species composition. The growth of the coccolithophore *Emiliania huxleyi* was preferentially stimulated over other species (Crawford et al. 2003). Sunda and Huntsman (1995a) found this alga has an unusually large cellular uptake and growth requirement for Zn/Co compared to the diatoms *Thalassiosira oceanica* and *Thalassiosira pseudonana*. Given *Emiliania huxleyi* and other coccolithophores are largely responsible for calcium carbonate formation and regulation of ocean water alkalinity, which in turn influences the air-sea exchange of CO_2, it was hypothesized that Zn (and possibly Co) could indirectly affect atmospheric CO_2 and global climate in oceans. Further studies are however required.

11 Emergent Concepts and Issues

11.1 Ecological Stoichiometry

One of the biggest ecological challenges is linking molecular biomarkers of pollution with ecologically relevant life history characteristics including growth, survival and reproduction

of aquatic organisms. The study of the balance of chemical elements in components, interactions, and processes in ecosystems is called ecological stoichiometry, which seeks to track the mass flow of elements from genes to ecosystems (Sterner and Elser 2002). For micronutrients and microalgae, this involves an examination of the bioaccumulation, biotransference and/or biomagnification of elements through food webs (see Quigg 2008; Bradshaw et al. 2012).

An implicit assumption associated with ecological stoichiometry is that the organism of interest can maintain homeostasis, that is, maintain constant "body" concentrations of elements despite changing concentrations in the environment and/or their resource supply (Sterner and Elser 2002). Stoichiometric homeostasis for a range of micronutrients has been shown in microalgae (see section above) such that a multi-elemental signature may be defined. Stoichiometrically, the most abundant micronutrient in microalgae is Fe, followed by Mn and Zn (Quigg et al. 2003a, 2011; Ho et al. 2003; Quigg 2008; Twining and Baines 2013). These are present in concentrations at least one to two orders of magnitude higher than those for the other essential elements (Cu, Co, Ni, Se, Mo), and several orders of magnitude higher than elements which are replaceable (e.g., V) or whose roles remain poorly defined (e.g. Cd). Differences in terms of cellular concentrations of Cu, Co, Ni, Se and Mo may be explained in terms of the evolutionary histories of microalgae (see above) and their corresponding cellular physiology and biochemistry, but in most cases, the known specialized roles for these elements do not readily explain their cellular concentrations or stoichiometric relationships with other nutrients – micro or macro – in microalgae (Quigg 2008).

Expanding the Redfield ratio ($C_{106}N_{16}P_1$) (Redfield 1934) to include micronutrients has the potential to improve our understanding of elemental cycling in ecosystems and food webs, homeostatic regulation of micronutrients, and consumer-resource mismatches. In essence, it will provide a mechanism to explore relationships between macro- and micro-nutrients from an ecological perspective. Early efforts to determine the multi-elemental composition of microalgae and expand the Redfield ratio began in the 1970s (e.g., Martin and Knauer 1973; Morel and Hudson 1985; Kuss and Kremling 1999; Ho et al. 2003) with ratios such as the following: $P_{1000}(Fe,Mn)_{10}(Zn, Cu, Ni, Cd)_1$ providing very basic information on the relatively proportions of micronutrients in microalgae. There are significant challenges to overcome to find a set of stoichiometries that may have the same kind of appeal as the Redfield ratio but new technologies and approaches may make that feasible in the future. Advances in genomics and proteomics will be key to characterizing microalgal genomes, and more specifically metalloproteomes, many of which remain largely uncharacterized at this time (see paper of Cvetkovic et al. 2010).

11.2 Micronutrients in Natural Populations of Microalgae

Analytical techniques to determine the micronutrient composition of natural populations, and more specifically, single cells in situ, have been developed in recent decades. At the population level (that is, bulk particulate matter), inductively coupled plasma mass spectrometry approaches (e.g., ICP-AES, ICP-SFMS) have enabled the simultaneous multi-elemental measurement of natural populations (e.g., Kumblad and Bradshaw 2008; Ho et al. 2010; Bradshaw et al. 2012). The major challenge with this approach however is subtracting interference of elements which maybe acting as scavengers on the organic material (e.g. Al, Pb) or be scavenged (e.g. Fe, Co), and/or the interference of lithogenic and detrital matter. Nonetheless, these studies compliment earlier findings such as those by Bruland (1980) and Bruland et al. (1991) for micronutrient profiles in seawater. Further, these studies have shown how the relationship between intra- and extra-cellular micronutrients change with depth – surface, mixed layer, below photic zone (e.g., Ho et al. 2010) or during an algal bloom (e.g., Bradshaw et al. 2012). The study by Bradshaw et al. (2012) also showed for the first time how high rates of incorporation of micronutrients (Co, Fe, Mn and Zn) to microalgae during a bloom can temporarily lower corresponding micronutrient concentrations in the surrounding seawater and thereby alter competition for these resources.

Synchrotron X-ray fluorescence (>6 keV) measures the concentrations of micronutrients such as Fe, Mn, Zn, Ni and P, at the single (nano- or pico-plankton) cell level. Studies using this approach (summarized in the review of Twining and Baines 2013) have revealed the localization and distribution of specific micronutrients in microalgal cells. Using this approach, Fe, Mn, Zn and Ni can be clearly seen to be localized in the chloroplasts of diatom and flagellate cells. Such studies also reveal regional (for temperate, equatorial, and Antarctic waters in the Pacific and Atlantic Oceans) variability in micronutrient concentrations, which have also been found to match trends in dissolved metal availability for micronutrients (e.g., Fe, Zn, Cu, Mn). An example from the review of Twining and Baines (2013) is that of Zn:P ratios – these were found to be differ for autotrophic flagellates depending on their presence south versus north of the polar front in the Southern Ocean; and in parallel with corresponding dissolved Zn concentrations in those waters.

One of the challenges with these new techniques is that although they can measure P concentrations simultaneously with micronutrients, they cannot do so for C or N. Hence, efforts at mass balance modeling cannot be directly based on element:C ratios or on carbon fluxes, unless additional assumptions are made. It is well understood that the Redfield ratio of C:N:P is affected by both biotic and abiotic factors

(Redfield 1934); hence model imbalances of micronutrient:P ratios will also be influenced by intra- and extra-cellular factors. Strategies to address this will be the focus of future studies.

11.3 Emergent Pollutants

Micronutrient concentrations occur in picomolar to nanomolar quantities in the aquatic environment. Enrichment of seawater may occur naturally through local environmental processes, such as volcanism or upwelling, but it may also occur as a result of human activities. Nanomaterials are diverse substances that are characterized by having one dimension that is <100 nm (Luoma 2008). They may be natural, incidental, or engineered and may be released through point and nonpoint sources, or introduced directly to the environment. Engineered nanoparticles are a class of nanomaterials with at least two dimensions between 1 and 100 nm (Nel et al. 2006; Luoma 2008); these are being used in a wide variety of applications including environmental remediation, pollution sensors, photovoltaics, medical imaging, and drug delivery. Of concern for microalgae (and other trophic levels) are nanoparticles formed from metal oxides (TiO_2, ZnO, CeO; natural and engineered), zero-valent metals (Fe, Ni, Au, engineered) and quantum dots such as those made with cores of cadmium selenide (CdSe), cadmium telluride (CdTe), and/or zinc selenide (ZnSe) (Nel et al. 2006; Klaine et al. 2008).

Research reveals that these emergent pollutants may cause toxicity to cells either directly (by becoming incorporated into the members or cellular space) or indirectly (by releasing chemical ions on and inside of cells) (reviewed in Navarro et al. 2008; Quigg et al. 2013). Silver engineered nanoparticles are the arguably the best studied nanomaterials to date in terms of their environmental impacts. It is known that with microalgae, it is less frequently the nanoparticles themselves causing toxicity and more frequently the released Ag ions that interact with cell membranes, proteins, DNA, RNA and inactivate vital cellular functions (e.g., Miao et al. 2009, 2010a). The cadmium used in quantum dots is known to have a similar effect on microalgae (e.g., Werlin et al. 2011; Zhang et al. 2012) as is the case for the zinc in metal oxides (e.g., Miao et al. 2010b; Ma et al. 2013). In a number of studies, it has been shown that microalgae have the ability to "protect themselves" from these potentially toxic materials by producing exopolymeric substances (see Chen et al. 2011; Quigg et al. 2013). Nonetheless, once inside cells, these materials have the potential to be bioaccumulated and/or biomagnified to higher trophic levels as described above for micronutrients. In a recent review, Hou et al. (2013) found that daphnia, fish, aquatic worms and earthworms are the most commonly studied ecological receptors of nanomaterials in freshwater systems. Hence, going forward, when studying the interactions of micronutrients and microalgae in the aquatic environment, an additional challenge will be the presence of these and other emergent pollutants which alter nutrient availability and/or may be toxic.

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Iron

Adrian Marchetti and Maria T. Maldonado

Preface

No other trace element has received more attention in relation to influences on microalgal physiology than iron (Fe). At the time of writing, a Web of Knowledge search using keywords "iron" and "phytoplankton" listed approximately 6800 references since the last edition of this textbook in 1974. To summarize all of these studies would be next to impossible. Rather, our goal in this chapter is to provide a comprehensive overview of key findings related to the acquisition and function of Fe within phytoplankton cells, emphasizing what we now know about how Fe is obtained, the important physiological roles of Fe and the ways in which microalgae have evolved to cope with widespread Fe limitation in aquatic environments. In addition, we have attempted to integrate a new wealth of information obtained through genomic approaches that are now common practice in physiological research with phytoplankton. Where possible, we provide reference to extensive literature reviews that will ultimately offer a more in-depth discussion on specific Fe-related topics. Although we have made efforts to include information on all phytoplankton functional groups, including some discussion of cyanobacteria, our expertise lie in diatom physiology, so there is undoubtedly a preferential focus on this group of microalgae. That being said, the vast majority of microalgal physiology studies within the scientific literature relating to transport and physiological adaptations to Fe limitation are focused on diatoms (and to some extent, freshwater green algae), as this group appears to be most affected by low Fe concentrations in the ocean and often exhibits the largest physiological response to both natural and anthropogenic Fe enrichments.

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1 Iron Sources and Distributions in Aquatic Environments

A review on Fe physiology of phytoplankton would not be complete without first describing the sources of Fe and how Fe is distributed throughout aquatic environments. Varying Fe concentrations in aquatic systems through space and time have largely shaped the evolutionary trajectories of many phytoplankton groups (Falkowski et al. 2004). Given that phytoplankton communities in an estimated 30–40 % of the oceans are chronically deprived of Fe (Moore et al. 2002), extensive efforts have been made to obtain high-resolution measurements of Fe concentrations in the sea. As such, we will primarily focus on Fe distributions in seawater, although there is growing evidence that Fe may also be an important regulator of phytoplankton growth in freshwater environments (Twiss et al. 2000; Sterner et al. 2004; Havens et al. 2012; Shaked and Lis 2012).

Iron exists in seawater in two oxidation states: soluble Fe (II) and sparingly soluble Fe (III), which has had a profound influence on iron concentrations in the ocean over geologic times. Since the first appearance of oxygen-evolving photosynthetic organisms ca. 3 billion years ago, alternating episodes of anoxic and oxic conditions in the oceans are evident throughout Earth's geological record with oxic conditions only prevailing since the start of the Phanerozoic era (~540 mya). Increased oxygen concentrations in seawater resulted in a massive decline in soluble Fe concentrations through the formation of insoluble Fe oxides that are rapidly precipitated and removed from the water column (Anbar and Knoll 2002). The end result is that Fe requirements in microalgae remain quite high due to evolutionary constraints relative to the supply, both of which being constrained by the chemistry of Fe. Dissolved Fe in contemporary oceans is typically present at concentrations of 0.02–1 nanomolar (10^{-9}) concentration range. In large areas of the ocean, complexation and speciation of this Fe results in bioavailable concentrations that are far below those needed to support

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maximum growth rates of many microalgal species (Wells et al. 1995) and have been influential in setting the maximum growth rates in many species (Sunda and Huntsman 1997, 2015).

Restricted availability of Fe to microalgae is largely a consequence of its complex chemistry. Iron is present in the ocean as inorganic soluble and insoluble chemical species, dissolved organic complexes, colloidal material, mineral particles and, of course, as components of living cells. The most stable form of Fe within oxygenated seawater is the oxidized ferric (Fe(III)) state. Dissolved forms of Fe(III) primarily exist as organic complexes with a very minimal amount existing as dissolved inorganic hydrolysis species (Rue and Bruland 1995). It is believed that these dissolved forms supply a significant proportion of the Fe flux to the cell. Ingestion of bacteria or colloidal (i.e. insoluble) forms may serve as other pathways for obtaining Fe for many groups of phytoplankton with the notable exception of diatoms, which unlike most microalgal groups are incapable of phagotrophy (Maranger et al. 1998; Nishioka and Takeda 2000). Most oceanic Fe-containing colloids are believed to consist of Fe in an organic matrix. Inorganic Fe oxyhydroxide mineral colloids appear to be rare in the open sea (Wells and Goldberg 1994). Indirectly, colloidal Fe may be accessible to phytoplankton by thermal, photochemical and possibly biological reduction (Hutchins 1995).

Iron inputs vary among regions and their proximity to land. Sources of Fe to marine euphotic zone waters include terrestrial run-off, atmospheric dust (dry deposition) and precipitation (wet deposition), upwelling of deep waters, and anthropogenic input. Such sources are crucial in determining Fe availability due to its high reactivity and low solubility (Johnson et al. 1997). Throughout the water column, Fe often exhibits a nutrient-like profile as it is significantly involved with the internal cycling of biologically derived particulate material, although in regions with high aeolian input Fe concentrations may be elevated at the surface (Bruland et al. 1994) (Fig. 1). More often, concentrations tend to be lowest in the euphotic zone due to rapid assimilation by phytoplankton and/or adsorption onto biogenic particles and then increase in the subsurface waters as sinking particles succumb to decomposition and dissolution. The residence time of Fe in seawater is relatively short (200–500 years) compared to other elements, eventually being removed from the water column through biological uptake and scavenging onto sinking particles (Bruland et al. 1994; Johnson et al. 1997).

Iron is the fourth most abundant element in the Earth's crust. Thus, the main inputs of new Fe to the ocean are from continental sources and, therefore, regions in close proximity to land or those that experience a high degree of upwelling of deep, nutrient rich waters tend to have higher Fe concentrations compared to open ocean regions that are far away from land (Fig. 1). Sources of Fe to surface waters vary depending on geographic location. Dominant inputs of Fe to coastal regions are river runoff and benthic inputs. Rivers are a primary source for most of the major ions in seawater, as well as trace metals such as Fe. Although Fe concentrations in rivers may be quite high, most of this Fe is likely removed by flocculation of strongly associated Fe-humic substances and may not make its way out of the estuary (Boyle et al. 1976). However, there are some measurements that suggest that riverine Fe inputs to the ocean can be quite high, constituting a significant portion of new Fe inputs to the adjacent open ocean (Wetz et al. 2006; Klunder et al. 2012).

Benthic inputs of Fe to the overlying water column are primarily from two different sources, the input of continental derived material that is released into the dissolved phase and biogenic material that is exported from the surface layer. For example, off the Oregon coast, the dominant form of Fe to surface waters is a combination of reduced Fe(II) from shelf sediments and sediment that is resuspended through upwelling (Johnson et al. 1999; Chase et al. 2007; Bruland et al. 2008; Lohan and Bruland 2008). The addition of dissolved Fe(II) to oxic seawater will quickly result in its oxidation to less-soluble Fe(III) with subsequent formation of colloidal and particular oxyhydroxide forms that are not readily biologically available. However the complexation of dissolved Fe(III) by strong Fe-binding ligands can maintain Fe(III) in the dissolved phase (see further details on siderophores below). Moore and Braucher (2008) modeled the global input of Fe from sediments and have shown the sedimentary sources along continental margins have a strong impact on open-ocean Fe concentrations, particularly in the Arctic and North Pacific and are equivalent to global estimates of Fe input from atmospheric dust (Fig. 1).

The primary source of Fe to surface waters in remote open ocean regions is through atmospheric deposition of dust from arid regions and anthropogenic emissions from the continents. Atmospheric inputs into the ocean are often spatially and temporally patchy with regions of particularly high atmospheric Fe input within the tropical and North Atlantic, Bay of Bengal, the Arabian Sea, and waters surrounding Australia and the southeastern continental margins of South America. Mineral aerosols consist of dust particles that are lifted into the atmosphere when high winds occur over arid erodible surfaces. These particles may be transported long distances before they are deposited into the ocean via settling, turbulent deposition and precipitation processes (Mahowald et al. 2005). The solubility of Fe in these various atmospheric forms depends on the source, with soluble Fe fractions ranging from 0.01 to 80 % (Mahowald et al. 2005).

Hydrothermal vents are considered another significant source of Fe to the ocean. In addition to emitting sulfur, high levels of trace metals, including Fe, are emitted by vents. Previously, vent-derived Fe was thought to be of limited importance due to rapid oxidation and precipitation around the vents. Yet it is now believed that hydrothermal vent

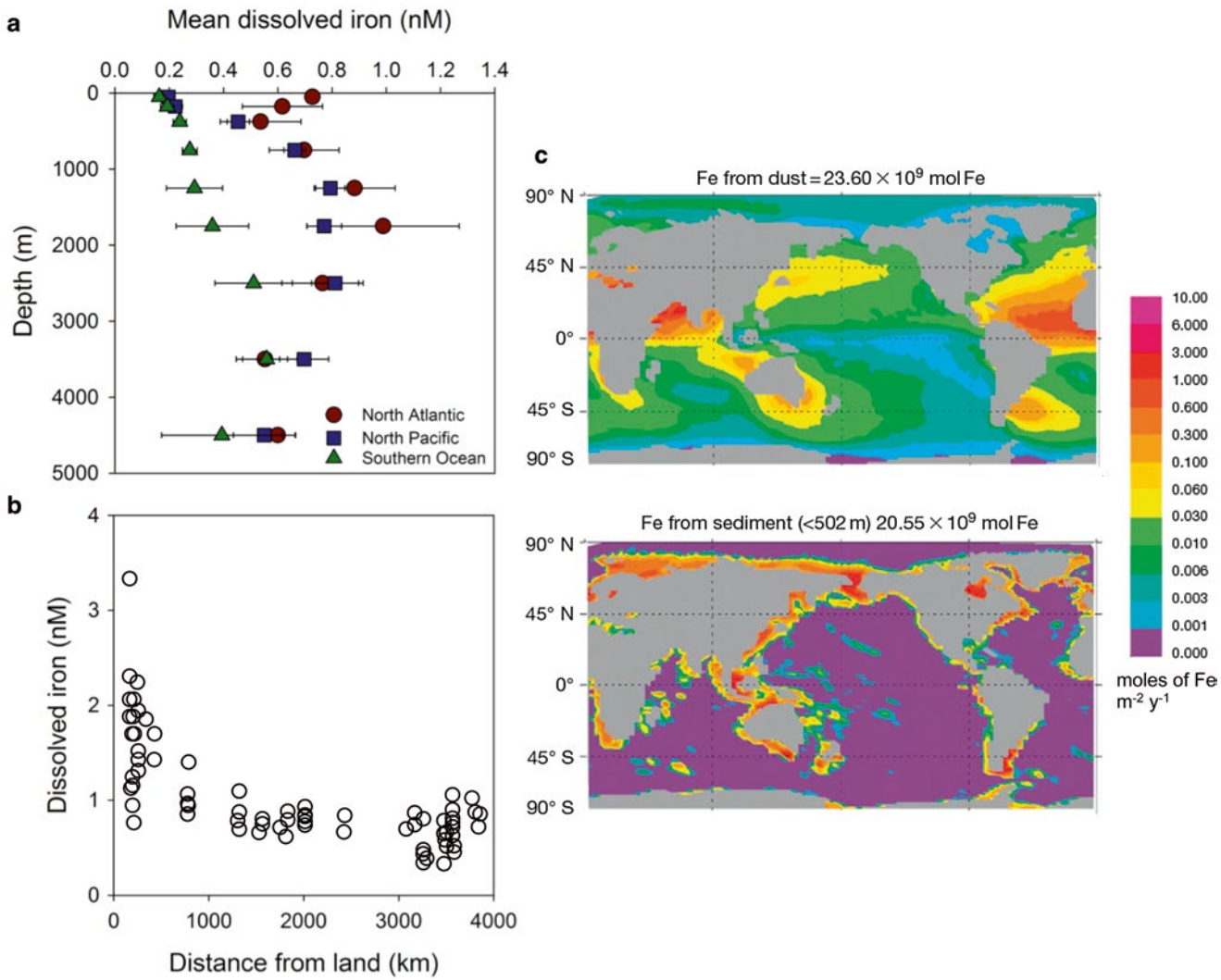

Fig. 1 Distributions of Fe in the world ocean. (**a**) Mean profiles of dissolved iron in the North Atlantic, North Pacific, and Southern Ocean averaged over the depth intervals: 0–100 m, 100–250 m, 250–500 m, 500–1000 m, 1000–1500 m, 1500–2000 m, 2000–3000 m, 3000–4000 m, and 4000–5000 m, (**b**) Observed Fe concentrations from depths >1000 m in the eastern subtropical Pacific Ocean (20–50°N) plotted as a function of distance from the continental land mass and (**c**) Modeled estimates of the two most important sources of Fe to the world ocean: mineral dust (24×10^9 mol Fe) and shallow (<502 m) sediments (21×10^9 mol Fe) (Figures are reproduced from Moore and Braucher (2008). Panel **c** is courtesy of Bruland et al. (2014))

inputs of Fe may in fact be long-lasting, contributing to the dissolved pool of Fe and eventually making their way to sunlit surface waters where they fuel primary productivity (Saito et al. 2013; Conway and John 2014). Stabilization of dissolved and particulate Fe in hydrothermal plumes is likely achieved through association with organic ligands. Modeling and observation-based studies have recently suggested hydrothermal vents may contribute substantially to overall Fe inputs into the ocean, likely being important in buffering the ocean's dissolved Fe inventory against shorter-term fluctuations in dust deposition. Recent estimates suggest hydrothermal input could provide 9–22 % of the Fe budget in the global deep ocean and upwards of 45–50 % in the tropical Pacific (Tagliabue et al. 2010).

In polar regions, glaciers may also be an appreciable source of Fe to the ocean. Glaciers can be considered as frozen rivers, and when they reach the ocean, they discharge in the form of icebergs and water. Icebergs often will transport significant amounts of continental debris that will be released into the ocean as they melt. In addition, sea ice can act as a cap on the ocean, which accumulates atmospheric sources of Fe and releases the Fe as it melts. Sea ice is believed to be an important mechanism for Fe delivery in high-latitude regions of the Southern and Arctic Oceans. The amount of bioavailable Fe supplied to the Southern Ocean by both aeolian dust and icebergs have been estimated to be similar (Raiswell et al. 2008). Free-drifting icebergs around Antarctica are often "hotspots" of enhanced biological production and

transport of organic carbon to the deep sea (Smith et al. 2007). This is likely a consequence of melting ice being a source of Fe that stimulates phytoplankton growth, although estimates on the contributions of Fe by icebergs to such regions are variable (Lin et al. 2011). The importance of melting ice as a source of Fe will likely increase as global temperatures continue to rise.

As in seawater, the Fe speciation in freshwater systems is highly affected by chemical variables (e.g. pH and O_2 concentrations), Fe inputs and removal processes, as well as internal recycling. In natural conditions, Fe is supplied from the products of weathered rocks and soil around the watersheds and is controlled by a number of factors including geological processes, soil composition, environmental temperature, precipitation and hydrology (Harris 1992). In addition to these sources, anthropogenic influences such as wastewater and storm-discharge are a major supply of Fe to freshwater lakes. Iron in freshwater environments is commonly orders of magnitude higher than that of seawater, although dissolved Fe concentrations have been suggested to influence rates of NO_3^- drawdown and Chl-*a* concentrations in some lakes (Havens et al. 2012). Iron may also play a regulatory role in the phytoplankton community where cyanobacteria tend be favored over green algae under high Fe conditions (Morton and Lee 1974; Pollingher et al. 1995; Hyenstrand et al. 2000). Similarly, Fe has been suggested to influence diatom composition in streams across the continental USA. Passy (2010) identified a relationship between stream diatom richness and wetland cover, where wetlands were suggested to provide an essential source of bioavailable, dissolved organic-bound Fe to stream ecosystems. More commonly, high Fe concentrations in freshwater systems, particularly rivers and streams, are a problem due to increased loads of Fe resulting from human activities such as mining of Fe enriched ores, intensified forestry, peat production and agricultural drainage.

2 Cellular Iron Requirements and Associated Physiological Processes

The transition metal iron exists in the environment in two oxidation states: Fe(III), the thermodynamically stable state in the presence of dioxygen and the reduced form Fe(II). Its facile redox chemistry has made Fe an essential metal for virtually all organisms, but it is particularly important for oxygenic photoautotrophs due to its essential role in photosynthetic and respiratory electron transport. Iron present in haeme or in the form of Fe-sulfur clusters occurs in a variety of metalloproteins that are involved in a myriad of metabolic pathways (Table 1).

Iron is particularly important in oxygenic photosynthesis. It is present in the two photosynthetic reaction centers (photosystems I and II, which contain respectively 12 and 2–3 iron atoms each) and the cytochrome b_6f complex (5 Fe), which is involved in ATP synthesis (Raven 1988; Strzepek and Harrison 2004). It also occurs in two smaller proteins within the photosynthetic electron transport chain: cytochrome c_6 (1 Fe) and ferredoxin (2 Fe) (Raven 1990). Based on iron use calculations (Raven 1988, 1990) and empirical data (Strzepek and Harrison 2004), 50–90 % of the metabolic Fe within phytoplankton occurs within the photosynthetic apparatus.

Phytoplankton experiencing Fe stress have reduced photosynthetic pigments and pigment binding proteins when compared to Fe-replete conditions (Greene et al. 1992; Sunda and Huntsman 1995; Geider and LaRoche 1994). Known as chlorosis, reduced chlorophyll content along with a decline in photosynthetic efficiency and reduced growth rates are the most noticeable symptom of Fe-deficiency in algae, (reviewed by Behrenfeld and Milligan 2013). Protein (Glover 1977; Rueter and Ades 1987; Doucette and Harrison 1991), lipid and carbohydrate (Milligan and Harrison 2000; van Oijen et al. 2004) content are also observed to vary with Fe nutritional state. A reduction in chlorophyll synthesis is likely the result of both a decrease in the number of photosynthetic units due to insufficient iron for their synthesis (Raven 1990; Sunda and Huntsman 1997) and a lower abundance of Fe-containing enzymes involved in chlorophyll synthesis (Behrenfeld and Milligan 2013). Due to iron's scarce supply to the oceans, microalgae have evolved unique strategies to reduce their photosynthetic Fe demand, a number of which are described below and have also recently been reviewed by Behrenfeld and Milligan (2013).

Iron also plays a large role in the reduction of oxidized chemical species of nitrogen, another essential element for cell growth. Phytoplankton require Fe for the assimilation of inorganic N species such as nitrate (NO_3), nitrite (NO_2) and nitrogen gas (N_2) into ammonium (NH_4). The assimilatory enzymes nitrate reductase (NR) and nitrite reductase (NiR) contain Fe (1 Fe: 1NR and 5–6 Fe: 1NiR). Under Fe limitation in the diatom *Conticribra* (*Thalassiosira*) *weissflogii*[1], the activity of NiR decreased by 50-fold and NO_2 was excreted because NO_2 assimilation was the rate-limiting step in the NO_3 assimilation pathway (Milligan and Harrison 2000). Based on biochemical calculations, cells growing on NO_3 should require 1.6 times more Fe than cells supplied with NH_4 due to the Fe demand associated with NO_3 reduction (Raven 1988; Morel et al. 1991). Experimental evidence supports this prediction since iron use efficiencies were, on average, 1.7 times higher for cells grown on NH_4 than those

[1]Wherever possible the currently accepted names for species are used. The name used in the paper cited is also indicated. For details of names see chapter "Systematics, Taxonomy and Species Names: Do They Matter?" of this book (Borowitzka 2016).

Table 1 Common Fe-related proteins in prokaryotic and eukaryotic phytoplankton

Protein	Abbreviation	Function[a]	Fe form	Microalgal Group[a]
Photosynthesis				
Cytochrome b6f	Cyt b6f	Electron transfer from PQ to Cyt c6 or PCY	H, Fe-S	All
Cytochrome c6	Cyt c6	Electron transfer from Cyt b6f to PSI	H	All except ch, some d
Ferredoxins	FDX or Fd	Electron transfer from PSI to FNR	Fe-S	All
NADP(+) reductase	FNR	Requires FDX in the synthesize NADPH	Fe-S	All
Photosystem I	PSI	Light reaction center complex	Fe-S, nH	All
Photosystem II	PSII	Light reaction center complex	H, nH	All
Plastid terminal oxidase	PTOX	Oxidation of PQ pool, carotenoid biosynthesis	nH	Many, but not all
Respiratory terminal oxidase	RTO	ATP formation through reduction of O2 to H2O	H	c,r
Cellular Respiration				
Aconitase	ACO	Isomerization of citrate to isocitrate	Fe-S	All
Alternative oxidase	AOX	Alternative route for electron transport	nH	All
Cytochrome c oxidase	COX1	RC IV; electron transfer for the formation of ATP	H	All
Cytochrome c	Cyt c	Electron transport	H	All
Ferredoxins	FDX or Fd	Electron transport	Fe-S	All
NADH: ubiquinone oxidoreductase	NQR	RC I; electron transfer from NADH to coenzyme Q10	Fe-S	All
Succinate:ubiquinone oxidoreductase	SQR	RC II; oxidation of succinate to fumarate	Fe-S, H	All
Ubiquinol-cytochrome c oxidoreductase	Cyt bc1	RC III, electron transfer from ubiquinol to ubiquinone	Fe-S, H	All
Nitrogen Assimilation				
Glutamate synthase	GLT	Requires FDX in the conversion of glutamine to glutamate	Fe-S	All
Nitrate reductase	NR	Reduction of nitrate to nitrite	H	All except some c
Nitrite reductase	NiR	Reduction of nitrite to ammonium	H	All
Nitrogenase	NifH	Fixes atmospheric nitrogen gas	Fe-S	Nitrogen-fixing c
Other				
Beta-carotene monooxygenase	BCMO	Final step in synthesis of retinal	nH	All
Biotin synthase	BIOB	Final step in synthesis of biotin	Fe-S	All
Coproporphyrinogen oxidase	CPOX	Synthesis of chlorophyll and heme	nH	All
Fe-superoxide dismutase	SOD	Dismutation of superoxide into oxygen and hydrogen peroxide	nH	Some but not all
Ferritin	FTN	Iron storage protein	nH	Some, but not all
Ferrochelatase	FECH	Final step in synthesis of heme	nH	All
Lipoxygenases	LOX	Dioxygenation of polyunsaturated fatty acids to lipids	nH	All

(continued)

Table 1 (continued)

Protein	Abbreviation	Function[a]	Fe form	Microalgal Group[a]
Peroxidases		Oxidation of a substrate by a peroxide	H	All
Xanthine oxidase	XO	Oxidation of hypoxanthine to xanthine and uric acid	Fe-S	All

Also provided are the protein abbreviations, their cellular function, the form of Fe associated with each protein and the phytoplankton groups in which these proteins are known to be present. The form of Fe and distribution of each protein within the taxonomic groups listed are tentative and based on available published data so should be interpreted with caution. *Fe* forms, *H* haeme, *nH* non-haeme, *Fe-S* iron-sulfur cluster

[a]*PCY* plastocyanin, *RC* respiratory complex, *ch* chlorophytes, *d* diatoms, *c* cyanobacteria, *r* rhodophytes

grown on NO_3 (Maldonado and Price 1996). Indirect support linking NH_4 use to a reduced iron requirement for growth was also provided for the cyanobacterium, *Synechococcus*, where half saturation constants of Fe for growth (the Fe concentration that decreases growth rate by half) were lower for NH_4 then NO_3- grown cells (Kudo and Harrison 1997). However, neither field nor laboratory experiments have observed significantly faster growth rates of Fe-limited eukaryotic phytoplankton when grown on NH_4 instead of NO_3 (Price et al. 1991, 1994; Maldonado and Price 1996). The lack of a N-dependent effect may, in part, be due to the profound influence of Fe limitation on photosynthesis. Conversely, Fe-stressed, low-light, NO_3-grown *Emiliania huxleyi*, a coccolithophore, grew significantly faster than Fe-stressed NH_4-grown cells (Muggli and Harrison 1996b). The difference in growth rates was attributed to the reduction in cell volume by NO_3-grown cells (see discussion below about Fe effects on cell size). Similarly, studies have shown that Fe-limited, NO_3-grown diatoms grew about 25 % faster than Fe-limited, NH_4-grown cells despite the higher iron use efficiency for cells grown on NH_4 (Price 2005). The higher growth for the iron limited NO_3-grown cells was due to a higher cellular Fe uptake rate, perhaps linked to increased rates of iron reduction at the cell surface by inducible transplasmalemma NO_3 reductases (Maldonado and Price 2000).

The ability to fix nitrogen also imparts a high Fe demand on diazotrophs, many of which are cyanobacteria (e.g. *Trichodesmium*) (Kustka et al. 2003; Berman-Frank et al. 2007). This is because the enzyme that catalyzes the conversion of N_2 gas to biologically available ammonium requires a large amount of Fe (38 Fe per enzyme). In addition N_2 fixation requires a large amount of ATP and reducing equivalents (NADPH) (16 and 8 mol, respectively, per N_2 molecule) which ultimately must be produced directly or indirectly from photosynthesis, further increasing the iron demand for the process (Kustka et al. 2003). Because of this increased Fe demand, the rate of N fixation by diazotrophs and thus the supply of fixed nitrogen to support phytoplankton growth, has been shown to be Fe-limited in many regions of the ocean, even when the growth rate of non-diazotrophs may not be directly limited by Fe (Moore et al. 2009; Sohm et al. 2011).

Other examples of cellular processes known to rely on Fe-containing enzymes and proteins include cellular respiration, vitamin synthesis, dehydrogenases and oxygenase reactions involved in fatty acid metabolism and detoxification of reactive oxygen species (e.g., hydrogen peroxide and super oxide radicals) (Table 1). Superoxide detoxification is catalyzed by superoxide dismutases (SODs) which catalyze the conversion of superoxide radicals to molecular oxygen and hydrogen peroxide. Four major groups of SODs are known and are distinguished by their metal co-factors: Fe, Mn, Cu/Zn, and Ni. The Fe/Mn family includes both single metal-binding and the rare cambialistic SODs which can bind either Mn or Fe. The Fe and Mn single metal-binding SODs are very similar but may be distinguished by two critical residues involved in the binding of the metal ion (Wolfe-Simon et al. 2005). Although many microalgae contain the Fe-SOD variants, interestingly, others completely lack Fe-SODs or down-regulate them when grown under low Fe conditions, which may thus contribute to lowering their cellular Fe demands (Allen et al. 2008; Marchetti et al. 2012).

The cellular Fe requirement is often expressed by the intracellular Fe content (or quota; Q) of a microalgal cell relative to its cellular C (or P) content. The cellular quota may be given as a ratio (Fe:C/P), which allows for a measure of Fe demand normalized to biomass and provides a means of comparison among different phytoplankton species of varying sizes and shapes. Recently, Quigg et al. (2003, 2011) suggested microalgal groups that inherited their plastids by endosymbiotic events from the green lineage tended to have higher trace metal:P ratios than those that derived their plastids through the red lineage. This suggested that this may be the result of the lineages originating in contrasting environmental conditions; the green lineage dominating under more reduced ocean conditions during earlier geologic times and the red lineage dominating later under more oxic conditions that are more similar to present day marine environments. To examine if this is also true specifically for Fe, we compiled published Fe:C (and Fe:P) ratios measured in various phytoplankton isolates and grouped them according to taxa. Comparing Fe quotas in phytoplankton grown under Fe-replete conditions is confounded by luxury consumption of Fe in certain groups of microalgae in excess of the cell

quotas needed to support maximum growth rates. This is especially true for diatoms where large but variable levels of luxury consumption are observed (see further discussion below). Therefore, we limited our data set to only those Fe:C ratios obtained when cells were grown under conditions of inorganic Fe concentrations that supported maximum or near maximum (μ/μ_{max} >75 %) growth rates, but were below levels that would result in a large amount of luxury Fe uptake.

Cyanobacteria, especially N_2-fixing species, and microalgae from the red primary plastid lineage (red algae) have relatively higher Fe requirements than the green algae and secondary red plastid lineage groups (e.g. diatoms, dinoflagellates and haptophytes); the latter groups appear to have somewhat similar Fe requirements (Fig. 2). Although our analysis does not necessarily support a clear distinction between red and green plastid lineages, it does suggest that certain phyla have reduced their Fe requirements more effectively than others. Members of two taxa in particular, the diatoms and haptophytes, possess the lowest Fe:C ratios, which may explain their persistence (along with picocyanobacteria that primarily benefit from their small size) in a number of low-Fe environments.

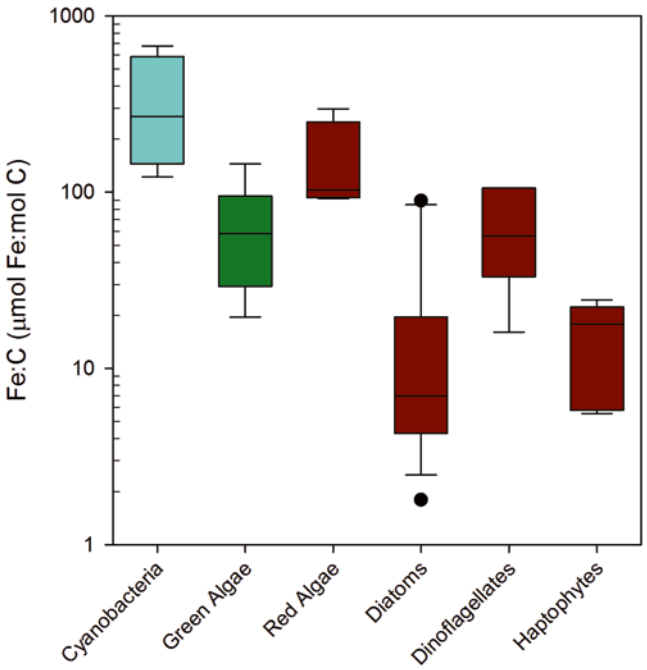

Fig. 2 Box and whisker plots of Fe requirements (presented in the form of Fe:C ratios) for cyanobacteria and microalgae grouped by phyla. Eukaryotic groups are separated by the green plastid lineage (*green*) and the red plastid lineage (*red*). All quotas were obtained from cultures grown in medium that enabled maximum or near maximum (μ/μ_{max} >75 %) growth rates and the Fe concentrations were below levels in which significant amounts of luxury uptake of Fe would occur ([Fe′] <0.25 nM) (Data are compiled from Sunda and Huntsman (1995), Maldonado and Price (1996), Ho et al. (2003), Marchetti et al. (2006), Lane et al. (2009), Quigg et al. (2011) and Strzepek et al. (2011))

2.1 Acclimation and Adaptation to Iron Limitation

There is considerable pressure on microalgae to evolve mechanisms to reduce their Fe requirements in order to reside in Fe-limited regions. Phytoplankton isolated from Fe-limited oceanic regions typically have lower Fe contents and higher Fe use efficiencies (the amount of C fixed per mole of cellular Fe per day) compared to their coastal congeners. There have been numerous investigations into the mechanisms by which oceanic phytoplankton, particularly diatoms, have acclimate and adapted to the low Fe availability in their environment. One of the most important evolutionary responses to low Fe in the open ocean is a substantial reduction in the cellular Fe requirement for growth (Sunda and Huntsman 1995). Given iron's involvement in many metabolic pathways, there are numerous strategies invoked by both eukaryotic (Fig. 3) and prokaryotic (Fig. 4) phytoplankton to adapt to low iron availability. These include reducing cell size, using intracellular Fe pools more efficiently, minimizing the use of metabolic pathways (or protein complexes) that require large amounts of Fe and replacing Fe-containing proteins with non-Fe containing ones that are more or less functionally equivalent.

A reduction in cell size often occurs when microalgae are acclimated to low light or various nutrient limitations, including Fe limitation (Sunda and Huntsman 1997; Marchetti and Cassar 2009). Nutrient requirements for growth per cell decrease as a function of the cube of the cell radius (r^3), whereas nutrient uptake decreases as a function of the available membrane area (r^2), and the diffusion-limited rate as a function of the radius (r) (Morel et al. 1991). Decreasing cell size would increase the surface area-to-volume ratio and maximize membrane transporters and subsequently uptake rates relative to Fe requirements. A decrease in cell size also decreases the diffusion boundary layer thickness, improving nutrient uptake kinetics. Such a physiological acclimation is observed in microalgae in both field and laboratory conditions. Many Fe-limited regions are populated primarily by small pico- and nanophytoplankton (<5 µm in diameter), suggesting that this small size-fraction has an advantage in coping with low [Fe] as compared to larger phytoplankton species (e.g. diatoms). This is due to the inherent overall lower Fe requirements for small phytoplankton (on a per cell basis) as well as their more effective uptake capabilities on a cell surface area basis. As a response to Fe limitation, many microalgae show a 20–50 % decrease in mean cell volume per cell (Sunda and Huntsman 1995; Muggli et al. 1996; Marchetti and Harrison 2007). This reduction would represent an increase in surface area-to-volume ratio of 8–26 %. Similarly, even cyanobacteria that benefit from being small, have been shown to decrease their size in response to Fe

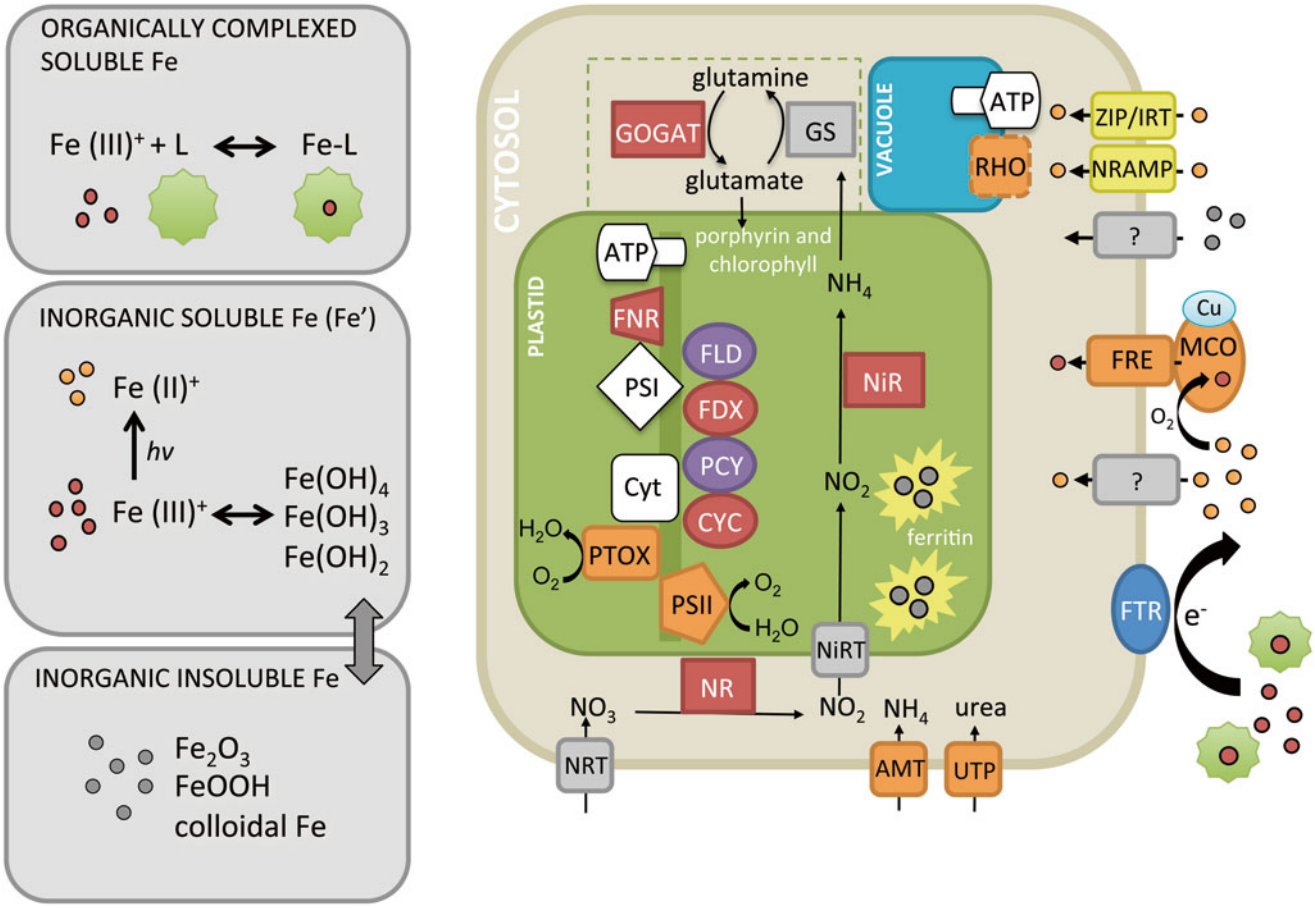

Fig. 3 Major forms of Fe in seawater and depiction of a eukaryotic microalgal cell. (**a**) Bioavailable Fe primarily exists as a dissolved ferric Fe (Fe(III)) ion or tightly bound to organic ligands (L). Ferrous Fe (Fe(II)) is supplied primarily either through wet deposition and/or photoreduction of ferric Fe. Due to the high oxygen content and short residence time, concentrations of free ferrous Fe are extremely low in seawater. Much of the Fe in oxic seawater will eventually form colloids that precipitate out of the water column. The bioavailability of Fe oxyhydroxides and colloidal Fe is not well known, but assumed to be minimal. (**b**) Fe uptake may occur through a number of mechanisms. Only Fe transport pathways into the cells are illustrated. In general, Fe bound to organic ligands must be reduced before taken up by the cell. Many microalgae have a high-affinity Fe uptake mechanism that couples the Fe reductase with a multicopper-oxidase and Fe permease. Ferrous Fe may also be taken up through non-Fe specific divalent transporters. There is no illustration for proteins involved in intracellular Fe tracking (which are listed in Table 3). The bulk of the Fe demand in eukaryotic microalgae is used within the photosynthetic reaction centers and for the assimilation of NO_3 (also shown). Iron containing proteins are indicated in *red*. Some non-Fe containing proteins that may substitute when Fe levels are low are indicated in *purple*. Low-Fe induced proteins and processes are indicated in *orange*, which include an increased dependence on reduced forms of nitrogen (e.g. NH_4 and urea) as well as an increased ratio of PSII: PSI reaction centers. In some (but not all) microalgae, the presence of rhodopsins may compensate for a decreased production of ATP from photosynthesis in Fe-limited cells. The localization of rhodopsins to the vacuolar membrane is speculative. Similarly, some microalgae contain ferritins for Fe storage that are located in the plastid. Full protein names are provided in Table 1 (Fe-related proteins) and Table 3 (Fe transport and associated proteins). Others: *AMT* NH_4 transporter, *ATP* ATP-synthase, *FLD* flavodoxin, *GS* glutamine synthetase, *NiRT* nitrite transporter, *NRT* nitrate transporter, *PCY* plastocyanin, *RHO* rhodopsin

limitation (Sherman and Sherman 1983; Wilhelm 1995). In addition to a reduction in total cell volume, a change in shape from spherical to elongated results in an increase in the SA:V ratio (Marchetti and Cassar 2009). However, there are likely to be constraints on the morphological changes that particular groups of microalgae may undergo, particularly groups such as diatoms with rigid cell walls. Similarly, although usually rare, the persistence of large cells in Fe-limited regions suggests there are benefits to large cell size, possibly in relation to nutrient (and Fe) storage capacities, periodic vertical migration and resistance to grazing (Smetacek et al. 2004).

Microalgae may also adjust their photosystems in response to Fe limitation. There is a preferential down-regulation of PSI relative to PSII (and Cyt b_6f) in prokaryotic and eukaryotic phytoplankton under Fe limitation of growth rate (Greene et al. 1991; Moseley et al. 2002; Strzepek and Harrison 2004; Behrenfeld and Milligan 2013). Under Fe deficiency, cyanobacteria may decrease their PSI:PSII ratio from 4:1 to 1:1 (Straus 2004), while the low-Fe adapted

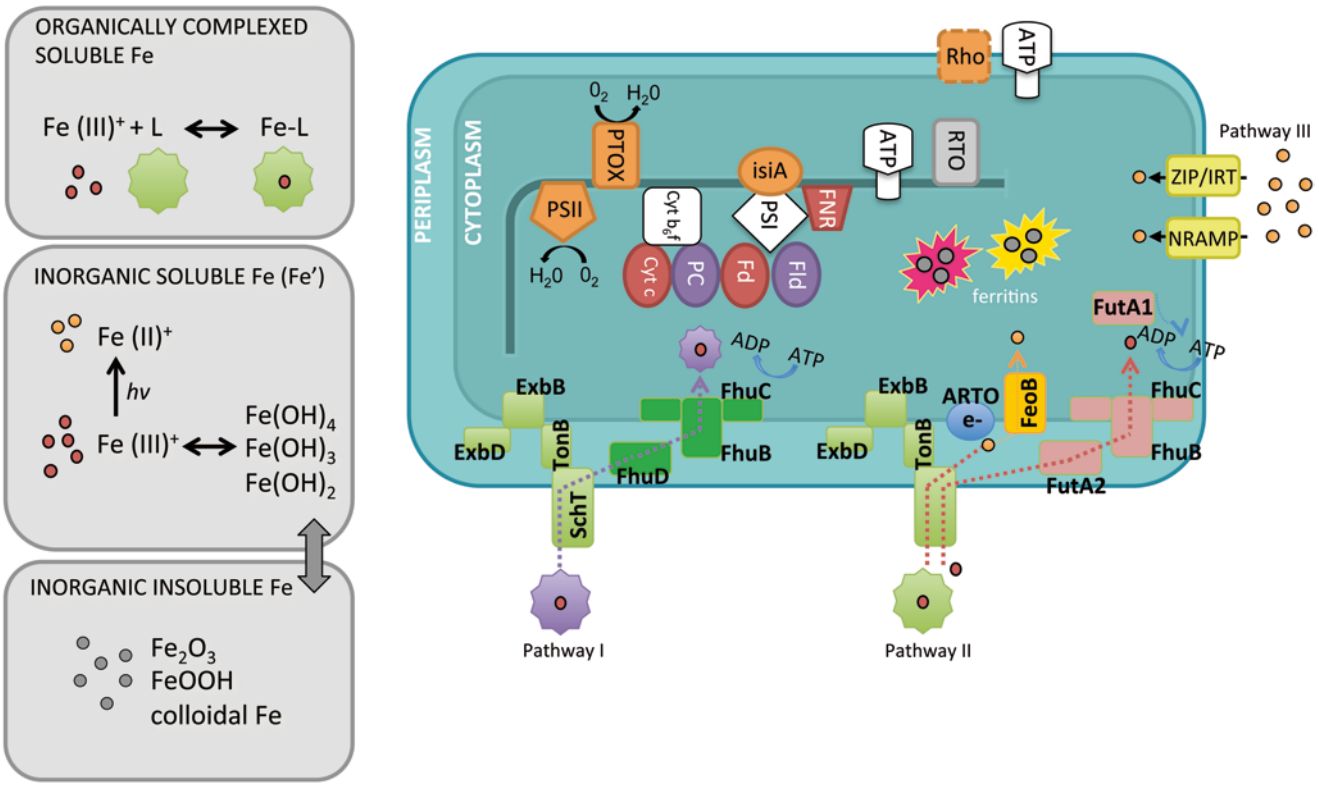

Fig. 4 Major forms of Fe in seawater and depiction of a cyanobacteria cell (**a**) See legend in Fig. 3. (**b**) Putative Fe transport pathways in cyanobacteria, as predicted (by genomic analyses, protein determination and physiological studies) for model organisms: Pathway I: Schizokinen transport by *Anabaena* sp., pathway II: reductive transport of organic and inorganic Fe(III) complexes (*Synechocystis*, PCC6803) and pathway III: Fe(II) transport in various cyanobacteria. Predicted locations and common substrates for each transporter are shown. The bulk of the Fe demand in cyanobacteria is used within the photosynthetic reaction centers and for the assimilation of N (not shown). Iron containing proteins are indicated in *red*. Some non-Fe containing proteins that may substitute when Fe levels are low are indicated in *purple*. Low-Fe induced proteins and processes are indicated in *orange*, which include an increased ratio of PSII: PSI reaction centers. Most cyanobacteria contain ferritins for Fe storage most commonly in the form of bacterioferritin or DPS ferritin. Full protein names are provided in Table 1 (Fe-related proteins) and Table 3 (Fe transport and associated proteins). Others: *Fld* flavodoxin, *isiA* Fe-starved induced protein, *PC* plastocyanin

oceanic diatom, *Thalassiosira oceanica* has been shown to have a constitutively low PSI:PSII ratio of 1:10 (Strzepek and Harrison 2004). Ratios of iron rich cytochrome b_6/f complex (5 Fe) to low iron PSII (2–3 Fe) are also reduced in this species (Strzepek and Harrison 2004). The decreased abundance of PSI (12 Fe) and cytochrome b_6f complex, however, may have resulted in a diminished ability by *T. oceanica* to acclimate to fluctuations in photosynthetically active radiation (see section of Fe-light interactions). A recent analysis of the *T. oceanica* genome and associated transcriptome under Fe-limitation demonstrated that this diatom possesses the potential to differentially adjust many of its iron-containing proteins and protein complexes (including PS1:PSII ratios) in response to Fe availability (Lommer et al. 2012). Major reductions in the PSI:PSII ratio, however may increase the cell's dependency on alternative electron pathways such as the plastoquinol oxidase (PTOX) pathway, which is present in both prokaryotic and eukaryotic phytoplankton (reviewed by Zehr and Kudela 2009; Behrenfeld and Milligan 2013). The PTOX pathway provides an electron shunt after PSII, enabling cells to bypass PSI and thereby produce ATP without the production of reducing equivalents (NADPH). Because the PTOX pathway has a low Fe requirement (2 Fe molecules per monomer), activation of this shunt under low Fe conditions would substantially reduce a cell's Fe demand while still being able to generate ATP, however, an alternative source of reductant must be present for carbon fixation to occur.

Cyanobacteria and red algae also express an induced protein (isiA) under Fe stress, which can become the most abundant chl-binding protein in the cell (Ryan-Keogh et al. 2012). The precise roles of isiA remain elusive. Despite strong sequence similarity between IsiA and the gene encoding the PSII core CP43 protein, IsiA is not thought to be a CP43 replacement nor antenna complex for PSII. Rather, during Fe stress, IsiA forms superstructures around PSI trimers and monomers, resulting in a significant increase in cross-sectional absorption area of PSI (σ_{PSI}) (Bibby et al. 2001;

Boekema et al. 2001). A continued up-regulation in isiA without any further increase in the σ_{PSI} led Ryan-Koegh et al. (2012) to speculate that this protein may also be involved in other secondary roles for coping with Fe limitation.

Many of the Fe-requiring proteins in microalgae may be reduced or substituted for non-Fe containing equivalents under low Fe conditions. Some of the protein substitutions represent acclimation strategies where the Fe-containing protein is preferred when cells are growing in Fe-replete conditions. Other protein substitutions represent evolutionary adaptations where species have adapted to chronically low [Fe] by replacing Fe-containing proteins with proteins that contain no metal cofactor or that use other redox active trace metals that are more readily available in the environment . The best example of Fe metalloprotein substitution is flavodoxin for ferredoxin (La Roche et al. 1993). Ferredoxins are Fe-sulfur cluster containing proteins which mediate electron transfer in a range of metabolic reactions. Flavodoxin, on the other hand, does not contain Fe but instead utilizes a single molecule of riboflavin 5′-phosphate as a cofactor. Although flavodoxin does not have as low a redox potential as ferredoxin, the expression of flavodoxin at suboptimal [Fe] would partially alleviate Fe stress. Flavodoxins are commonly found in cyanobacteria, although not all species are found to contain them. For example, *Anabaena* ATCC 29211 lacks the potential to synthesize flavodoxin, and simply decreases the content of ferredoxin at very low Fe concentrations (Sandmann et al. 1990). The diatom *Phaeodactylum tricornutum* increases the expression of flavodoxin 25–50-fold under Fe limitation (Allen et al. 2008). The flavodoxin to ferredoxin ratio has been considered to be an *in situ* marker for Fe stress in phytoplankton (Doucette et al. 1996; La Roche et al. 1996). However, it should be used with caution given that some microalgae have several copies of flavodoxin, with some gene variants not regulated by Fe availability (Whitney et al. 2011) whereas other phytoplankton groups may express flavodoxin constitutively (Pankowski and McMinn 2009). In *T. oceanica* there has been a permanent transfer of the ferredoxin gene from the chloroplast genome to the nuclear genome (Lommer et al. 2010). This relocation is thought to allow *T. oceanica* to better coordinate gene expression for its low-Fe response.

Another well-characterized substitution is that of the copper-containing electron transport protein plastocyanin for the Fe-containing cytochrome c_6 in photosynthetic electron transport. Where plastocyanin is more commonly used in green algae (Hill and Merchant 1995), in haptophytes, primarily cytochrome c_6 is found whereas in diatoms and cyanobacteria either transport protein may be present, and is largely dependent on growth conditions and biogeography (Sandmann et al. 1983). For example, while some oceanic diatoms have replaced cytochrome c_6 with plastocyanin, many coastal diatoms continue to use cytochrome c_6. In the oceanic diatom *T. oceanica*, the constitutive use of plastocyanin is believed to reduce the cellular Fe demand by as much as 10 % (Peers and Price 2006).

3 Iron Acquisition Mechanisms

Cellular transport of an essential element refers to the ability of a cell to transfer that element across the plasma membrane, from the outside of the cell to the inside. In general, the word *transport* and *uptake* are used interchangeably. Fe acquisition refers to the ability of microalgae to access Fe within a variety of complex chemical species (both inorganic and organic) before it is transported across the plasma membrane. In contrast, assimilation of an element refers to the intracellular incorporation of that element into cellular biomolecules.

Ions of essential elements enter the cell by moving across the cell's diffusive boundary layer toward the cell surface, then passing through the cell wall (if present) and plasmalemma into the cytoplasm. The rate of diffusion through the surface boundary layer is inversely related to the cell diameter. Hence, large cells are much more prone to suffer from diffusion limitation of uptake of iron, or other nutrients, than smaller cells. The cell wall is not a barrier to ion entry. In contrast, the plasma membrane—which consists of polar lipid bilayers interspersed with proteins—does not allow the free diffusion of charged ions, polar hydrophilic molecules or large neutral molecules. These species must be transported across the plasma membrane via facilitated diffusion (also called passive transport) or active transport. Some of the common characteristics of facilitated diffusion and active transport include: (a) facilitation by a transmembrane transport protein (either a carrier protein or a channel protein), (b) unidirectional and often ion-specific transport, (c) Michaelis-Menten saturation kinetics, and (d) competitive and non-competitive inhibition of ion transport.

The greatest difference between facilitated diffusion and active transport is that in the latter, the ion is transported against an electrochemical gradient, and thus energy input is required. In both, facilitated diffusion and active transport, the substrate first binds to the receptor sites on the outer surface of the transport protein. These carrier proteins (also called permeases or transporters) then undergo a series of conformational changes to transfer the bound solute across the membrane. In contrast, channel proteins are only involved in facilitated diffusion. Channel proteins form an aqueous pore in the membrane through which a specific solute passes, and thus these proteins interact only weakly with the solute.

In phytoplankton, as in all other organisms, Fe uptake is an active transport process. The mechanisms of Fe transport in phytoplankton are extremely diverse and complex, and point to unique Fe uptake mechanisms acquired via horizon-

tal gene transfer (Morrissey and Bowler 2012). We will describe general mechanisms involved in Fe transport by discussing prokaryotic and eukaryotic phytoplankton separately. So far, the vast majority of work on phytoplankton has focused on Fe acquisition and transport, and not on Fe assimilation. The following sections thus focus on these two topics.

There are physiological and molecular aspects of Fe acquisition and transport. From a physiological perspective, the following questions can be addressed: (1) What are the Fe species (oxidation state and chemical complexes) that are bioavailable for uptake by phytoplankton? (2) What are the Fe species that bind to receptor sites on the transporters? (3) What is the Fe species that is transported into the cell? (4) If Fe transport involves various proteins, what are their activities and functions? (5) What is the limiting step in Fe acquisition? and, (6) Does Fe uptake follow Michaelis-Menten kinetics?

3.1 Physiological Aspects of Iron Transport

The vast majority of physiological Fe transport studies in phytoplankton have used marine diatoms as model organisms. However, we expect that most of the findings apply to many other microalgal taxa, and freshwater algae. There is evidence for two Fe uptake systems, a low-affinity and a high-affinity transport system. The low-affinity Fe transport mechanism is utilized when inorganic Fe species are available for uptake, and cells are Fe sufficient. The high-affinity Fe transport system is operational when the cells are Fe-stressed (but before their growth rate is actually reduced due to low Fe level) or growth limited by Fe and where the concentrations of dissolved inorganic Fe species are extremely low, but there is a larger pool of organically bound Fe (e.g., in the nM range). In the low-affinity system, Fe uptake is a function of the labile dissolved inorganic Fe(III) concentration (Fe´) in the bulk medium (Hudson and Morel 1990), while in the high-affinity system, Fe uptake is best predicted by the concentration of organically bound Fe (Maldonado and Price 2001; Strzepek et al. 2011). These two Fe transport systems have been reconciled in a general kinetic model for Fe acquisition by marine phytoplankton (Shaked et al. 2005), where Fe(II) is an obligate intermediate in both systems. The presence of a low- and a high-affinity transport system is often termed biphasic uptake kinetics for short-term uptake rates as a function of nutrient concentration (best fit using a double rectangular hyperbola equation). Biphasic uptake kinetics have been shown for Zn in the haptophyte *Emiliania huxleyi* (Sunda and Huntsman 1992) and Cu in diatoms (Guo et al. 2010). For Fe, such data are still rare, though hints of biphasic uptake kinetics have been shown in the Southern Ocean prymnesiophyte, *Phaeocystis antarctica* (Strzepek et al. 2011). These two Fe acquisition mechanisms are believed to share some components/proteins, which are likely to be up-regulated under Fe limiting conditions (see below).

3.1.1 Low-Affinity Acquisition Mechanisms for Inorganic Iron

Before describing Fe transport mechanisms in depth, it is essential to understand how Fe uptake is controlled by the Fe conditions in the growth medium (or the environment), as well as by intracellular Fe levels. Most studies investigating Fe physiology use a chemically well-defined medium such as Aquil (Price et al. 1988/89; Sunda et al. 2005). In this medium, a large excess of aminocarboxylate chelating agents are added to buffer a (nearly) constant concentration of dissolved inorganic ferric iron hydrolysis species ([Fe´]), which in turn controls cellular iron uptake rates, intracellular iron levels and specific growth rate in iron limited algal cultures (Hudson and Morel 1990; Sunda and Huntsman 1995). A typical medium might have 100 μM ethylenediaminetetraacetic acid (EDTA) and nM to μM concentrations of Fe, depending on what degree of Fe limitation or sufficiency needs to be imparted to the phytoplankton (see further discussion below). The Fe′ concentration is maintained by steady state dissociation and formation reactions of the organically bound Fe, and thus is dependent on the total iron concentration [Fe_T], the chelator concentration and the affinity of the ligand for Fe(III). Photochemistry might also induce an increase in the Fe′ pool, due to photo-redox cycling of the organically complexed Fe (Sunda and Huntsman 2003).

When the concentrations of all the nutrients are in excess, except for Fe, the concentration of labile inorganic Fe [Fe′] determines microalgal growth rates. Following the Monod equation (Monod 1942), the specific growth rate is determined by the labile dissolved inorganic Fe concentrations ([Fe′]), as well as the half-saturation constant for Fe for growth (K_μ), and the maximum specific growth rate (μ_{max}) so that:

$$\mu = \mu_{max} * [Fe'] / ([Fe'] + K_\mu) \quad (1)$$

The specific growth rate can also be related to intracellular Fe content (Harrison and Morel 1986), using the Droop equation (Droop 1970). In this case, the maximum growth rate is only achieved when the microalgae are able to fulfill their cellular Fe demand:

$$\mu = \mu'_{max} \left[1 - \left(Q_{Fe}^{min} / Q_{Fe}\right)\right] \quad (2)$$

where Q_{Fe}^{min} is the minimal intracellular Fe content needed to allow any growth, and Q_{Fe} is the intracellular Fe level.

Here μ'_{max} refers to the 'impossible' growth rate at infinite quota, but in general $\mu'_{max}/\mu_{max} \sim 1$ and μ_{max} is achieved when the optimal cellular Fe content (Q_{Fe}^{max}) is reached (Harrison and Morel 1986).

We can also write this equation in terms of Q_{Fe}^{max}, which is the optimal cellular Fe content when μ_{max} is achieved, thus

$$\mu = \left(\mu'_{max}\left(Q_{Fe} - Q_{Fe}^{min}\right)/Q_{Fe}^{max}\right) \quad (3)$$

When phytoplankton are growing under steady-state conditions (or exponential growth), and the rate of growth is limited by the Fe supply, the growth rate is directly proportional to the steady-state Fe uptake and inversely proportional to the cellular Fe content, so that

$$\mu = \rho^{ss}/Q_{Fe} \quad (4)$$

The steady-state Fe uptake rates also follow Michaelis-Menten kinetics and can be described as:

$$\rho^{ss} = \rho^{ss}_{max} * [Fe'] / \left(K_{\mu Q} + [Fe']\right) \quad (5)$$

where $K_{\mu Q}$ stands for half-saturation constant for steady-state Fe uptake. Equations 1, 2 and 4 are related to each other quantitatively according to $\mu = \rho^{ss}/Q_{Fe}$ (Eq. 3). We can solve for ρ^{ss} and take the logarithm of both sides of the equation so that $\log \rho^{ss} = \log\mu + \log Q_{Fe}$. This implies that the log of the steady-state Fe uptake rates is the sum of logarithm of μ and the logarithm of Q_{Fe} (Morel 1987). A graphic representation of this is given in Fig. 5.

The net result is that the half-saturation constant for steady-state Fe uptake rates is higher than that for growth, and that the quantitative relationship between $K_{\mu Q}$ and K_{μ} is related to the ability of the cells to modify their cellular Fe quotas (Harrison and Morel 1986; Morel 1987), such that:

$$K_{\mu Q}/K_{\mu} = Q_{Fe}^{max}/Q_{Fe}^{min}, \text{ and thus, the } K_{\mu Q} >>> K_{\mu}$$

For a phytoplankton cell growing under low Fe, a practical implication of Eq. 3, is that fast growth rates can be maintained by decreasing the Q_{Fe} (Harrison and Morel 1986; Morel 1987), even though at low [Fe′] ρ^{ss} will decrease according to Eq. 4.

Short-term Fe uptake rates reflect the rate of Fe uptake under non steady-state conditions. For example, a culture can be grown under a specific Fe limiting concentration, and can be examined for its ability to take up Fe at various [Fe]. Normally, these short-term Fe uptake experiments should not last more than the time it takes a cell to synthesize new proteins. Typically, approximately <6 h is the recommended time for laboratory Fe uptake experiments, but this depends on the microalgal growth rate (i.e. should be increased for slower growing algae). For exponentially growing cells, when the [Fe] in the short-term Fe uptake experiments is equal to the [Fe] in the growth medium, the short-term Fe uptake rates coincide with the steady-state Fe uptake rates since they are determined by the concentration of Fe′ (see Eq. 9 below). However, in the presence of higher [Fe] (but not saturating) in the uptake medium, the short-term Fe

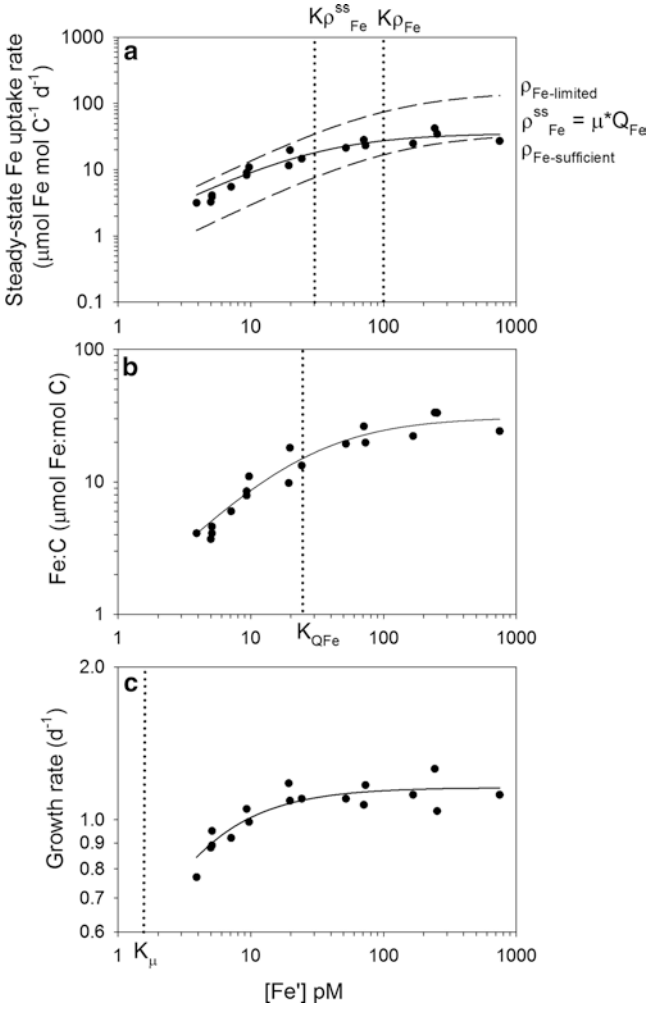

Fig. 5 (**a**) Steady-state Fe uptake rates, (**b**) Fe quotas and (**c**) growth rates as a function of inorganic Fe concentration ([Fe´]; 10^{-12} M) for the coccolithophore, *Emiliania huxleyi* (Sunda and Huntsman 1995). These log-log graphs show how the half-saturation constant for growth, steady-state and short-term Fe uptake rates are related to each other, so that the half-saturation constant for growth is much lower than those for steady-state and short-term Fe uptake rates (Morel 1987). The short-term Fe uptake rates for Fe-limited and Fe-sufficient *Emiliania huxleyi* are computed, based on our knowledge in other marine phytoplankton species (Harrison and Morel 1986). Our calculated K values are: for growth: $K_{\mu} = 1.46$ pM (Monod equation); for Fe quota: $K_{QFe} = 26$ pM (Droop equation); for steady-state Fe uptake rates: $K_{\rho}^{ss} = 29$ pM [$\rho^{ss} = \rho^{ss}_{max}$ * [Fe´]/(K_{ρ}^{ss} + [Fe´])]; and for short-term Fe uptake rates: $K_{\rho} = 100$ pM (Michaelis-Menten kinetics). See text for equations

uptake rates will be faster than the steady-state Fe uptake rates due to the higher Fe′ in the uptake medium compared to the growth medium.

In general, inorganic Fe acquisition (low-affinity Fe uptake system) is observed when concentrations of Fe′ are in the nanomolar range, and a typical oceanic species is either Fe sufficient or slightly Fe-stressed. Under this condition, short-term Fe transport in phytoplankton follows typical Michaelis-Menten uptake kinetics, where the rate of Fe uptake is determined by the maximum rate of uptake (ρ_{max}), the concentration of labile dissolved inorganic Fe species ([Fe′]), as well as the half-saturation constant for Fe uptake (K_ρ) (Hudson and Morel 1990).

$$\rho = \rho_{max} * [Fe'] / (K_\rho + [Fe']) \quad (6)$$

The half-saturation constant for short-term Fe uptake (K_ρ) is species specific and its inverse ($1/K_\rho$) provides a measure of the affinity of transporter for Fe. The K_ρ for short-term Fe uptake is less plastic than ρ_{max}, which may vary significantly, depending on the number of Fe transporters at the cell surface (Harrison and Morel 1986, 1990). Fe-limited phytoplankton have been observed to increase ρ_{max} values by up to 20-fold, presumably by increasing the number of Fe transporters on the outer cell membrane (Harrison and Morel 1986). This can be graphically represented by two kinetic curves (ρ^{low} and ρ^{high}) that have a similar K_ρ but different ρ_{max}, so that $\rho_{max}{}^{low}$ and $\rho_{max}{}^{high}$ represent the ρ_{max} for the Fe-sufficient and Fe-limited phytoplankton, respectively (Morel 1987).

When the [Fe] in the uptake experiment is equal to that in the growth medium, the short-term Fe uptake rates are equal to the steady-state Fe uptake rates. Therefore, for a Fe- sufficient culture, the short-term and steady-state Fe uptake rate should be the same at high [Fe]. Similarly, for a Fe-limited cell, the short-term and steady-state Fe uptake rate should be identical at low [Fe]. As a result, the half-saturation constant for short-term Fe uptake is higher than for steady-state uptake by a factor of $\rho_{max}{}^{high}/\rho_{max}{}^{low}$, so that

$$K_\rho / K_{\mu Q} = \rho_{max}{}^{high} / \rho_{max}{}^{low}, \text{ and thus, the } K_{\mu Q} <<<< K_\rho$$

We can thus compare half-saturation constants for growth, short-term Fe uptake and steady-state Fe uptake (Fig. 5). As predicted by Morel (1987), Fig. 5 shows how the half-saturation constants for growth, short-term Fe uptake and steady-state Fe uptake vary in magnitude for *E. huxleyi* (K_ρ = 0.1, $K_{\mu Q}$ = 0.029, and K_μ = 0.0014 nM Fe′; using data from Sunda and Huntsman 1995), so that

$$K_{\mu <<<} K_{\mu Q <<<} K_\rho$$

Thus, the ratio of the half-saturation constants for growth and short-term Fe uptake rates is directly determined by the lower and upper limits of the Fe intracellular levels and of the maximum uptake rates, according to Eq. 7 (Morel 1987):

$$K_\mu / K_\rho = \left(Q_{Fe}{}^{min} / Q_{Fe}{}^{max}\right) * \left(\rho_{max}{}^{low} / \rho_{max}{}^{high}\right) \quad (7)$$

We compiled data for a wide variety of phytoplankton to compare half-saturation constants for growth, short-term Fe uptake and steady-state Fe uptake in microalgae, as previously done by Morel (1987). As predicted by Morel (1987), Table 2 shows how the half-saturation constants for growth, short-term Fe uptake and steady-state Fe uptake vary by orders of magnitude (for cultures K_ρ = 3.7, $K_{\mu Q}$ = 0.37, and K_μ = 0.041 nM Fe′; for field studies K_ρ = 2.96 vs. K_μ = 0.00032 nM Fe′). The order-of-magnitude difference among these half-saturation constants (K_ρ vs. $K_{\mu Q}$ vs. K_μ) is related to the plasticity of the cells to regulate intracellular Fe levels (lower under Fe limitation), as well as the number of Fe transporters at the cell surface (higher under Fe limitation) under various Fe conditions.

These half-saturation constants shown in Table 2 can also be used to define the Fe-limited condition of a phytoplankton culture or an in situ population (Morel et al. 1991). Under Fe sufficiency, when $[Fe'] > K_{\mu Q}$, near maximum growth rates are observed, and neither the number of Fe transporters are maximized, nor the Q_{Fe} is minimized. Under Fe stress, when $K_\mu < [Fe'] < K_{\mu Q}$, near maximum growth rates are observed, but the number of Fe transporters is maximized and the Q_{Fe} is minimized. Under Fe limitation, when $[Fe'] < K_\mu$, the Fe uptake rate is too slow to fulfill the Fe requirement despite maximizing the number of Fe transporters and minimizing Q_{Fe}, thus cellular growth rate decreases.

3.2 Physical, Chemical and Biological Factors Controlling Fe Transport

Physical factors that may influence rates of Fe uptake include light, temperature and diffusion constraints. Light availability has been observed to affect Fe uptake rates by either generating changes in aquatic Fe chemistry (i.e. changes in [Fe′] due to photo-redox cycling of iron; Hudson and Morel 1990; Barbeau et al. 2003; Sunda and Huntsman 2003, 2011; Maldonado et al. 2005; Fujii et al. 2011), and/or influencing the energy supply to the cells (ATP and/or NADPH) for Fe acquisition (Strzepek et al. 2011). In addition, a physiological interaction between light and Fe availability during growth has been shown to affect steady-state Fe transport rates (see biological factors below). The temperature effects on Fe uptake are associated with the typical microalgal temperature coefficient (Q_{10} value is ~2), which means that, in general, the rate of Fe uptake will be two times faster for every 10 °C increase in temperature, as found in the coastal

Table 2 Kinetics of Fe transport and growth in phytoplankton

Organism or Field location	Growth conditions or dominant microalgae	Cultures			Field (or no chelators added to cultures)	References
		K_ρ (nM Fe′)	$K_{\mu Q}$ (nM Fe′)	K_μ (nM Fe′)	K_μ (nM Fe_{diss})	
Marine microalgae						
Synechococcus sp.	High light (110 µmol photons m^{-2} s^{-1}, 100 nM EDTA, NO_3^-)				0.74	Kudo and Harrison (1997)
	High light (110 µmol photons m^{-2} s^{-1}, 100 nM EDTA, NH_4^+)				0.18	
	Low light (26 µmol photons m^{-2} s^{-1}, 100 nM EDTA, NO_3^-)				2.08	
	Low light (26 µmol photons m^{-2} s^{-1}, 100 nM EDTA, NH_4^+)				1.29	
Thalassiosira oceanica	High light (75 µmol photons m^{-2} s^{-1}, 14L:10D)			0.00129		Bucciarelli et al. (2010)
	Low light (7.5 µmol photons m^{-2} s^{-1}, 14L:10D)			0.00301		
Ditylum brightwellii	High light (75 µmol photons m^{-2} s^{-1}, 14L:10D)			0.0254		
	Low light (7.5 µmol photons m^{-2} s^{-1}, 14L:10D)			0.0365		
Thalassiosira weissflogii[a]		7.5		0.025		Harrison and Morel (1986)
Chrysotila (Pleurochrysis) carterae		0.7				
Thalassiosira weissflogii		3.1				Hudson and Morel (1990)
Thalassiosira weissflogii		22		0.033		Morel (1987)
Thalassiosira weissflogii (estimated from graph)		1.4		0.008		Morel et al. (1991)
Thalassiosira pseudonana				0.1		Sunda et al. (1991)
Emiliania huxleyi (Fig. 4)		0.1	0.029	0.00146		Sunda and Huntsman (1995)
Thalassiosira oceanica			0.182	0.00314		
Pelagomonas calceolata			0.0048	0.0007		
Thalassiosira pseudonana			1.031	0.07678		
Thalassiosira weisflogii			0.237	0.051		
Prorocentrum minimum (= *P. cordatum*)			0.744	0.01738		
Emiliania hyxleyi	0.16 µM total FeEDTA	*200*				Hartnett et al. (2012a)
Thalassiosira oceanica		1.18				Maldonado and Price (2001)
Prorocentrum micans				0.03		Sunda and Huntsman (1997)
Prorocentrum minimum (= *P. cordatum*)				0.015		

(continued)

Table 2 (continued)

Organism or Field location	Growth conditions or dominant microalgae	Cultures			Field (or no chelators added to cultures)	References
		K_ρ (nM Fe′)	$K_{\mu Q}$ (nM Fe′)	K_μ (nM Fe′)	K_μ (nM Fe_{diss})	
Synechococcus				0.07		
Thalassiosira pseudonana				0.04		
all together, normalized to SA				0.51		
Phaeocystis antarctica (Southern Ocean)	w/ various EDTA & DFB additions			0.00001		Strzepek et al. (2011)
Proboscia (Southern Ocean)				0.0000163		
Fragilariopsis kerguelensis (Southern Ocean)				0.118		
Eucampia (Southern Ocean)				0.01436		
Thalassiosira antarctica (Southern Ocean)				0.00915		
Actinocyclus sp. (Southern Ocean)					1.14	Timmermans et al. (2004)
Fragilariopsis kerguelensis (Southern Ocean)					0.2	
Corethron pennatum (Southern Ocean)					0.57	
Thalassiosira sp. (Southern Ocean)					0.62	
Chaetoceros dichaeta (Southern Ocean)					1.12	Data from Timmermans et al. (2001), as cited in Timmermans et al. (2004)
Chaetoceros brevis (Southern Ocean)					0.0006	Data from Timmermans et al. (2001), as cited in Timmermans et al. (2004)
Cylindrotheca fusiformis					0.02	Bucciarelli et al. (as cited in Timmermans et al. 2004)
Thalassiosira pseudonana					0.21	Bucciarelli et al. (as cited in Timmermans et al. 2004)
Thalassiosira pseudonana					0.08	Data from Sunda and Huntsman (1995), as cited in Timmermans et al. (2004)
Thalassiosira oceanica					0.04	Data from Sunda and Huntsman (1995), as cited in Timmermans et al. (2004)
Synechococcus	HNLC water w/ DFB			0.00139		Timmermans et al. (2005)
Pelagomonas calceolata				0.000031		

(continued)

Table 2 (continued)

Organism or Field location	Growth conditions or dominant microalgae	Cultures			Field (or no chelators added to cultures)	References
		K_ρ (nM Fe′)	$K_{\mu Q}$ (nM Fe′)	K_μ (nM Fe′)	K_μ (nM Fe_{diss})	
Prasinomonas capsulatus				0.000376		
Phaeocystis antarctica (solitary)	20 µmol photons m^{-2} s^{-1}				0.26	Garcia et al. (2009)
	40 µmol photons m^{-2} s^{-1}				0.045	
	90 µmol photons m^{-2} s^{-1}				0.19	
Phaeocystis antarctica (colonial)	20 µmol photons m^{-2} s^{-1}				0.45	Sedwick et al. (2007)
Pseudo-nitzschia delicatissima				0.013		LeLong et al. (2013)
Pseudo-nitzschia granii				0.005		Marchetti et al. (2008)
Pseudo-nitzschia multiseries				0.023		
Freshwater microalgae						
Dunaliella salina	Fe-replete (Fe citrate additions)				*800*	Paz et al. (2007b)
	Fe-limited (Fe citrate additions)				*200*	
Chlamydomonas reinhardtii	w/ 6 µM HEDTA				*30,000*	Eckhardt and Buckhout (1998)
Parachlorella (Chlorella) kessleri					*7500*	Middlemiss et al. (2001)
Microcystis aeruginosa	for Fe(II)’	*0.000018*		*0.0000036*		Dang et al. (2012)
Tetraselmis suecica	for FeEDTA	*310*				Hartnett et al. (2012b)
Synechocystis sp. PCC 6803		0.27				Kranzler et al. (2011)
Modelled						
Fe(II) model work (using *T.weissflogii* data)		0.23				Sunda and Huntsman (1995)
Fe(II) model work (using *P. minimum* data)		0.12				Sunda and Huntsman (1995) (Marine chemistry)
Field populations						
SERIES (NE Pacific) 12 °C	Nanoplankton				0.08	Kudo et al. (2006)
SERIES (NE Pacific) 16 °C					0.3	
SEEDS (NW Pacific) 9 °C					0.4	
SERIES (NE Pacific) 12 °C	Microplankton				0.1	
SERIES (NE Pacific) 16 °C					0.19	
SEEDS (NW Pacific) 9 °C					0.58	
Humboldt Current					0.17	Hutchins et al. (2002)
Peru upwelling					0.26	
Polar Front Zone (PFZ, 0.33 nM Fe)	Small pennate diatoms				0.41	Blain et al. (2002)

(continued)

Table 2 (continued)

Organism or Field location	Growth conditions or dominant microalgae	Cultures			Field (or no chelators added to cultures)			References
		K_ρ (nM Fe´)	$K_{\mu Q}$ (nM Fe´)	K_μ (nM Fe´)	K_μ (nM Fe_{diss})			
					0.45			
Confluence subantarctic & subtropical front (SAF/STF, 0.29 nM Fe)	Mixed assemblage				0.055			
					0.086			
Southern Subtropical zone (STZ, 0.09 nM Fe)	Small pennate diatoms				0.0925			
Ross Sea					0.09			Cochlan et al. (2002)
Equatorial Pacific Ocean					0.12			Coale et al. (1996)
Equatorial Pacific Ocean					0.12			Fitzwater et al. (1996)
High Si water of the ACC								Coale et al. (2003)
	Phaeocystis				0.27			
	Diatoms (*Thalasiossira & Fragilariopsis*)				0.13			
	Other phytoplankton				0.05			
Ross Sea (PII3 & PII4) community					0.004			
					0.021			
ACC in spring, community					0.013			
					0.111			
ACC in summer, community					0.03			
					0.237			
Southern Ocean, diatoms dominated, Pacific sector					0.038			Cullen et al. (2003)
Southern Ocean Fe enrichment	>20 µm size-fraction	3.3						Maldonado et al. (2001)
	20-2 µm size-fraction	2.6						
		Cultures			**Field (or no chelators added to cultures)**			
		K_ρ (nM Fe´)	**$K_{\mu Q}$ (nM Fe´)**	**K_μ (nM Fe´)**	**K_μ (nM Fe_{diss})**	**K_μ (nM Fe´)**	**K_ρ (nM Fe´)[b]**	
AVERAGE		42.0	0.37	0.040	802.37	0.802		
Average excluding values in italics		3.7	0.37	0.041	0.32	0.00032	2.96	

Half-saturation constants for steady-state Fe uptake rates ($K_{\mu Q}$, $[Fe´]_{nM}$), short-term Fe uptake rates (K_ρ, $[Fe´]_{nM}$), as well as Fe-limited growth (K_μ, $[Fe´]_{nM}$ or $[Fe_{diss}]_{nM}$). In some instances the kinetic parameters were not determined in the original manuscript, thus the available data were used to calculate K, using the hyperbolic, Michaelis-Menten kinetic function [$\rho Fe = \rho_{max} * [Fe´]/(K_\rho + [Fe´])$, or the Monod equation [$\mu_{Fe} = \mu_{max} * [Fe´]/(K_\mu + [Fe´])$. Most culture studies utilized 100 µM EDTA. Otherwise, the chelator used and the concentrations are noted under "growth conditions or dominant phytoplankton". For culture studies where [Fe´] was not specified, concentrations were calculated using MINEQL

[a]The currently accepted name for *Thalassiosira weissflogii* is *Conticribra weissflogii*

[b]Obtained from Maldonado et al. (2001)

diatom *Thalassiosira pseudonana* (Sunda and Huntsman 2011). Low temperature may also enhance Fe uptake by increasing the residence time of photo-chemically produced Fe′ in the presence of photolabile ferric chelates (Sunda and Huntsman 2011).

The effects of diffusion limitation of Fe uptake have been discussed in detail in Morel et al. (1991). In essence, theoretical calculations suggest that even with a very low Fe demand for growth, a phytoplankton cell with a radius ≥10 µm, and dividing once a day, would be diffusion limited for Fe′ uptake in the open ocean. This is due to the relationship between Fe demand of a cell and its Fe uptake rate. The former is proportional to the cellular volume and specific growth rate, while the latter is a function of the number of Fe transporters in the outer cell membrane, and the rate at which Fe diffuses to the cell surface. At a given Fe transporter density on the cell membrane (mol μm^{-2}), a smaller cell will achieved faster Fe uptake rates per unit of cell volume (mol Fe $L^{-1}{}_{cell\ volume}\ h^{-1}$) than a larger cell, and thus would be better able to fulfill its Fe demand for growth. To enhance Fe transport, a larger cell (or one grown at low [Fe]) may increase the number of Fe transporters at the cell surface. However, eventually, the maximum rate of Fe uptake will be limited, not by the number of Fe transporters, but instead by the rate at which Fe′ diffuses to the cell surface, and this effect will be more pronounced for a bigger cell. Indeed, a large cell (r ≥ 30 µm; Sunda and Huntsman 1997) requires such a large number of Fe transporters at the cell surface to fulfill its Fe demand, that even if it only divides once a day, its rate of Fe uptake is diffusion limited.

The chemical factors that affect Fe uptake include: the total Fe concentration in the environment, the oxidation state of Fe (Fe(II) vs. Fe(III)), its chemical speciation (dissolved inorganic species versus organic Fe complexes), the concentration and binding strength of ligands in the environment, as well as pH effect on Fe complexation (Schenck et al. 1988; Sunda and Huntsman 2003; Shi et al. 2010). In essence, strong Fe binding ligands in seawater (or the medium) directly compete with cell surface proteins involved in Fe transport for dissolved inorganic Fe.

The biological factors that influence Fe uptake rates are cellular Fe demand (i.e. the minimum intracellular Fe needed for maximum growth rate), the Fe limited condition of the cells, and the cell population density. The Fe-limited condition of the cells (i.e. the extent to which the cellular Fe content falls short of the minimum intracellular Fe needed for maximum growth rate, and thus, their relative growth rates (μ/μ_{max}) is below 1) exerts a negative feedback regulation of iron transport systems. In addition, Fe transport has been shown to be affected by the physiological condition of the cells especially with regards to Cu (Peers et al. 2005; Maldonado et al. 2006). Extensive discussion of these chemical and biological factors follows below.

In the 1980s and 1990s a series of studies investigating the kinetics of Fe transport in marine phytoplankton provided the foundation of what we know about Fe transport in these organisms (e.g., Anderson and Morel 1982; Harrison and Morel 1986, 1990), and established the Fe′ model (Hudson and Morel 1990; Sunda and Huntsman 1995, 1997). In essence, the Fe′ model predicts a dependency of Fe uptake rates on the concentrations of labile dissolved inorganic Fe species (Fe′), which consist of a mixture of dissolved iron hydrolysis species ($Fe(OH)_2^+$, $Fe(OH)_3$, and $Fe(OH)_4^-$), whose composition is highly dependent on pH (Sunda and Huntsman 2003). These studies also established that Fe uptake occurs via specialized active transport proteins on the plasmalemma (Hudson and Morel 1990, 1993). Indeed, binding of Fe to the transport proteins must first occur, followed by internalization. Thus, these Fe transporters behave as surface ligands with a very high affinity for reaction with Fe′ ($k_L = 2 \times 10^6\ M^{-1}\ s^{-1}$, Hudson and Morel 1990). Iron uptake by these transporters is regulated by a series of reactions:

$$Fe' + L_{surface} \underset{k_L}{\overset{k_L}{\leftrightarrow}} FeL_{surface} \xrightarrow{k_{in}} Fe_{cell} \tag{8}$$

Based on the limited data available, iron uptake by the iron transport system follows typical Michaelis-Menten uptake kinetics, where the rate of Fe uptake is determined by the maximum rate of uptake (ρ_{max}), the concentration of labile dissolved inorganic Fe species ([Fe′]), as well as the half-saturation constant for Fe uptake (K_ρ) (Harrison and Morel 1986, 1990):

$$\rho = \rho_{max} * [Fe'] / (K_\rho + [Fe']) \tag{9}$$

Indeed [Fe′] is the total concentration of labile dissolved inorganic Fe species whose 'effective' reaction rate with the uptake L determines uptake. As a result, K_ρ is determined by the rates of metal-ligand binding (k_L) and dissociation (k_{-L}), as well as the rate of Fe internalization (k_{in}), such that $K_\rho = (k_{-L} + k_{in})/k_L$. Hudson and Morel (1990) demonstrated that Fe transport was under kinetic control, meaning that the rate of internalization of the Fe bound to the cell surface ligands is much faster than its rate of dissociation from the surface ligands, thus the rate of dissociation can be ignored and $K_\rho = k_{in}/k_L$. As discussed above, the maximum rate of Fe uptake (ρ_{max}) is highly affected by the number of transport ligands (L_T) at the cell surface, which can increase by more than 20-fold when cells are experiencing Fe limitation (Harrison and Morel 1986). The expected increase in Fe transport due to higher density of Fe transporters (up to 20-fold) is far greater than that expected from a decrease in cell size (~2 fold, see section above).

When phytoplankton are Fe-limited, their maximum short-term Fe uptake rates (in the presence of non-limiting

[Fe′]) are determined by the number of Fe transporters at the cell surface and the rate of internalization, so that

$$\rho_{max} = k_{in*} L_T^{max} \quad (10)$$

In addition, $K_\rho = k_{in}/k_L$, and under Fe-limiting conditions [Fe′] <<< K_ρ, so the upper limit on their steady-state Fe uptake rates can be simplified from the Michaelis-Menten equation above (Eq. 9) to

$$\rho^{ss} = k_L * L_T^{max} * [Fe] \quad (11)$$

Thus, under steady-state Fe limiting conditions, the growth rate is defined as:

$$\mu = k_L * L_T^{max} * [Fe'] / Q_{Fe} \quad (12)$$

In summary, the Fe′ model predicts that Fe uptake rates are dependent on dissolved inorganic Fe(III) species (Fe′), and that these Fe′ species (be it Fe(II)' or Fe(III)') bind the Fe surface transporters before the Fe is internalized. This Fe′ model has been extremely useful in the last 25 years for laboratory trace metal algal physiology and toxicity studies.

3.2.1 High-Affinity Acquisition Mechanisms for Organically Bound Iron

The development of extremely sensitive analytical techniques in the 1990s allowed measurements of organic complexation of Fe in the sea. The finding that the vast majority of dissolved Fe (>99.9 %) is bound to very strong organic complexes (Gledhill and van den Berg 1994; Rue and Bruland 1995; for a review see Gledhill and Buck 2012) led to the reexamination of the Fe′ model because the calculated Fe′ in the open ocean, in the absence of photochemical reactions, was shown to be too low to support sufficient Fe′ to meet cellular requirements of open ocean phytoplankton (Rue and Bruland 1995). At the same time that Fe speciation in the ocean was being unraveled, laboratory experiments were also revealing new insights into Fe transport in phytoplankton (e.g. Allnutt and Bonner 1987a, b; Jones et al. 1987; Soria-Dengg and Horstmann 1995; Hutchins et al. 1999b; Maldonado and Price 2000, 2001).

Physiological studies of Fe nutrition in phytoplankton were conducted using model siderophores, such as desferrioxamine B (DFB), to induce severe Fe limiting conditions in the growth medium. Siderophores (from the Greek "iron carriers") are some of the highest affinity ferric chelators known in nature (Neilands 1995). These molecules have a moderately low molecular mass (usually <1000), and are secreted mainly by Fe-limited bacteria, fungi and grasses to scavenge Fe from the environment. Depending on the Fe(III) binding group, siderophores can be classified as hydroxamates, catecholates or mixed-ligand types (containing another Fe binding group such as α-hydroxy-carboxylate, in addition to hydroxamate or catecholate ligand groups). Once the siderophore complexes a Fe(III) ion, the ferrisiderophore is transported into the cell via specific membrane bound siderophore transporters. In the early 1990s, eukaryotic phytoplankton had not been shown to be able to internalize ferrisiderophores. Therefore, siderophores were added to the medium to achieve extreme in situ Fe-limiting conditions, where Fe′ is practically undetectable.

Surprisingly, these physiological studies showed that Fe-limited phytoplankton could access Fe from these strong organic complexes, using an enzymatic reductive mechanism at the cell surface (Allnutt and Bonner 1987a, b; Soria-Dengg and Horstmann 1995; Maldonado and Price 2000, 2001; Weger et al. 2002; Matz et al. 2006). The ability of the phytoplankton to access these strong organic ligands was induced under Fe limitation (Allnutt and Bonner 1987a; Maldonado and Price 1999, 2001; Weger et al. 2002; Strzepek et al. 2011), suggesting that this was a high-affinity Fe transport system similar to that of other well studied eukaryotes (i.e. the yeast *Saccharomyces cerevisiae*) (for a review, see Van Ho et al. 2002). It is now evident that the high-affinity Fe transport system in phytoplankton involves the activity of Fe permeases in the outer cell membrane, as well as ferric reductases and multi-Cu containing ferroxidases (Fig. 3). Indeed, physiological evidence for the existence of a Fe reductive pathway in microalgae is widespread (see Table A1 in Shaked and Lis 2012).

The ferric reductases are transmembrane proteins in the plasmalemma that transfer an electron from cytosolic NAD(P)H to the iron complexes outside of the cell, including Fe strongly bound in organic chelates. Since most organic Fe chelators have a much higher affinity for Fe(III) than Fe(II), Fe reduction results in a dissociation of the Fe(II) from the ligand, and thus an increase of inorganic Fe(II) at the cell surface. The rates of Fe reduction of the organic complexes are inversely related to the stability of the Fe(III) coordination complex (Maldonado and Price 2001). However, once the Fe is reduced, the fraction of this Fe taken up into the cell depends critically on the relative concentration (and affinities) of the free organic ligands in solution and the free Fe transporters at the cell surface (see the Fe(II)s model below).

Once the Fe(III) is reduced to Fe(II) and dissociates from the organic complex, the free Fe(II) then binds with a receptor site on an iron membrane transport-complex (which consists of a multi-Cu containing oxidase and the permease). The Fe(II) is subsequently (and likely rapidly) oxidized to Fe(III) by multi-Cu oxidases associated with the permease, followed by internalization of the Fe(III) by the permease. These reduction and oxidation steps facilitate the transfer of

iron from ligands in the external medium to the receptor site on the iron membrane transport-complex. They also impart specificity and selectivity to the Fe transport system, which is crucial in the case of essential trace elements. Physiological studies with Fe-limited diatoms have measured comparable rates of Fe(III) reduction of organically bound Fe and Fe(II) oxidation (Herbik et al. 2002a, b; Maldonado et al. 2006). The coupling between these oxidation and reduction rates may ensure that, just after the Fe(III) is reduced by the reductase, the Fe(II) is rapidly oxidized by the putative multi-Cu oxidase, before it is internalized by the permease as Fe(III). The proximity of these putative reductases, oxidases and permeases at the cell surface of microalgae may contribute to an efficient cascade of redox reactions. Close proximity of the reductases and oxidases may also allow the formation of a ternary complex, ferric reductase-Fe(III)siderophore-putative Fe(II) oxidase, which may facilitate the reductive dissociation of Fe from very strong organic complexes (Boukhalfa and Crumbliss 2002).

The occurrence of the reductive Fe uptake pathway to acquire Fe from strong organic Fe complexes is widespread in freshwater and marine microalgae (see Table A1 in Shaked and Lis 2012). In many instances, physiological data have been complemented with genomic and proteomic data. One field study also measured Fe reduction rates of organically bound Fe by in situ phytoplankton (Maldonado and Price 1999). Most recently, data from the Global Ocean Survey (GOS) metagenomes have shown that ferric reductases are characteristic of marine eukaryotic phytoplankton Fe uptake systems (Desai et al. 2012). Physiological evidence for the multi-Cu containing oxidases is still limited to a few species, including *Chlamydomonas reinhardtii*, *Thalassiosira pseudonana* and *T. oceanica* (Herbik et al. 2002a, b; La Fontaine et al. 2002; Peers et al. 2005; Maldonado et al. 2006). Molecular and genomic evidence for the occurrence of multi-Cu-containing oxidases is starting to emerge (Maldonado et al. 2006; Kustka et al. 2007; Paz et al. 2007a; Guo et al. 2015).

Recently, a limited set of studies has demonstrated that Fe reduction is also a necessary step for the acquisition of inorganic Fe in the presence of weak organic ligands (such as EDTA), where the dissolved inorganic Fe(III) pool is significant (Shaked et al. 2005); in this case the ferric reductase reduces Fe(III)' to Fe(II)'. As discussed above in the "inorganic Fe acquisition section", Fe uptake in this case is still a function of the concentrations of labile dissolved inorganic Fe(III), because the production of Fe(II)' depends on concentration of Fe(III)', and can be predicted with the Fe′ model (or Fe(III)' model). However, these studies have concluded that Fe(II) is an obligate intermediate in phytoplankton Fe acquisition, regardless of whether the cells are acquiring Fe(III) from inorganic or organic Fe(III) complexes (Shaked et al. 2005). Yet, it is important to emphasize that the high-affinity Fe permeases ferry Fe(III) across the plasma membrane, and not Fe(II). Thus, if Fe(II) is an obligate intermediate for Fe acquisition of inorganic Fe (at non-saturating Fe′ concentrations), this Fe(II) is either oxidized by a multi-Cu-containing oxidase-Fe permease complex, like that of the high-affinity Fe transport system for organically bound Fe, or alternatively, is directly internalized as Fe(II) via lower affinity divalent transporters such as ZIP and NRAMPS (see below). Further research is needed to elucidate the mechanism of uptake of Fe(II) after the enzymatic reduction of Fe complexes.

Based on the new findings that organically complexed Fe is bioavailable via a reductive mechanism at the cell surface, and that inorganic Fe acquisition also requires a reductive step, Shaked et al. (2005) proposed a new conceptual model for Fe transport by phytoplankton, "the Fe(II)s model" (Fig. 6). The name of this model was chosen to emphasize that reduction of Fe at the cell surface is an obligate intermediate in Fe acquisition. Unfortunately, to the non-specialist, this name gives the false impression that Fe(II) is the Fe species that is internalized by the phytoplankton.

The Fe(II)s model builds on the previous kinetic model of Fe uptake (Hudson and Morel 1990), "the Fe′ model", which predicted Fe uptake rates based on the concentration of labile dissolved inorganic Fe(III) in the bulk medium. The Fe′ model is valid for well-buffered Fe media, in the presence of excess concentrations of aminocarboxylate chelating agents. However, the Fe′ model is incomplete because it is unable to predict rates of Fe uptake when the ratio of [Fe(III)'] to organically bound iron is extremely low; as observed in the presence of very strong organic Fe complexes, such as DFB. The Fe(II)s model is meant for calculating rates of Fe uptake when the concentrations of Fe(III)' in the media or seawater are non-saturating. This model reconciles the Fe′ model with the new data on the extracellular, biologically mediated Fe reduction of, not only, strong organic Fe complexes, but also weak organic complexes and inorganic Fe species (Shaked et al. 2005). The Fe(II)s model is not mechanistic, but is based on the premise that the Fe acquisition mechanism in marine eukaryotic phytoplankton is similar to that of yeast and involves Fe reductases at the cell surface, followed by the activity of a Fe transport complex, which contains a multi-Cu-containing Fe oxidase and a permease. It aims to predict Fe uptake rates based only on reduction rates of Fe(III), either bound within organic or inorganic complexes. Specifically, the Fe(II)s model predicts Fe uptake based on the concentration of Fe(II) at the cell surface, which is determined by (a) reduction of organically bound Fe (FeY) at the cell surface, (b) reduction of Fe(III)' at the cell surface, and (c) the competition for free or inorganic Fe (*assigned a nomenclature of Fe(II)s, but includes inorganic and free Fe(II) or Fe(III) species*) between the cell surface Fe transport complexes and the free organic ligand in the medium (Z). [Note: in cases where only one ligand is present in the medium, Y = Z]. Thus, only three parameters need to be

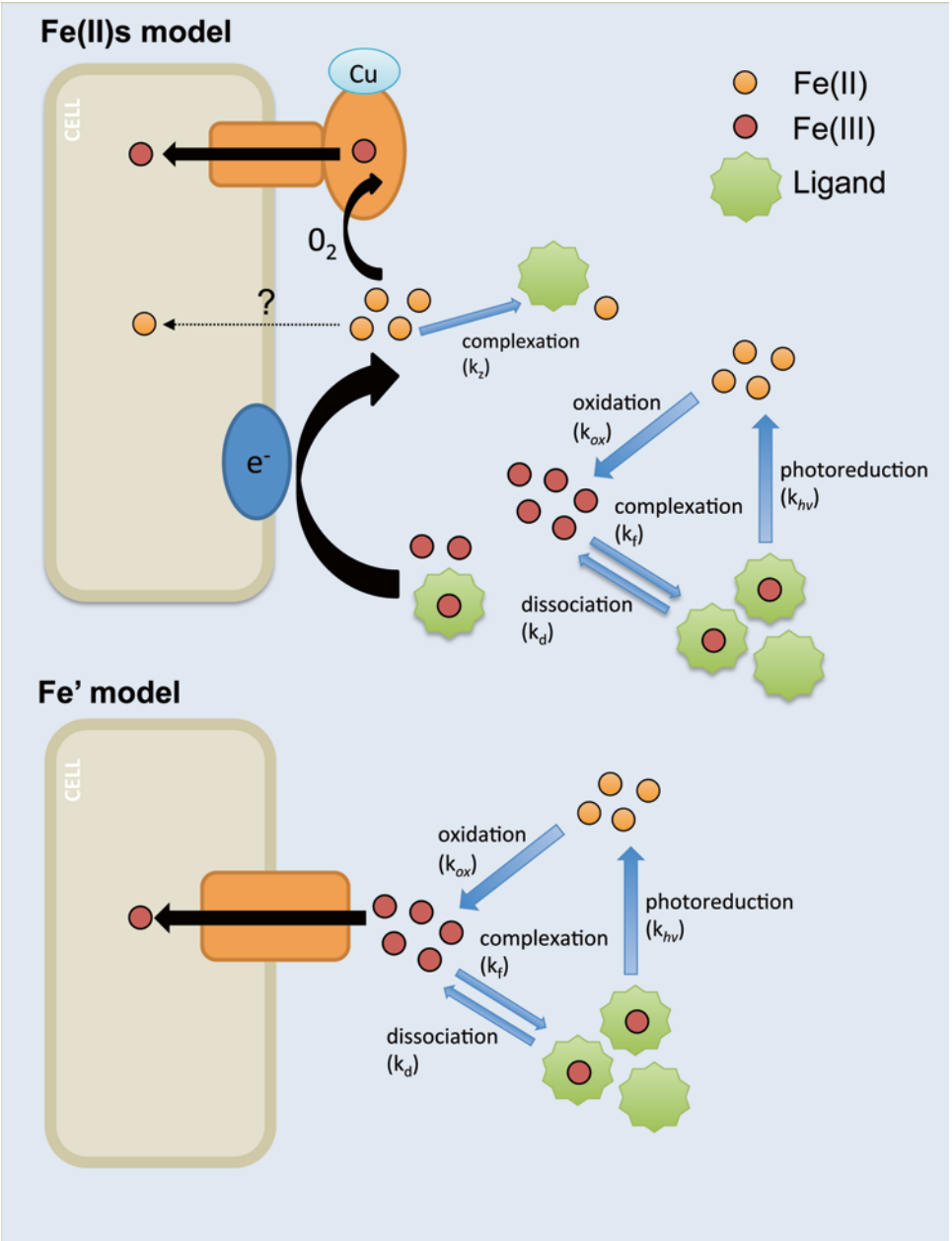

Fig. 6 Cartoon schematic of the most common Fe transport kinetic models in microalgae: Fe´ model (Hudson and Morel 1990) and the Fe(II)s model (Shaked et al. 2005). These kinetic models are for a medium with an excess of a photolabile organic ligands, inorganic Fe(III)', and inorganic Fe(II)'. Fe(III)' is more chemically stable than Fe(II)' and is the dominant species for Fe transport. The concentration of Fe(III)' is maintained at equilibrium by dissociation (rate constant k_d) and complexation (rate constant k_f) reactions with the organic ligands, as well as photoreduction of Fe(III) bound to photolabile organic ligands (rate constant k_{hv}) to Fe(II)', which quickly oxidizes to Fe(III)' (rate constant k_{ox}) at seawater pH (~8.1). In the Fe´ model, Fe(III)' are the species reacting with the Fe transporter. In the Fe(II)s model, both the Fe(III)' and the organic Fe have to be enzymatically reduced to Fe(II) before Fe is transported into the cell through the Fe(III) transporter complex. This complex is hypothesized to contain a multi-Cu containing oxidase and an Fe permease. Fe(II)' may also be directly internalized, but these divalent transporters are low affinity and are not illustrated here. For details of the Fe(II)s model and the Fe´ model, see Shaked et al. (2005), and Hudson and Morel (1990), respectively. Most model studies have used eukaryotes, though some prokaryotes have proven to have similar kinetics

specified: (a) cellular FeY reduction constant (k^{FeY}_{red}; L cell^{-1} h^{-1}), (b) cellular Fe(III)' reduction constant ($k^{Fe(III)'}_{red}$; L cell^{-1} h^{-1};), and (c) the ratio (k_z/k_{up}; M^{-1}) of effective formation constant of Fe(II)s complexation by excess ligand Z (k_z; M^{-1} h^{-1}) and the rate constant of Fe(II)s complexation by the membrane Fe transport complex under non-saturating [Fe(III)'] (k_{up}; h^{-1}).

In the presence of weak organic Fe complexes, such as EDTA (i.e. [EDTA] = 5–100 µM & Fe at ~100 nM), Fe′ is relatively high and reduction of Fe(III)' dominates. Since EDTA cannot compete effectively with the cell Fe transport complex for the Fe(II)s, Fe uptake rate is simply a function of $k^{Fe(III)'}_{red}$ and the [Fe(III)'], so that Fe uptake (ρFe; mol Fe cell^{-1} h^{-1}) is defined as

$$\rho = k^{Fe(III)'}_{red} * \left[Fe(III)'\right] \quad (13)$$

In the presence of very strong organic ligands, such as FeDFB, [Fe(III)'] is practically non-existent, thus reduction of FeY dominates, and competition for Fe(II)s between the cell surface Fe transport complexes and the free organic ligand in the medium (Z) has to be incorporated into the equation (note that Z can be DFB or some other organic ligand), so that

$$\rho = k^{FeY}_{red} * [FeY] / \left(\left(k_z [Z] / k_{up} \right) + 1 \right) \quad (14)$$

The Fe(II)s model is able to reconcile the Fe(III)' model (because in the presence of weak organic Fe complexes, the inorganic Fe(III) is the substrate for cellular surface Fe reduction and uptake) with the laboratory and field data showing Fe uptake from strong Fe organic complexes, such as siderophores. This is a kinetic model for Fe acquisition under non-saturating Fe concentrations, in which the activities of the Fe reductases and the Fe-transport complexes (multi-Cu oxidases and Fe permeases) increase proportionally to the concentrations of Fe(III) and Fe(II), respectively. These non-saturating conditions are prominent in the open ocean, as well as in laboratory experiments under Fe limiting conditions. Processes able to increase [Fe′] in surface oceanic waters, such as photoreduction of organic Fe complexes (Barbeau et al. 2001), photodissolution of Fe oxides (Waite and Morel 1984) or superoxides (reviewed by Rose 2012) would potentially increase Fe(III)' for cellular reduction at the cell surface. Shaked et al. (2005) estimated that in the open ocean, the Fe needed for phytoplankton growth is supplied by cellular reduction of mainly FeY (2/3rds) and some Fe(III)' (1/3rd).

3.3 Molecular Aspects of Fe Transport

From a molecular perspective, the following questions can be addressed: (1) Is Fe uptake achieved by a single Fe transporter or a Fe transport complex (i.e. a transport system involving multiple proteins)? (2) What are the expression patterns of the proteins involved in Fe acquisition? (3) How are these proteins arranged in the plasmalemma? and, (4) How many transmembrane domains do these transporters have?

3.3.1 Eukaryotic Microalgae

Though we are starting to learn more about molecular aspects of Fe transport in marine eukaryotic microalgae (reviewed by Blaby-Haas and Merchant 2012) so far, the best characterized Fe transporters in microalgae are those of the freshwater green alga *Chlamydomonas reinhardtii* (reviewed by Merchant et al. 2006). Fe(III) transport occurs through FTR (Fe TRansporter) (Table 3). These FTRs are localized to the plasma membrane and form a complex with multi-Cu oxidases (referred to as FOXs, FET3Ps, or MUCOXs depending on the organisms). In addition, these Fe transport complexes are associated with ferric reductases (or FREs).

Three types of ferric reductases are involved with Fe(III) reduction in eukaryotes: NAD(P)H oxidases (NOX; cytochrome b_{558}), cytochrome b_5 reductases, and cytochrome b_{561}. In *C. reinhardtii*, FRE1 (a NOX enzyme) is localized in the plasma membrane and is highly induced under Fe deficiency; thus, this Fe reductase is hypothesized to be involved in Fe acquisition (Allen et al. 2007). In diatoms, the following Fe reductases have been identified: *T. pseudonana* (TpFRE1 and TpFRE2, Kustka et al. 2007), *Phaeodactylum tricornutum* (PtFRE1-4, Allen et al. 2008), and *T. oceanica* (ToFRE1, Lommer et al. 2012). TpFRE1 is similar to cytochrome b_5 reductase, while PtFRE3 and PtFRE4 are similar to cytochrome b_{561} (Allen et al. 2008), and TpFRE2, PtFRE1 and PtFRE2 to NOX proteins (Blaby-Haas and Merchant 2012). The transcript abundance of many of these diatoms' FREs increases under Fe limitation (Kustka et al. 2007; Allen et al. 2008), thus Fe reduction is believe to be an integral component of the high-affinity Fe transport system. In addition, Fe reduction is also a required step for Fe uptake of inorganic Fe into the cell (Shaked et al. 2005), as well as mobilization through intracellular membranes, such as into the chloroplast or out of the vacuole (reviewed by Nouet et al. 2011).

Putative multi-Cu oxidases have been identified in many genomes, including FOX in *C. reinhardtii*, *Chlorella variabilis*, *Coccomyxa* sp. C-169, and *Volvox carteri*; FET3P in *Fragilariopsis cylindrus* and *Thalassiosira pseudonana*; and MUCOX2 in *Thalassiosira oceanica* (Blaby-Haas and Merchant 2012). As with the ferric reductases, the transcript abundance of these genes is enhanced in Fe-limited cultures of *C. reinhardtii* (La Fontaine et al. 2002; Allen et al. 2007) and *T. oceanica* (Maldonado et al. 2006). Surprisingly, gene homologs to these multi-Cu containing ferroxidases have not been found in *P. tricornutum* (Allen et al. 2008), nor is its expression always enhanced in Fe-limited *T. pseudonana* (see Maldonado et al. 2006; Kustka et al. 2007). Multi-Cu oxidases contain four Cu atoms, and are divided into two types: FET3p-type and ceruloplasmin. While the multi-Cu oxidases in green algae are similar to the second type, the ones from diatoms seem more comparable to FET3p. Interestingly, the MCO of the pelagophyte *Aureococcus anophagefferens* is different from these two types, and *Cyanidioschyzon merolae* is lacking a MCO (Blaby-Haas and Merchant 2012). A physiological interaction between Fe and Cu has been shown in eukaryotic algae (Herbik et al. 2002a, b; La Fontaine et al. 2002; Peers et al. 2005; Maldonado et al. 2006; Annett et al. 2008; Guo et al. 2010, 2012), as well as cyanobacteria (Nicolaisen et al. 2010). For example, when diatoms are co-limited by Cu and Fe, the oxidation rates of Fe are slower, resulting in slower Fe uptake (Maldonado et al. 2006).

In yeast, internalization of Fe by the multi-Cu oxidase-Fe permease complex has been proposed to occur by a Fe-channeling mechanism which is activated when the multi-Cu oxidase oxidizes Fe(II) to Fe(III) and transfers it to the Fe permease (Stearman et al. 1996). In addition, the maturation of the FET3 and FTR proteins is coupled in the

Table 3 Most common proteins involved in Fe transport separated between prokaryotic and eukaryotic phytoplankton

Protein		Abbreviation	Localization	Fe oxidation state	Microalgal group
Eukaryotes					
Group A: Fe transport into the cytoplasm (from outside of cell or from the vacuole)					
Fe(III) transport systems (from inorganic and organic Fe(III) complexes)					
Ferric reductase (coupled to NAD(P)H oxidase)		FRE (Ferric REductase)	Plasma membrane	Fe(III)	All
	Cytochrome b558	NOX (RBOL)			Green, diatoms
	Cytochrome b5 reductase	CBR1 (Cytochrome b5 reductase)			Diatoms
	Cytochrome b561 (ascorbate as electron donor)				Diatoms
	Riboflavin				
High-affinity Fe(III) Transporter Complex (associated with ferric reductases, see above)			Plasma membrane		
	Multi-Cu containing ferroxidase	MCO/FOX/MUCOX, FET3P		Fe(II)	
	Fe(III) permease	FTR (Fe TRansporter)		Fe(III)	All, except prasinophytes and some chromalveolates
Uncommon/Novel Fe(III) transporters					
	Fe(III) binding proteins in the extracellular space, may deliver Fe to FTR	FEA-like	Plasma membrane	Fe(III)	*C. reinhartii*, *M. pusilla*, *and O. lucimarinus*, chromalveolates
	Iron-starvation induced protein	ISIP (Iron-Starvation Induced Protein)	Plasma membrane	Fe(III)	Diatoms
	Transferrin-like Fe(II) transport protein	TRF (TRansFerrin-like)	Plasma membrane	Fe(III)	*Dunaliella*
	Ferrichrome binding protein	FBP (Ferrichrome Binding Protein)	Plasma membrane	Fe(III) chelates	*Phaeodactylum*
Fe(II) transport systems					
ZIP-IRT	Passive divalent transporters (non-Fe specific, Zn transporter)	(ZIP, ZInc Transporter; IRT, Iron Regulated Transporter)	Plasma and vacuolar membrane and secretory system	Fe(II)	*C. reinhardtii*, *P. tricornutum*
NRAMP	metal/hydrogen antiporter or symporter (non-Fe specific; Fe/Mn transporter)	NRAMP (Natural-Resistance-Associated-Macro-Phage Protein)	Plasma and vacuolar membrane and secretory system	Fe(II)	All, except *P. tricornutum*
	Subgroup I				Major lineages
	Subgroup II				Prasinophytes and *C. merolae*
Group B: Fe trafficking within the cell, to sites of Fe assimilation or storage					
CDF	intracellular Fe(II) transporters	CDF (Cation Diffusion Facilitator)	Various intracellular compartments	Fe(II)	Diatoms, *Ectocarpus*, *Aureococcus*
	FieF-like				*Micromonas*
MSC	intracellular Fe(II) transporters	MSC (Mitochondrial Solute Carrier)	Mainly localized to mitochondria	Fe(II)	
	MFL1 (MitoFerrin-Like 1)				*C. reinhardtii*
	MML1 (MTM1 subgroup)				

(continued)

Table 3 (continued)

Protein		Abbreviation	Localization	Fe oxidation state	Microalgal group
VIT1/Ccc1	intracellular Fe(II) transporters	VIT (Vacuolar Iron Transporter) and Ccc (Cation-Chloride Co-transporter)	Vacuolar membrane	Fe(II)	All
	CVL1/ CVL2 (Pcl1- subgroup)				*C. reinhardtii*
Prokaryotes					
Ferric reductase					
	Cytochrome c oxidase (associated w/FeoB)	ARTO (Alternate Respiratory Terminal Oxidase)	periplasmic membrane	Fe(III)	*Synechocystis*
Fe(III) transport systems (ABC transport system, from inorganic and organic Fe(III) complexes)					
	Fe(III) binding protein	IdiA/FutA/AfuA	Periplasmic space	Fe(III)	*Trichodesmium*, *Prochlorococcus*, *Synechococcus*, *Synechocystis*, *Crocosphaera* (abundant in picocyanobacteria)
	Fe(III) permease	IdiB/FutB	Periplasmic membrane	Fe(III)	
	ATPase	IdiC/FutC		Fe(III)	
Fe(II) transport systems					
	Small soluble protein	FeoA		Fe(II)	
	Predicted Fe(II) permease	FeoB	periplasmic membrane	Fe(II)	*Synechococcus*, *Trichodesmium*, *Synechocystis*, rare in *Prochlorococcus*
	Predicted regulator	FeoC		Fe(II)	
	NRAMP				
	ZIP				
TonB-dependent siderophore transporters (TBDTs)				Fe(III) chelates	
For schizokinen transport into the cell (SchT)		Periplasmic binding protein (FhuD/FutA/FecA)			*Anabaena* sp.
		Membrane embedded permease (FhuB/FutB/ FecCD)			
		ATP-binding protein (FhuC/ FutC/FecB)			
		TonB-energy translocation system (ExbB/ExbD)	periplasmic membrane		
For heme		TonB & energy translocation system (ExbB/ExbD)			*Prochlorococcus*
Siderophore synthesis					
NRPS pathway and PKS (polyketide synthases)		Non-Ribosomal Peptide Synthase (NRPS)			More prevalent in filamentous and heterocyst cyanobacteria, picocyanobacteria *P. marinus*
	Condensation				(Absent in *Prochlorococcus, Synechococcus* and *Synechocystis*)

(continued)

Table 3 (continued)

Protein		Abbreviation	Localization	Fe oxidation state	Microalgal group
NIS synthesis pathway for hydroxamate and mixed-hydroxamate sideophores		Nrps-Independent Siderophore synthesis (NIS)			*Anabaena* sp., *Synechococcus* and *Prochlorococcus* (prasinophyte *O. lucimarinus*, an eukaryote)
	Acetyl transferase (AlcB)				
	Siderophore synthetase for Aerobactin (IucA/ IucC & IucD)				
	Pyridoxal 5′-phosphate dependent decarboxylase for Rhizobactin (RhhB)				*P. marinus*
	diaminobutyrate-2-oxoglutarate amonotransferase for Rhizobactin (RhhA)				Cyanobacteria

Also provided are the protein abbreviations, their localization within the cell, the Fe oxidation states and the phytoplankton groups in which these proteins are known to be present. The phytoplankton groups identified for each protein are tentative, based on available genomic data and should therefore be interpreted with caution

secretory pathway, so that only the complex containing both proteins is present in the plasma membrane (Stearman et al. 1996). FTR1 homologs are common in microalgal genomes, except in prasinophytes and some chromalveolates (Blaby-Haas and Merchant 2012). Fe deficiency induces FTR1 genes in *T. pseudonana* (Kustka et al. 2007) and *C. reinhardtii* (La Fontaine et al. 2002; Allen et al. 2007). When neither FOX1 nor FTR1 are present, as in prasinophytes or *P. tricornutum*, direct uptake of organic Fe complexes might occur (Blaby-Haas and Merchant 2012). Indeed, a gene encoding a putative siderophore binding protein was recently identified in *P. tricornutum* (Allen et al. 2008).

In general, algal high-affinity Fe transporters are structurally divergent from those well characterized in *Saccharomyces cerevisiae*. Novel proteins have been hypothesized to be involved in Fe transport, such as FEA1- and FEA2-like protein in *C. reinhardtii* (Allen et al. 2007), FEA1-like protein in *Micromonas pusilla* and *Ostreococcus lucimarinus* and "Iron-starved-Induced Proteins" (ISIPs) in diatoms (Lommer et al. 2012). Since *M. pusilla* and *O. lucimarinus* lack typical, high-affinity Fe transport FTR1 homologs, these FEA1 proteins may facilitate Fe-specific transport in these organisms (Blaby-Haas and Merchant 2012).

In addition to Fe(III) transporters, Fe(II) transporters such as those belonging to the ZIP (reviewed by Gaither and Eide 2001) and NRAMP (reviewed by Nevo and Nelson 2006) families are found in microalgae (Table 3). An important difference between the Fe(III) transporters discussed above and the ZIP and NRAMP Fe(II) transporters is their specificity. In essence, ZIP and NRAMP proteins may transport Fe(II) in addition to other divalent metals. Thus, these Fe(II) transporters have been hypothesized to be: (a) low(er)-affinity plasma membrane Fe permeases, or (b) proteins involved in the trafficking of intracellular Fe to various cellular compartments. The ZIP family was named after the first discovery of a Zn transporter (Zrt1p and Zrt2p; Zhao and Eide 1996a, b) in *S. cerevisiae* and the Fe transporter IRT1 in *Arabidopsis* roots (Eide et al. 1996). ZIP transporters may be localized to plasma and vacuolar membranes or the secretory system. ZIP metal transporters are believed to be passive, mediating metal transport via concentration gradients (Lin et al. 2010). Within the ZIP family, there are four groups of transporters, depending on their phylogenetic relationships: Subfamilies I (with mainly fungal and plant proteins) and II (with plant and animal proteins), GufA (with prokaryotic and eukaryotic proteins) and LZT (with human LIV-1 Zn transporter as the founder) (Gaither and Eide 2001). ZIP transporters are not metal specific but are able to transport a variety of divalent cations, such as those of Fe, Cu, Zn, and Mn. In *C. reinhardtii*, there are 13 ZIP family members, including IRT1 and IRT2 (from the GufA family), both of which are up-regulated under Fe deficiency (Allen et al. 2007). In addition, IRT1 and IRT2 are induced under Zn and Cu limitation (Castruita et al. 2011), respectively. Since IRT2 is also a potential target for the Cu responsive transcription factor CRR1, IRT2 has been hypothesized to be an intracellular Fe(II) transporter delivering Fe to cytochrome c_6, or an alternative plasma membrane Fe transporter when Cu is limiting and the high affinity Fe transporter-complex is not functional (Blaby-Haas and Merchant 2012). The diatoms *P. tricornutum* (Allen et al.

2008) and *T. pseudonana* (Kustka et al. 2007) also have ZIP homologs. As in *C. reinhardtii*, the gene transcript of a ZRT, IRT-like protein, belonging to the ZIP family, was enriched under Fe deficiency in *P. tricornutum* (Allen et al. 2008). In *T. pseudonana*, five genes encoding ZIP-like transporters have been identified, and the expression of some of these genes are enhanced under Fe deficiency or repressed after a Cu addition to a low Cu culture (Guo et al. 2015).

Another family of metal permeases able to transport Fe(II) is the NRAMP family (reviewed by Cellier et al. 2001). This family is named after its first identified member, a Natural-Resistance-Associated Macro-Phage protein 1. Most NRAMP permeases are able to transport divalent cations, mainly Fe and Mn, into the cytoplasm. As with ZIP proteins, NRAMPs may be localized to the secretory system, plasma and vacuolar membranes. NRAMP proteins rely on a proton gradient for metal transport. Phylogenetically, the NRAMP family is divided into four subfamilies: prokaryotic MntH group A, B, and C, as well as a eukaryotic group (Cellier et al. 2001). Within the eukaryotic group, three clusters are found: subgroup I (with NRAMPs from major eukaryotic lineages), II (NRAMPs from plants, prasinophytes and *C. merolae*), and III (fungal proteins). Most microalgal genomes to date contain one NRAMP homolog (Blaby-Haas and Merchant 2012), except for *P. tricornutum*, which lacks an NRAMP homolog (Kustka et al. 2007). The *Chlamydomonas* genome encodes four NRAMPs: NRAMP1, NRAMP2, RET1 and NRAMP4, although some of these proteins do not have metal transport activity (Blaby-Haas and Merchant 2012). An ortholog of NRAMP4 is highly up-regulated in Fe-limited *T. pseudonana*, however whether this permease is located to the plasma or vacuolar membrane remains to be established (Kustka et al. 2007). In a more recent study, the expression of TpNRAMP was up-regulated not only by Fe limitation, but also by low Cu (Guo et al. 2015). Once Fe enters the cell, it must be taken to its sites of incorporation or storage (for a review, see Nouet et al. 2011).

3.3.2 Prokaryotic Phytoplankton

Because siderophore production is more prominent in prokaryotic organisms than in eukaryotes, since the 1970s many cyanobacteria have been tested for their ability to produce siderophores. Cyanobacteria, both marine and freshwater, are able to produce hydroxamate- (i.e. synechobactin A–C, (Ito and Butler 2005); schizokinen, (Simpson and Neilands 1976)) and catecholate-type siderophores (i.e. anachelin-H, and anachelin-1 and -2 (Beiderbeck et al. 2000)), though the former is more commonly synthesized (reviewed by Gademann and Portmann 2008). Interestingly, some of the marine cyanobacterial siderophores are photolabile, and have a fatty acid tail, which is believed to enhance the affinity of the siderophore for the bacterial membrane (Ito and Butler 2005), as previously shown for marine heterotrophic bacteria (Barbeau et al. 2003; Martinez et al. 2003). These two characteristics might aid in Fe acquisition in the dilute oceanic environment.

Siderophores can be synthesized by the non-ribosomal peptide synthase (NRPSs) pathway or by the NRPS-independent siderophore (NISs) biosynthesis pathway. NRPSs are normally associated with polyketide synthases (PKSs) (Table 3). Several genes coding for NRPSs and PKSs have been found in cyanobacteria genomes (Silva-Stenico et al. 2011), but in some cases the specific siderophore that is produced by these synthases is unknown. Both NRPSs and PKSs genes are more prevalent in filamentous and heterocystous cyanobacteria, but were absent in many unicellular strains, including *Synechocystis*, *Prochlorococcus*, and *Synechoccocus* (Ehrenreich et al. 2005). In general, NRPSs genes seem to be more prominent in cyanobacteria genomes than NISs (Hopkinson and Morel 2009). However, many NRPSs pathways synthesize secondary metabolites, instead of siderophores. Thus, the presence of NRPSs genes only suggests the potential for siderophore synthesis. In contrast, finding genes encoding proteins in NIS pathways are indicative of siderophore biosynthesis (mainly hydroxamates and mixed-hydroxamate ligands). In a study of marine prokaryotic genomes, genes for the NIS siderophore biosynthesis pathway were only found in 15 % of the marine prokaryotic genomes, and were completely absent from the oceanic environmental samples (Hopkinson and Barbeau 2012). The low abundance of siderophore biosynthesis genes in the ocean suggests that many marine cyanobacteria utilize non-siderophore iron uptake systems or may be involved in siderophore Fe piracy by stripping the Fe off ferrisiderophores at the cell surface (via a reductive mechanism or a ternary complex formation). Recent studies on the diversity of iron uptake systems in the ocean examined sequenced genomes of marine microbes (eukaryotes and prokaryotes), as well as metagenomes from GOS (Desai et al. 2012). They demonstrated the presence of some of the components of rhizobactin siderophore synthesis in cyanobacteria and eukaryotic phytoplankton genomes, as well as in the metagenomes (though at much lower abundance). One of the few eukaryotic microalgae previously shown to have a siderophore biosynthesis pathway is the prasinophyte *O. lucimarinus* (Palenik et al. 2007).

Little is known about how cyanobacteria secrete siderophores to the environment after intracellular synthesis. The only siderophore secretion pathway studied in detail is that of *Anabaena* sp. PCC 7120. Export through the inner membrane (the plasmalemma) is suggested to be mediated by a transporter from the Resistance, Nodulation and cell Division (RND), the Major Facilitator (MFS) or the ATP-binding cassette (ABC) superfamilies, while transport through the outer membrane is suggested to occur by a TonlC-type protein, as in other bacteria (Bleuel et al. 2005; and reviewed by Miethke

and Marahiel 2007). In *Anabaena* sp. PCC 7120, siderophore schizokinen secretion is mediated by the MFS-type protein SchE in the inner membrane and by the TonlC-type protein HgdD in the outer membrane (Nicolaisen et al. 2010).

Siderophore acquisition has been studied in detail in *Escherichia coli* and *Pseudomonas aeruginosa*. Siderophore-specific transporters are found in the outer membrane of Gram-negative bacteria, and are part of the TonB-dependent transporter (TBDT) family. Although TBDTs are known to transport a variety of substrates, their structure consists of a membrane bound 22-stranded β-barrel (poorly conserved), and a N-terminal plug domain (highly conserved) located within the barrel. Molecular dynamic simulations indicated that binding of siderophores, such as ferrichrome, initiates a signaling mechanism that ultimately leads to the TonB-mediated partial or total removal of the core domain from the β-barrel, thus opening up a permeable pore (Faraldo-Gomez and Sansom 2003; Faraldo-Gomez et al. 2003). In many cases, the substrate-binding site in the extracellular pocket of the transporter lacks sequence conservation (Chimento et al. 2005), thus allowing TBDTs to have specificity for a variety of substrates, besides siderophores, such as haeme, vitamin B_{12} and Fe-citrate (reviewed by Schauer et al. 2008).

Even though many TBDTs have been identified in cyanobacteria (Mirus et al. 2009), their function in Fe transport has only been shown in a few species. For example, a TBDT belonging to *Anabaena* sp. PCC 7120 has been identified (SchT) for the transport of a specific siderophore, schizokinen (Fig. 4) (Nicolaisen et al. 2008). SchT is encoded by *alr*0379; it spans across the outer membrane, and it is fueled by a "Ton" system anchored in the periplasmic membrane. Bacterial TonB-dependent transporters consists of an energy transducing unit, TonB, and two stabilizing units, ExbB and ExbD (reviewed by Noinaj et al. 2010). The interaction between TBDT and the "Ton" system is mediated by TonB. The TBDT contains a TonB-box in front of its N-terminal plug domain, which is recognized by TonB. In the absence of a ferrisiderophore, the plug domain is blocked. The interaction between a ferrisiderophore-loaded TBDT and TonB results in a conformational change in the plug domain, allowing passage of the siderophore through the TBDT pore (Fig. 4).

Other components of siderophore-mediated iron transport were recently identified in *Anabaena* sp. PCC 7120, namely TonB3, and the ExbB3/ExbD3 and the Fhu systems (Stevanovic et al. 2012) (Table 3). The transcript abundance of these genes is enhanced under iron-limiting conditions, as is typical of high-affinity Fe transport systems (Stevanovic et al. 2012). In general, transport of ferrisiderophore across the periplasmic and inner membrane is then accomplished by a three-component system: a periplasmic Fe binding protein (e.g. FhuD/FutA/FecA), a membrane-embedded permease (e.g. FhuB/FutB/FecCD) and an ATP-binding protein (e.g. FhuC/FutC/FecB). The FhuD/B/C-system that allows transport of ferrichrome in *E. coli* is well established (reviewed by Krewulak and Vogel 2011). So far, in *Anabaena* sp. PCC7120, five gene clusters similar to Fut-, Fec- and Fhu-system have been annotated, but only the Fhu-cluster has been linked to intracellular ferrisiderophore transport (Stevanovic et al. 2012). The expression of some of these genes in *Anabaena* sp. is enhanced by not only low Fe, but also by high Cu (Stevanovic et al. 2012).

Genes encoding putative TonB-dependent receptors are found in many freshwater cyanobacterial genomes, but are much less common in marine cyanobacteria, and practically absent in picocyanobacteria (Hopkinson and Morel 2009; Hopkinson and Barbeau 2012). Interestingly, the only TBDT-like gene found in picocyanobacteria in this latter study, is a TBDT-type heme transporter (as well as its associated TonB energy translocation system, ExbB/Exbd) in the genome of *Prochlorococcus* sp. MIT9202. These results suggest that direct internalization of ferrisiderophores might not be a widespread physiological strategy to acquire Fe in the ocean. However, the most recent genomic study by Desai et al. (2012) found that TBD hydroxymate uptake components were relatively abundant in the metagenomes, in agreement with the abundance of hydroxymate siderophores in the open ocean.

Axenic cultures of cyanobacteria, as well as natural populations of marine *Synechococcus* have been shown to use a reductive mechanism at the cell surface, similar to that of eukaryotic microalgae, to access the Fe within siderophore complexes (Lis and Shaked 2009; Kranzler et al. 2011). This strategy is advantageous in the ocean because it allows access to Fe from a wide variety of naturally occurring strong organic Fe complexes. One of the pending questions in this research is where does the reduction of Fe bound to strong organic complexes occur in cyanobacteria? Iron reduction is most likely to occur either on the surface of the outer membrane or in the periplasmic space. Studies investigating reduction of Fe in strong organic Fe complexes use the Fe(II) trapping agent ferrozine (FZ). These studies measure Fe(II) FZ in solution, suggesting that $Fe(II)FZ_3$ is produced outside the cell. Since $Fe(II)FZ_3$ is not available for uptake, it is possible that FZ crosses the outer membrane, traps the Fe(II) produced in the periplasmic space and then the resultant $Fe(II)FZ_3$ diffuses back across the outer membrane into the surrounding medium.

Recently, a reductive pathway for the transport of inorganic Fe has also been proposed for the freshwater *Synechocystis* strain (PCC6803) (Kranzler et al. 2014) (Fig. 4). Inorganic Fe(III) (i.e., Fe(III)') is first transported across the outer membrane, possibly through a non-specific porin (Fujii et al. 2011). Once in the periplasma, Fe(III) is complexed by the high-affinity Fe binding protein FutA2,

thus establishing a chemical gradient for Fe(III) influx through the outer membrane. Subsequently iron in the Fe(III) within the Fe(III)FutA2 protein is reduced to Fe(II) by the integral plasma membrane cytochrome c oxidase (Alternate Respiratory Terminal Oxidase). The reductive step results in the release of Fe(II) from the Fe(II)FutA2 complex and the subsequent Fe(II) transport across the plasma membrane via the Fe(II) transporter FeoB (Kranzler et al. 2014). The Fe(II)-specific transporter FeoB genes have been identified in freshwater and coastal cyanobacteria, but appear to be absent in oceanic picocyanobacteria and eukaryotic microalgae (Desai et al. 2012). One coastal strain of *Synechococcus* has genes encoding a Fe(II)-specific transporter FeoB (Palenik et al. 2006). This might be an adaptation to the faster redox cycling of Fe in coastal waters due to photo-reduction of Fe(III) in the presence of hydroxycarboxylic acids (Kuma et al. 1992). Other divalent transporters, such as ZIPs and NRAMPs have been identified in marine picocyanobacteria, and thus may mediate their Fe(II) uptake, as previously demonstrated in other organisms. In oceanic metagenomes, Fe(II) transporters are not very abundant, but ZIPs, NRAMPs are more common than FeoB-type.

Even though Fe reduction and a plasma membrane Fe(II) transporter seem to be the dominant Fe transport mechanism in the model freshwater cyanobacterium *Synechocystis* (PCC6803), the presence of an Fe(III) transport system, such as FutABC is also expected in freshwater and marine cyanobacteria, given that some of these genes have been identified (Katoh et al. 2001a, b). This transport system consists of a soluble Fe(III)-binding periplasmic protein (FutA2), a permease (FutB), a peripheral plasma membrane associated ATPase (FutC), and an intracellular FutA1 (Fig. 4) (Katoh et al. 2001a, b). The intracellular subunits FutA1 and FutC of this Fe(III) transport system are believed to regulate the reductive Fe(II) uptake pathway (Kranzler et al. 2014). Genomic analyses have revealed the presence of Fe(III)-specific ABC transport systems in many marine cyanobacteria, as well as picocyanobacteria (Desai et al. 2012; Hopkinson and Barbeau 2012), suggesting that Fe(III) uptake is very common, in agreement with the thermodynamic stability of Fe(III) and the prevalence of Fe(III) organic complexing ligands in seawater. In addition, in oceanic metagenomes Fe(III)ABC transport systems are much more common than Fe(II) uptake systems (Desai et al. 2012; Hopkinson and Barbeau 2012).

4 Iron Storage and Luxury Uptake

Iron storage is another strategy that enables microalgae to cope with low and intermittent Fe supplies. Because many of the new Fe inputs to the ocean are sporadic, being able to rapidly take up and store Fe from ephemeral inputs would be particularly advantageous. The capacity to take up more Fe beyond than required to satisfy biochemical functions for maximum growth is often termed luxury uptake and likely an important Fe-acquisition strategy of microalgae, in particular diatoms. The Fe storage capacity can be approximated by the ratio between the intracellular Fe requirements when microalgae are grown in excessively high [Fe] and the cellular concentration in which growth starts to decrease due to Fe-limiting conditions. The resulting ratio reflects a phytoplankter's potential to store intracellular Fe. Among the phytoplankton examined, the general trend is that oceanic microalgae have lower minimum cellular iron requirements than coastal microalgae (Fig. 7) (Sunda and Huntsman 1995; Maldonado and Price 1996; Marchetti et al. 2006). Microalgae, particularly bloom-forming species, may benefit from being able to take up and store large quantities of Fe during periods of surplus Fe availability because these reserves can then be drawn upon as Fe concentrations become exhausted. The relatively high storage capacities observed in many diatom isolates from the Fe-limited regions of the Equatorial Pacific relative to capacities in other coastal and oceanic diatom isolates were suggested to be an evolutionary adaptation for living in low Fe environments with sporadic Fe inputs (Maldonado and Price 1996).

The Fe storage capacities of pennate diatoms belonging to the genus *Pseudonitzschia*, a cosmopolitan diatom genus found in both coastal and oceanic environments, have also been examined (Marchetti et al. 2006). Members of this genus are near-universal responders to artificial Fe enrichment in Fe-limited regions of the ocean (Marchetti et al. 2008; Trick et al. 2010). In Fe-limited oceanic waters, *Pseudonitzschia* abundance is usually low compared to other phytoplankton although species richness may be high. Upon Fe-enrichment, *Pseudonitzschia* numerically dominates the Fe-induced diatom bloom and can make up a significant proportion of the phytoplankton biomass. The potential Fe storage capacities (estimated by dividing the maximum Fe quota by the minimum Fe quota) for the oceanic *Pseudonitzschia* spp. examined were markedly higher than those calculated for several oceanic *Thalassiosira* spp. Differences in Fe:C ratios were primarily a consequence of a higher mean maximum iron quota rather than a lower mean minimum quota (Fig. 7), suggesting that *Pseudonitzschia* spp. have exceptionally high Fe storage capabilities.

A molecular basis for the enhanced Fe storage in *Pseudonitzschia* was provided with the identification of ferritin (*FTN*) genes within several pennate diatoms (Marchetti et al. 2009). Due to its facile redox chemistry and tendency to generate toxic hydroxyl radicals via Fenten chemistry, extensive intracellular concentrations of unchelated Fe could be detrimental to cells. Ferritin is a Fe-storage protein used by plants, animals, cyanobacteria and other microorganisms to safely concentrate and store Fe, thereby minimizing

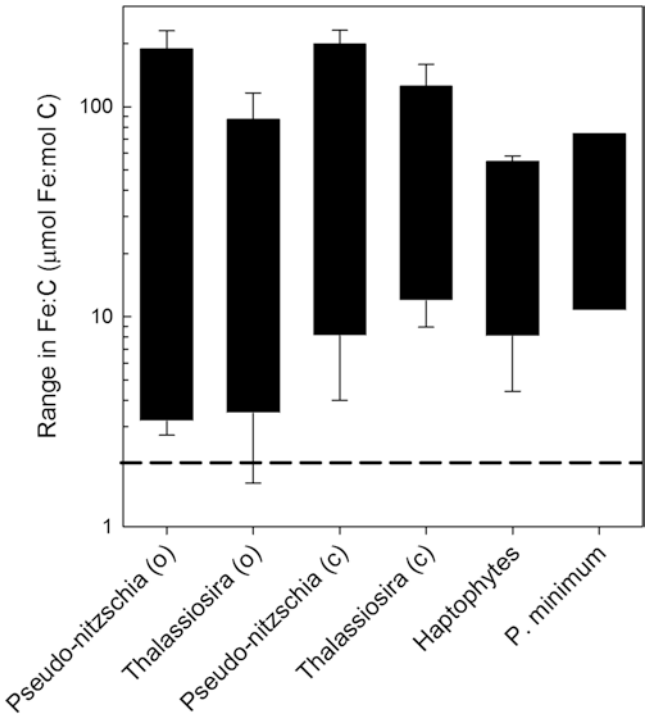

Fig. 7 A comparison of Fe quotas ranges (as measured by the Fe:C ratios) in marine microalgae grown under Fe-limited (low range) and Fe-replete (high range) conditions. Shown are the average ranges for oceanic (o) and coastal (c) isolates of the diatom genera *Pseudonitzschia* and *Thalassiosira*, several haptophytes (*Emiliania huxleyi*, *Phaeocystis pouchetti* and *Chrysochromulina polylepis*) and the dinoflagellate *Prorocentrum minimum* (=*Prorocentrum cordatum*). The dashed horizontal line represents the Fe:C ratio for phytoplankton from low Fe regions (Derived from Sunda et al. 1991) that is commonly used in marine biogeochemical models (e.g. see Moore et al. 2002) (Data are compiled from Sunda and Huntsman (1995), Maldonado and Price (1996), Marchetti et al. (2006) and Lane et al. (2009))

potential cell damage from reactive oxygen species and oxidative stress. In cyanobacteria, there are three different types of ferritin family proteins that have been identified, including bacterioferritins, ferritins and DNA-binding ferritins (DPS proteins) (Kranzler et al. 2013). Ferritins and bacterioferritins oxidize intracellular Fe(II) to Fe(III), thereby functioning as ferroxidases while generating hydrogen peroxide. Bacterioferritins differ from ferritins by the presence of a haeme molecule, anchored at the interface between two adjacent protein subunits. In eukaryotic ferritins (24 subunits, 480 kDa), up to 4500 Fe(III) atoms can be stored as an Fe oxide mineral and releases reduced Fe(II) on cellular demand (Liu and Theil 2005). Such storage compounds allow Fe to be readily released and utilized when needed, thus acting as a buffer against Fe deficiency. Although ferritins appear to be ubiquitously present in green algae and cyanobacteria, the discovery of ferritin in diatoms was the first indication of the Fe storage protein in any member of the Stramenopile species with whole genomes sequenced. Thus, the acquisition of a ferritin gene in diatoms, likely resulted from a horizontal gene transfer event. The diatom ferritin genes contain signal and target peptides that predicted the protein to be targeted to the chloroplast to control the supply of iron for synthesis of critical iron containing proteins in the photosynthetic apparatus (e.g., those in PSII, PSI, and the b6/f complex) (Marchetti et al. 2009).

The absence of a ferritin gene in *T. pseudonana* in combination with the significantly reduced iron storage capacity of *T. oceanica* compared to *Pseudonitzschia* led to the speculation that many diatoms do not contain ferritin. Although centric diatoms lacking ferritin can certainly store Fe (Sunda and Huntsman 1995; Maldonado and Price 1996), the mechanism as to how this is achieved is not well understood. For example, in the centric diatoms *T. pseudonana* and *C. weissflogii*, Nuester et al. (2012) observed the elemental stoichiometry of cellular Fe to be consistent with a potential vacuolar Fe storage pool mechanism. Recent transcriptomic sequencing of a number of centric and pennate coastal diatoms has now identified the sporadic presence of ferritin-like genes within some centric diatoms (Groussman et al. 2015). In addition, multiple homologs of *FTN* genes have been identified in *Chlamydomonas reinhardtii* and several diatom genomes, including *Pseudonitzschia multiseries*. Whether these homologs perform different functions is unknown. This patchy diversity of ferritins throughout the diatom lineage necessitates a more systematic examination into the differences in the Fe storage capabilities of diatoms that either contain or lack ferritin.

While the results from bulk studies with natural phytoplankton assemblages suggest that some microalgae can accumulate Fe following pulsed inputs, these experiments do not clarify which species are accumulating the Fe, or whether the accumulated Fe is simply adsorbed onto the outside of the cells or actually accumulated internally. Synchrotron x-ray fluorescence microscopy (SXRF) has provided this missing information, as it enables quantitative elemental analysis of individual cells, as well as spatial information about where the elements are located within the cells (Twining et al. 2003; Twining and Baines 2013). During the SOFeX Fe fertilization experiment in the Southern Ocean, diatoms increased their Fe:C ratios nearly fivefold after two Fe additions, while autotrophic flagellated cells increased their Fe quotas by only threefold (Twining et al. 2004). Additionally, two-dimensional element maps showed that the extra Fe was accumulated largely in the regions of the chloroplasts, indicating that uptake was internal and not the result of abiotic precipitation and adsorption. SXRF has also been used to measure Fe accumulation by microalgae in the Equatorial Pacific. Baines et al. (2011) found Fe quotas of *Pseudonitzschia*-like diatoms to increase 24-fold in 48-h following addition of 2 nM Fe to deckboard incubations. Further, this Fe was largely localized in Fe storage bodies adjacent to the chloroplasts, as predicted

for ferritin (Marchetti et al. 2009). Over the final 2 days of the 96-h incubation, cellular Fe contained within the storage bodies dropped exponentially at a rate approximating the growth of the diatom population, while overall cellular Fe quotas decreased more slowly (Twining et al. 2011). These data demonstrate the potential for stored Fe to support subsequent population growth, as well as the unique ability of SXRF to quantify luxury uptake of Fe by phytoplankton in natural systems.

5 Iron and Light Co-limitation

Light and Fe availability interact in a complex and bi-directional manner, and have a profound effect on the ability of phytoplankton to photosynthesize. Indeed, Fe limitation (especially at low light) limits the biosynthesis of iron-containing components of the photosynthetic apparatus and impairs the capacity of the cells to absorb light energy, as well as convert light energy into chemical energy. Some phytoplankton acclimate to limiting Fe levels by lowering the abundance of the Fe-rich PSI relative to PSII, as previously discussed. High light may enhance Fe availability under low Fe concentrations by promoting photochemical reactions that increase the pool of inorganic Fe ([Fe′]) in the presence of photolabile organic Fe complexes (Anderson and Morel 1982; Sunda and Huntsman 2011). In addition, under low Fe, high light may enhance Fe uptake by providing more photosynthetically derived NAD(P)H, which is needed for the functioning of the membrane reductases involved in Fe uptake (Askwith et al. 1996).

Phytoplankton acclimate to low photon flux by changing the ratio of accessory pigments to chl *a*, as well as the abundance and stoichiometry of Fe-rich photosynthetic electron carriers (Falkowski et al. 1981). Under low light conditions, phytoplankton may modify their PSUs to enhance light harvesting by increasing their numbers and/or their size. These two physiological strategies alter distinctively their Fe requirements for photosynthesis. A higher number of PSUs will increase their Fe demand, as the photosynthetic electron transport protein and protein complexes associated with PSUs are Fe-rich. In contrast, an increase in the size of PSU involves higher concentrations of chlorophyll and light-harvesting accessory pigments per PSU, and does not necessarily increase Fe demand (Geider et al. 1993).

Support for the first strategy has been predicted theoretically (Raven 1990) and documented empirically by showing higher intracellular Fe:C ratios needed to support a given iron-limited specific growth rate under low light intensity or shortened photoperiod, both in laboratory cultures (Sunda and Huntsman 1997, 2011; Strzepek and Price 2000; Strzepek and Harrison 2004; Finkel et al. 2006; Weng et al. 2007), and field populations (Maldonado et al. 1999; Hopkinson and Barbeau 2008). These higher Fe quotas are allocated towards the synthesis of additional Fe-rich photosynthetic electron transport protein complexes to maintain high rates of C fixation at low light (Sunda and Huntsman 1997). Indeed, in the case of the diatom *C. weissflogii*, the relative increase in cellular Fe growth requirements under low light matches the predicted relative increase in abundance of PSI relative to PSII during photoacclimation (Strzepek and Harrison 2004). However, the higher Fe quotas observed under low light are partly a consequence of slower growth rates (p^{ss}_{Fe} = Fe quota * growth rate, Sunda and Huntsman 1997, 2011). In addition, significantly higher half-saturation constants for Fe (K_μ) have been shown for cyanobacteria and diatoms grown under low light than under high light (Kudo and Harrison 1997; Bucciarelli et al. 2010). The higher K_μ under low light is partly explained by lower [Fe′] in a medium with photolabile organic Fe complexes ($[Fe']_{HL} = [Fe']_{LL} * 1.14$, Bucciarelli et al. (2010)). The remaining difference between these K_μs (e.g. for *T. oceanica* $K_{\mu HL}$ and $K_{\mu LL} = 1.29$ and 3.01 pM, respectively; Bucciarelli et al. (2010)) is likely due to higher Fe demand by low-light-adapted phytoplankton.

The second low-light acclimation strategy, increasing the size of PSUs, has been shown in cyanobacteria, the green alga *Dunaliella salina*, and Southern Ocean diatoms and haptophytes. Southern Ocean phytoplankton are extremely well adapted to limiting Fe levels and cope with low light by an unprecedented change in the size (sixfold larger) of the effective absorption cross section (σ_{PSII}) of their PSII reaction centers, without an increase in cellular Fe (Strzepek et al. 2012). In a similar manner, Fe-limited cyanobacteria have fewer Fe-rich PSIs, but promote the expression of IsiA antenna proteins associated with PSI. This increases the PSI absorption cross-section and maintains rapid electron flow through PSI (Chauhan et al. 2011). Likewise, under low Fe, the green alga *D. salina* induces the expression of chlorophyll *a*/*b*-binding proteins associated with PSI (Varsano et al. 2006). However, this physiological photo-acclimation strategy has its limitations, as the size of the PSU has an optimal maximum, beyond which the energy transfer efficiency between the light harvesting pigments and PSUs decreases sharply (Raven 1990).

6 Iron Limitation Effects on Elemental Composition

Phytoplankton are responsible for approximately half of the carbon fixation on Earth. Thus minor alterations in their elemental stoichiometry from the ideal Redfield composition (i.e. 106C:16N:1P) due to Fe limitation could have large influences on global biogeochemical cycles. Diatoms, in particular account for approximately one-half of marine primary

productivity (Nelson et al. 1995). One important effect of Fe limitation on the elemental composition of diatoms is a relative increase in their Si content. A study in the California coastal upwelling region by Hutchins and Bruland (1998) noted a two to threefold increase in the silicic acid-to-nitrate ($Si[OH]_4$:NO_3) and silicic acid-to-dissolved inorganic carbon ($Si[OH]_4$:DIC) drawdown ratios of diatom-dominated communities in Fe-limited controls relative to Fe-enriched seawater samples. The observed decrease in the dissolved nutrient drawdown ratios upon the alleviation of Fe limitation was primarily due to the rapid increase in NO_3 utilization with little or no change in $Si(OH)_4$ assimilation. This lead to the speculation that Fe-limited diatoms utilize more Si per cell or unit biomass relative to Fe-replete diatoms and thus were more heavily silicified (Boyle 1998). At the same time, in addition to studying the response to Fe enrichments in natural assemblages, Takeda (1998) found that Fe-limited Antarctic diatoms had higher Si:N ratios relative to Fe-replete diatoms. In a *Nitzschia* sp., the increase in the ratio was due to more Si per cell, whereas in *Chaetoceros dichaeta* less N per cell drove the increase in the ratio. Since then, additional studies have documented changes in the Si:N and $Si(OH)_4$:NO_3 uptake/consumption ratios in Fe-limited diatoms or diatom-dominated natural assemblages and have attributed these changes to enhancements in silicification and/or reductions in N and C, or have suggested that no changes in the ratios occur when both new (NO_3-based) and regenerated (NH_4-based) forms of N are considered (reviewed by Marchetti and Cassar 2009). In addition, coordinated gene expression responses to Si and Fe limitation within *T. pseudonana* suggest both elements have a similar influence on diatom cell wall processes (Mock et al. 2008).

Discrepancies in the causal element influencing the Si:N ratios among these laboratory and field studies may be due to varying levels of Fe deficiency and hence reductions in the specific growth rates, differences in growth conditions, implemented methodology, shifts in microalgal species composition and/or the intrinsic variability among different diatoms (reviewed by Marchetti and Cassar 2009). Despite the variety of possible causes behind changes in the element stoichiometries, the observed outcome of Fe-limited diatoms is generally consistent—an increase in the cellular Si:N ratios, although there are exceptions with certain diatoms under severe Fe limitation (Bucciarelli et al. 2010). This result supports the assertion that Fe-limitation is an important factor in contributing to the preferential export of Si relative to N (Dugdale and Wilkerson 1998; Hutchins and Bruland 1998). However, the specific physiological mechanism driving the changes in elemental stoichiometry of diatoms (i.e., an increase in cellular Si content and/or a decrease in both N and C contents) are important for predicting diatom biogeochemical fluxes to the ocean floor. Cell size and the degree of silicification may affect how effectively a certain diatom species can sequester carbon due to the possibility of relatively larger and thicker-shelled diatoms sinking faster (Smayda 1970; Smetacek 1985, 1999; Raven and Waite 2004; Assmy et al. 2013), being less susceptible to grazing (Smetacek 1999; Hamm et al. 2003; Pondaven et al. 2007) and more effectively preserved in ocean sediments.

Although the C:N ratios of phytoplankton are relatively unaffected by nutrient limitation, including Fe, there are reports of a decrease in N:P (and by inference C:P) ratios in Fe-limited phytoplankton. In the diatom *C. weissflogii*, this decrease was primarily a result of accumulation of P under low-Fe conditions due to a more rapid decline in specific growth rate than in steady-state uptake rate of phosphorus (Price 2005). Under the most severe Fe-limiting conditions, *C. weissflogii* contained 1.5 times more P per liter cell volume than when Fe-replete. The trend observed in the laboratory isolate is supported by field observations from Fe-limited natural assemblages dominated by diatoms, where lower N:P and C:P ratios have been observed. Thus, as Fe inputs to Fe-limited regions around the globe vary in space and over geological timescales, considerable changes in the elemental composition of sinking phytoplankton debris would be anticipated.

Iron status may also influence the stoichiometry of other trace elements. For example, as previously discussed, diatoms that contain a multi-copper oxidase as part of their high affinity Fe uptake pathway have an increased demand for copper when this pathway is induced under low Fe-limiting conditions (Peers et al. 2005; Maldonado et al. 2006). Even under Fe-replete conditions, the copper requirements of many oceanic diatoms are higher due to their dependence on the copper-containing electron transport protein plastocyanin, whereas many coastal diatoms use cytochrome c_6, which is a Fe-containing protein (Peers and Price 2006).

7 Phytoplankton Response to Iron Fertilization

In vast expanses of our world's oceans, the biomass of phytoplankton is low even though macronutrient concentrations are quite high. These areas are termed "high nutrient (nitrate), low chlorophyll" or HNLC regions. Until recently, the factors most responsible for minimizing new production in HNLC regions remained unknown, forcing oceanographers to speculate about possible causes. Competing hypotheses that emerged in the late 1980s attempted to explain these paradoxical HNLC regions. As reviewed by Cullen (1991), factors believed to control primary productivity from a "bottom-up" approach included the effects of low temperature (Tilzer et al. 1986), light limitation and/or water column instability and strong turbulence (Chavez et al. 1991; Miller et al. 1991; Mitchell et al. 1991), "shift-up" in NO_3 assimila-

tion (Dugdale and Wilkerson 1991) and micronutrient (Fe) limitation (Martin and Fitzwater 1988). "Top-down" or "grazer" control was also suggested as the primary factor regulating phytoplankton biomass in these oceanic regions, as these regions were dominated by small-celled phytoplankton that were rapidly grazed by microzooplankton (McAllister et al. 1960; Banse 1991; Miller et al. 1991). The possibility that the regulation of primary productivity in HNLC regions may be a combination of all these factors rather than a single one was also considered (Harrison et al. 2004).

Martin and Fitzwater (1988) proposed that low Fe concentrations may limit growth of phytoplankton in HNLC regions of the ocean. Although early investigators had proposed the possibility of Fe limitation (Hart 1934), supportive experimental data was difficult to obtain due to the lack of trace metal clean sampling methods that inevitably resulted in Fe contamination. Martin et al. (1989) showed that surface concentrations of dissolved Fe in these HNLC regions were very low (<<1 nM) and dissolved Fe exhibited a nutrient-like profile. Furthermore, bottle experiments consisting of the addition of Fe to the HNLC Subarctic Pacific water samples resulted in an increase in chlorophyll *a* and a subsequent drawdown of NO_3 (Martin and Fitzwater 1988). In particular, the growth rate of large phytoplankton increased, resulting in a species composition shift from cyanobacteria and small flagellates to large diatoms. These experiments provided compelling evidence that Fe may indeed limit phytoplankton growth and validated the "Iron Hypothesis" (Martin 1990). The idea that iron limits the growth of phytoplankton in the vast HNLC regions of the ocean was confirmed convincingly by a series of in-situ iron fertilization experiments conducted in patches of surface seawater tens of km in surface area in major HNLC regions (see discussion below). There are three main regions of the world's oceans where the phytoplankton have been shown to be consistently or periodically limited by the supply of Fe. These include parts of the Equatorial Pacific, the Subarctic Pacific and most of the Southern Ocean (de Baar et al. 2005; Boyd et al. 2007). There is also compelling evidence for Fe limitation of phytoplankton in coastal upwelling regions (Hutchins et al. 1998; Firme et al. 2003), as well as seasonal Fe limitation primarily of N_2 fixation rates in tropical and subtropical regions the Pacific and South Atlantic Oceans receiving low inputs of iron from continental source (Sohm et al. 2011). In addition, iron-light co-limitation has been suggested to limit phytoplankton growth and influence phytoplankton community composition in the deep chlorophyll maximum at the bottom of the euphotic zone in vast regions of thermally stratified subtropical ocean waters, where both light and iron concentrations are very low (Sunda and Huntsman 1997, 2015; Hopkinson and Barbeau 2008).

The confirmation that phytoplankton growth is limited by a trace metal that is very abundant on land has led to the concept of performing mesoscale open ocean Fe enrichment experiments to mitigate increases in atmospheric CO_2 caused by the burning of fossil fuels. In addition, since the molar stoichiometry of most phytoplankton is ~10^5 C:1Fe, compared to ~7C:1N, it is considerably very economical to add Fe to large areas of the ocean rather than N to stimulate algal growth. Though met with considerable debate and skepticism by many scientists (Strong et al. 2009), human-induced Fe enrichment is viewed by some as a possible solution to dealing with climate change and reductions in commercial fish stocks. The unprecedented rate of increase in atmospheric levels of CO_2 is considered to be the major contributor to global warming, although methane, nitrous oxides and chlorofluorocarbons also play a role. A number of such Fe enrichment experiments have been performed in the equatorial Pacific (IronEx I and IronEx II), in the Southern Ocean (SOIREE, EisenEx, SOFex, Eifex and Lohafex) and North Pacific (SEEDS I and II and SERIES) (Fig. 8) (Boyd et al. 2007; Smetacek et al. 2012). Each of these experiments demonstrated varying degrees of increased phytoplankton growth in response to Fe additions to surface waters that were dependent on the initial environmental conditions and the initial phytoplankton community composition. With the exception of a few experiments, Fe enrichments resulted in increased diatom growth that subsequently partially or fully depleted macronutrient concentrations leading to bloom termination. The resulting diatom blooms are often large enough to be visible form space (Fig. 8).

Following the initial Fe enrichment studies performed in each of the well-characterized HNLC regions, subsequent rounds of large-scale in-situ Fe addition experiments have focused on the Southern Ocean, as it is the largest of the HNLC regions containing a vast pool of underutilized macronutrients. The SOFeX experiments added Fe to two separate north and south patches, with the north patch containing low $Si(OH)_4$ concentrations and the south patch containing high $Si(OH)_4$ concentrations (Coale et al. 2004). Although an increase in phytoplankton biomass was observed in both patches, the phytoplankton assemblage differed, as the north patch contained a significantly lower proportion of diatoms due to $Si(OH)_4$ limitation. During Eifex, Fe was added to a vertically coherent, mesoscale eddy of the Antarctic Circumpolar Current (Smetacek et al. 2012). An ensuing large diatom bloom was monitored for 5 weeks and at least half of the phytoplankton bloom biomass sank to a depth of 1000 m with a substantial portion likely reached the sea floor. Similar to natural Fe fertilization observations in the Southern Ocean (Blain et al. 2007; Pollard et al. 2009), Eifex verified a substantial deep carbon export associated with the ensuing Fe-induced diatom bloom.

A molecular basis for the phytoplankton response to Fe enrichment and their mechanism of recovery was achieved through a comparative metatranscriptome analysis of a Fe-limited plankton community in HNLC waters of the NE Pacific Ocean (Marchetti et al. 2012). Consistent with the

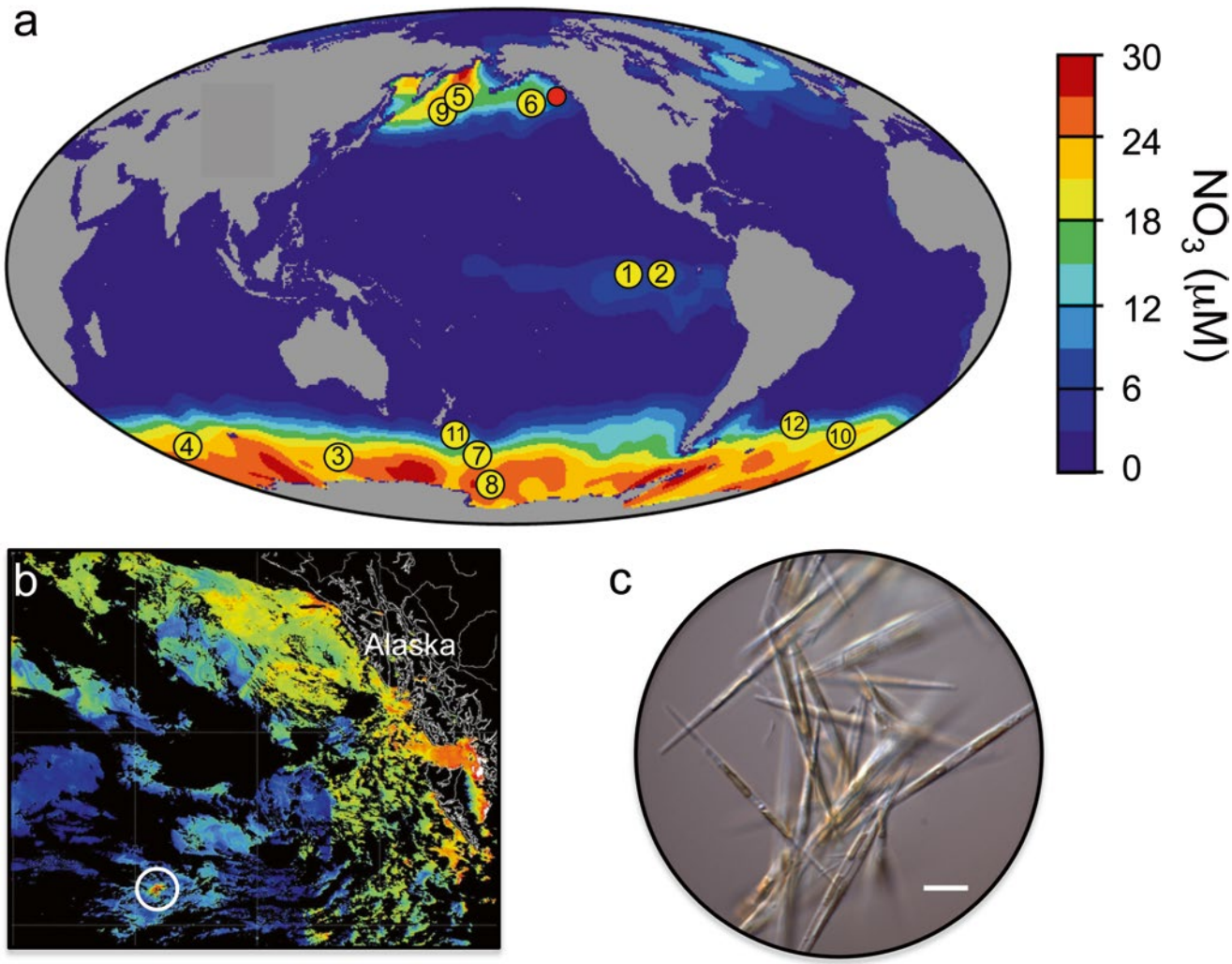

Fig. 8 (**a**) Map of global surface NO_3 concentrations along with the locations of 12 Fe enrichment experiments (indicated by *yellow circles*). *1* IronEx-I (1993), *2* IronEx-II (1995), *3* SOIREE (1999), *4* EisenEx (2000), *5* SEEDS-I (2001), *6* SERIES (2002), *7* SOFeX North (2002), *8* SOFeX South (2002), *9* SEEDS-II (2004), *10* EIFEX (2004), *11* SAGE (2004), *12* LOHAFEX (2009). Also indicated is an Fe addition experiment performed by the Haida Salmon Restoration Corporation in 2012 that was not government approved (*red circle*). NO_3 are World Ocean Atlas climatological spring values. (**b**) SeaWiFs satellite image of the phytoplankton bloom resulting from the SERIES Fe enrichment experiment in the northeast Pacific Ocean (indicated by the number 6 in panel **a**). The coast of Alaska is shown. Warm colors (*reds* and *yellows*) indicate high concentrations of chlorophyll *a* and thus high phytoplankton biomass; cool colors (*blues*) indicate low chlorophyll *a* concentrations. *Dark areas* over the ocean result from cloud cover. The *white-circled* region indicates the 700 km^2 region of high chlorophyll *a* concentrations resulting from the addition of Fe. (**c**) Light microscope image of the oceanic diatom *Pseudonitzschia granii*; members of this genus are common responders to Fe enrichment in all HNLC regions. Scale bar is 10 μm (Adapted from figures in Behrenfeld et al. (2009), Armbrust (2009) and Morrisey and Bowler (2012). SERIES satellite image courtesy of J. Gower, Orbimage/NASA)

large-scale Fe enrichment experiments, a large diatom bloom ensued after Fe addition to mesocosms. The immediate diatom response to Fe enrichment was to continue to express genes encoding non-Fe containing proteins rather than carrying out a widespread replacement of these proteins with their Fe-containing counterparts. For example, transcripts associated with the Fe-free electron transfer protein flavodoxin were abundant in all libraries and over-represented after Fe enrichment, suggesting that some or all diatoms continue relying on this protein for photosynthetic electron transfer rather than switching entirely to use the Fe-containing version, ferredoxin. Additionally, diatom transcripts for copper-containing plastocyanin were highly abundant under all conditions whereas very few transcripts for cytochrome c_6, the Fe-requiring counterpart, were readily identified. Many oceanic phytoplankton have likely adapted to life under continuous iron-limitation by permanently using the iron-free equivalents of these proteins and may have lost the genes to synthesize ferredoxin and cytochrome c_6. Continued dependence on Fe-free proteins may also provide diatoms with a more rapid acclimation to a return to low-Fe conditions.

The addition of Fe to diatoms stimulated what appears to be a system-wide pattern of gene expression in alleviation of chlorosis and preparation for rapid cell division. Specifically,

significant increases were observed in transcripts for genes encoding proteins that catalyze the biosynthesis of nucleic acids, amino acids, sugars, chlorophyll (and its precursors, i.e. porphyrin) as well as the prerequisites for long-chain polyamines, which may play an important role in silica deposition in diatom cell wall formation, were observed. Components of the diatom urea cycle were suggested to likely play a significant role in facilitating the Fe response leading to bloom formation following the alleviation of Fe limitation and/or low-Fe-induced NO_3 limitation. The ability of diatoms to divert their newly acquired Fe towards nitrate assimilation may underlie why diatoms consistently dominate Fe enrichments in HNLC regions. The distinctiveness of the diatom response was even more apparent when compared to the haptophytes, the other dominant member of the phytoplankton community after Fe enrichment, which displayed a more typical response to Fe enrichment (i.e. increased transcripts of ferredoxin, Fe-SODs and other Fe-requiring protein-encoding genes). In addition, rhodopsin transcripts assigned to diatoms, haptophytes and dinoflagellates were highly abundant in the Fe-limited libraries, and decreased in abundance following Fe enrichment. Rhodopsins are light-driven proton pumps that may be associated with ATP generation (Beja et al. 2000; Fuhrman et al. 2008), which suggested a role for rhodopsins in eukaryotic phytoplankton to deal with low-Fe conditions (Marchetti et al. 2015).

8 Climate Change Effects on Iron-Related Phytoplankton Physiology

As the amount of CO_2 in the atmosphere continues to increase, equilibration of higher atmospheric CO_2 levels with the surface ocean will acidify ocean waters and decrease their pH, typically referred to as ocean acidification. An extensive amount of research has been conducted examining ocean acidification effects on the calcification processes of marine organisms, including microalgae (e.g. coccolithophores) (for a review, see Doney et al. 2009). As seawater pH is reduced, the most prominent effect is a decrease in the carbonate ion concentration that could impede calcification rates, although the responses of marine organisms appear to be varied. Less is known about other potential effects of ocean acidification and warming on marine ecosystems, including how the speciation and distributions of trace metals such as Fe will be affected (Hoffmann et al. 2012). In addition to changes in carbonate chemistry (i.e. a reduction in carbonate ion concentrations), a decrease in pH will result in a decrease in hydroxide ion concentrations in most natural surface waters. Decreases in pH may have a profound influence on the concentrations and chemical speciation of iron, and hence on iron availability to phytoplankton due to a number of factors: the existence of dissolved inorganic iron (FeIII)') in seawater as soluble iron hydroxide species, the polymerization and precipitation of iron hydroxides (which removes iron from sea water), and to the fact that many organic complexation reactions are sensitive to changes in pH (Shi et al. 2010). In addition decreasing pH will promote rates of reduction of Fe(III) to soluble Fe(II), and retard the reoxidation of Fe(II) to Fe(III) (Millero et al. 2009).

To some degree, potential increases in Fe solubility along with proposed changes in kinetics could lead to increased iron concentrations and hence in primary productivity in Fe-limited regions in the ocean. However as the vast majority of Fe_{diss} is bound to strong organic ligands (Gledhill and van den Berg 1994; Rue and Bruland 1995), the changes in complexation of Fe with these organic ligands at a reduced pH may largely drive how ocean acidification affects Fe availability. The effects of reduced pH on Fe availability to marine phytoplankton when Fe is bound to a variety of organic ligands were recently examined (Shi et al. 2010). In all examined species, which included both coastal and oceanic diatoms as well as the coccolithophore *E. huxleyi*, Fe uptake rates were related to Fe′, indicating that the effect of pH is largely due to a change in the chemical speciation of Fe and not a physiological response of the organisms. When Fe was bound to chelators with acidic binding groups that are not protonated in seawater, the extent of decrease in Fe′ with decreasing pH is dependent on the number of protons released upon dissociation of Fe. Likewise, if the uptake of Fe is dependent on enzymatic reduction of the Fe-chelator complex, a decrease in pH could affect rates of the enzymatic reaction if protons are liberated. This was proposed to be the case for a decrease in Fe uptake rates with decreasing pH when Fe is bound to the trihydroxomate siderophore, DFB (Shi et al. 2010). In contrast, there was a negligible effect of pH on Fe uptake when Fe was bound to chelators that are protonated in seawater (e.g. catechols). Thus, in general, it was determined that the availability of Fe to phytoplankton decreases at lower pH and are largely dictated by the acid-base chemistry of the chelating ligands.

Field experiments have shown mixed responses to alteration in CO_2 and Fe concentrations within natural phytoplankton assemblages from HNLC regions. In Fe-limited waters within the Gulf of Alaska, a marginal increase in biomass and production was observed at high CO_2 and was attributed to the energy savings associated with a down-regulation of the carbon-concentrating mechanisms (CCM) and, in some cases, a reduction of Fe-rich photosynthetic proteins (Hopkinson et al. 2010). Alternatively, under Fe-enriched conditions, increases in growth and biomass at high CO_2 compared to low CO_2 treatments were suggested to be the result of lower rates of respiration, leading to reductions in carbon loss. Similarly, in coastal waters off Norway, at high CO_2 levels, increases in Fe_{diss} and Fe(II) concentrations were observed within mesocosm experiments, suggesting

increased Fe availability as a result of CO_2 enrichment (Breitbarth et al. 2010). In contrast, experiments conducted with natural seawater samples in coastal and oceanic waters in the North Atlantic (Shi et al. 2010) and Fe-limited waters in the Bering Sea (Sugie et al. 2013) inferred decreases in Fe uptake rates and growth of phytoplankton with increasing CO_2 levels. In the Bering Sea study, these CO_2-mediated influences disappeared under Fe-replete conditions. Such discrepancies could be due to a number of factors, including differences in the Fe concentrations, Fe speciation and the phytoplankton community composition.

Apart from affecting Fe availability, interactive effects of ocean acidification and Fe have been shown to influence other aspects of microalgal physiology and community dynamics. For example, in the coastal diatom *Pseudonitzschia pseudodelicatissima*, Si:C and Si:N elemental ratios decreased, whereas N:P ratios increased with increasing pCO_2 (Sugie and Yoshimura 2013). Thus, in a high CO_2 world, these patterns could buffer the changes in elemental ratios that occur when diatoms are Fe-limited, which tend to be in the opposite direction. Within Fe-enriched natural assemblages present in the Weddell Sea, Antarctica, CO_2-dependent changes in productivity were accompanied by a taxonomic transition from weakly to heavily silicified diatoms (Hoppe et al. 2013). These transitions did not occur under Fe-limiting conditions, suggesting differences in carbon acquisition strategies and/or pH-mediated cellular physiologies among diatoms that were only evident after the alleviation of Fe stress.

Although our knowledge of ocean acidification effects on cyanobacteria and microalgae has advanced tremendously over the last decade, ocean acidification is only one of multiple factors that will be altered within marine systems as a consequence of elevated atmospheric CO_2 levels and resultant climate change. Significant warming of the land and oceans are also projected to occur which will result in changes to water column stratification, salinity and ocean circulation (Doney et al. 2012). In addition, changes in nutrient content and oxygen input to the ocean surface waters are predicted. All of these factors will undoubtedly effect Fe distributions and speciation, perhaps more so than ocean acidification, which will then have downstream biological consequences.

9 Techniques for Studying Iron-Related Microalgal Physiology

9.1 Achieving Iron Limitation in the Laboratory

One of the primary means by which physiologists investigate ways in which microalgae acclimate to varying Fe availabilities is by attaining steady-state Fe-limitation of growth rates in laboratory cultures. Achieving steady-state Fe limitation is now common practice provided that there is access to trace metal clean culturing facilities and implementation of certain culturing protocols (Price et al. 1988/89; Sunda et al. 2005). These protocols include the use of chemical-resistant plastics that contain lower concentrations of trace elements for culturing (such as polycarbonate, low-density polyethylene and teflon), acid-cleaning of all culture tubes and bottles, chelexing of the artificial seawater media—to remove trace metal impurities—and performing all preparation of medium and culture transfers within a trace metal clean laminar flow hood. Either synthetic ocean water (i.e. Aquil or ESAW; Harrison and Berges 2005) or low-Fe natural seawater can be used as growth medium. In most seawater medium recipes, synthetic aminocarboxylate chelating agents, such as EDTA are commonly used to regulate trace metal speciation. Concentrations of EDTA vary among the different types of medium, but typically range between 5 and 100 μM. In the presence of high concentrations of EDTA, virtually all of the iron in the medium is chelated by this ligand, and an insignificant fraction of Fe is bound to strong natural organic ligands (in cases where natural seawater is used); hence binding by the latter are unlikely to have a significant influence on metal speciation. In addition, without EDTA, $[Fe]_{diss}$ exceeding *ca*. 0.07 nM is expected to precipitate and is mostly unavailable to phytoplankton (Johnson et al. 1997), whereas in the presence of EDTA, Fe′ concentrations can reach up to 0.7 nM before exceeding the empirically observed threshold for precipitation of Fe hydroxides (Sunda and Huntsman 1995). By adjusting the concentrations of Fe and EDTA in the culture medium, the Fe′ concentrations can be lowered to the point where the availability of Fe regulates the growth rate of the phytoplankton. As described previously, EDTA will continuously buffer the concentration of Fe′ until the biomass becomes sufficiently high to increase culture pH (which decreases iron complexation in EDTA buffers in seawater) or the cells take up Fe′ from the medium at a faster rate than it is resupplied from dissociation of Fe-EDTA chelates (which causes Fe′ concentrations to decrease) (Sunda et al. 2005). Thus, to maintain a constant Fe′ concentration in the culture it is essential to not let the culture biomass get too high. The total Fe, Fe′ and Fe(III) concentrations reportedly used in laboratory cultures to achieve Fe replete (e.g. pFe 18 and 19) and Fe-limited (pFe >20.5) growth of microalgae are listed in Fig. 9.

Although useful for laboratory studies, the relevance of using synthetic chelators such as EDTA to buffer trace metals within culture medium to determine the Fe requirements and Fe-related physiology of microalgae has limitations. As previously discussed, the use of large amounts of EDTA results in the bioavailability of Fe to be primarily controlled by concentrations of Fe′. Yet our current understanding is that the dissolved Fe pool in the ocean is predominantly bound by natural organic ligands and the computed equilib-

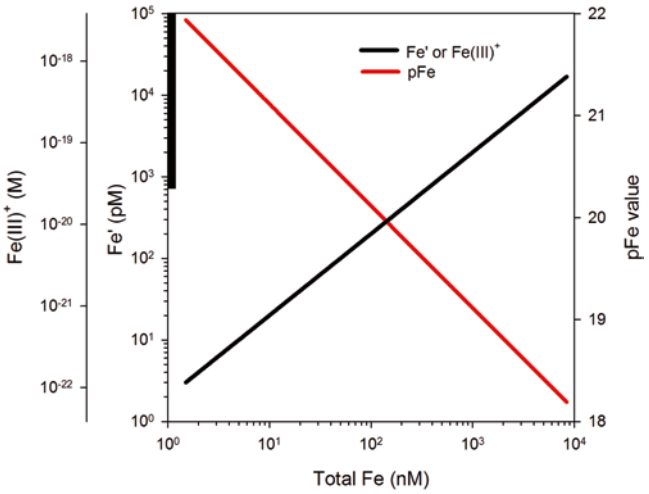

Fig. 9 Predicted dissolved inorganic Fe (Fe´), free ferric Fe (Fe(III)) concentrations, and pFe values for growth mediums as a function of total Fe concentrations. The conditional stability constants used to predict Fe´ and Fe(III) were obtained from Sunda et al. (2005), with a 100 µM EDTA metal ion buffer system in seawater at 20 °C, pH of 8.2, and salinity of 36. pFe values are the -log[Fe(III)]. At Fe´ above ~750 pM (*black bar*), significant iron hydroxide precipitation is likely to occur (Sunda and Huntsman 1995). For simplicity, the Fe´ and Fe(III) are also shown above this maximum

rium Fe′ concentrations are too low to support the observed growth of phytoplankton (Gledhill and van den Berg 1994; Rue and Bruland 1995). This observation suggests that some of these natural organic Fe complexes are bioavailable (e.g. Strzepek et al. 2011). The question can then be posed whether EDTA provides for an accurate representation of these natural organic ligands (Muggli and Harrison 1996a)? Although it is unlikely, it is difficult (but certainly not impossible) to perform Fe physiological investigations without the use of such chelators when growing phytoplankton in natural low-Fe seawater unamended with additional Fe complexes. Therefore, there are only a few studies examining Fe physiology without the use of synthetic chelators such as EDTA (e.g. Timmermans et al. 2001, 2004). Alternatively, natural organic ligands such as DFB can be used (see section below). If the objective is to grow phytoplankton without the use of such chelators, practicing strict trace metal clean procedures, in addition to quantifying and characterizing the naturally present organic and Fe ligand pools are essential.

9.2 Probing the Iron Nutritional Status of Natural Phytoplankton

The physiological state of phytoplankton with regards to Fe nutrition can be assessed by measuring rates of Fe uptake in the presence of DFB or EDTA using Fe radionuclides (i.e. ^{55}Fe or ^{59}Fe). The transport of Fe in the presence of excess DFB at sub-saturating Fe concentrations is indicative of Fe limitation. In contrast, the transport of Fe at saturating concentrations, achieved by nM additions of Fe bound to EDTA (to prevent Fe precipitation), are a measure of the number of Fe transporters at the cell surface. The reason is that at saturating Fe levels, maximum rates of Fe transport are observed and are determined by the number of Fe transporters at the cell surface and the rate of internalization, so that $\rho_{max} = k_{in*} L_T^{max}$.

9.2.1 Determining Sub-saturating Rates of Iron Uptake in the Presence of DFB

The hydroxamate siderophore desferrioxamine B (DFB) has a conditional stability constant for Fe similar to those of in situ strong organic ligands (Rue and Bruland 1995). Most recently, hydroxamate siderophores have been identified and quantified in the Atlantic, and seem to be widely distributed in the ocean (Mawji et al. 2008). Therefore, DFB is good model for the strong naturally occurring organic ligands for Fe in seawater. Many studies (e.g. Hutchins et al. 1999a; Eldridge et al. 2004; Taylor et al. 2013) have used DFB to induce Fe limitation in bottle incubations, by adding high concentrations of DFB that significantly decrease the concentration of inorganic Fe and result in an excess of free DFB. Whether additions of DFB induce Fe limitation depends on the molar ratio of [Fe] to [DFB]. If the concentration of DFB greatly exceeds that of Fe, the free DFB competes directly with the cell surface Fe transport complexes for the biologically generated free Fe, and thus the ability of the cells to take up Fe is hampered, and results in Fe-limited growth. For example, many oceanic and Southern Ocean species are able to grow at maximum rates when Fe and DFB are at equimolar concentrations (e.g. 4 nM each), while coastal species are Fe-limited in these growth conditions. As the concentration of DFB is increased, oceanic and Southern Ocean species start to experience Fe limitation to various degrees. For example, *T. oceanica* grew at 50 % of μ_{max} in the presence of 4 nM Fe and 40 nM DFB, while to achieve similar reductions in growth rates in Southern Ocean isolates (*Phaeocystis antarctica* and *Proboscia inermis*) additions of 400 nM DFB (bound to 4 nM Fe) were needed (Strzepek et al. 2011). In another study, *T. oceanica* did not achieve Fe limitation at Fe:DFB ratios of 12.9 nM Fe: 1.29 µM DFB, nor at ratios of 40 nM Fe: 4 µM DFB. However at ratios of 120 nM Fe:12 µM DFB, a 50 % reduction in growth rates was observed (Maldonado and Price 2001). In the Arctic, at in situ $[Fe]_{diss}$=0.15 nM, Fe limitation was induced with additions of 1 nM DFB (Taylor et al. 2013). In the Weddell Sea, at in situ $[Fe]_{diss}$=1.12 nM, Fe limitation was induced with a 10 nM DFB addition (Hoppe et al. 2013). Therefore, it is essential to know the initial concentration of dissolved Fe when conducting DFB addition experiments. As a rule of thumb, a DFB addition at a concentration 10- to 20-fold in excess of that of Fe should be sufficient to induce Fe limitation.

However, Fe uptake in the presence of DFB can also be used to test the Fe-limited conditions of phytoplankton

growth. This is due to the fact that phytoplankton are only able to acquire significant amounts of Fe bound within FeDFB when their growth rates are Fe-limited, and thus, have up-regulated their high-affinity Fe transport system (Maldonado and Price 1999; Strzepek et al. 2011). Although the rates of Fe uptake in the presence of FeDFB are orders of magnitude slower than those in the presence of only inorganic Fe or FeEDTA, these FeDFB uptake rates can be used as a proxy for the Fe-limited condition of the phytoplankton (Maldonado and Price 1999). Thus, the rates of ^{55}Fe uptake from ^{55}FeDFB (ρFeDFB) can be determined in order to establish whether plankton in the various treatments or locations are Fe-limited. The addition of Fe and DFB can vary depending on the study. For example, we have used mixtures of 2 nM Fe and 20 nM DFB (100 % of the Fe as ^{55}Fe, Maldonado and Price 1999; Maldonado et al. 2005), or 100 nM Fe and 105 nM DFB (5 % of the Fe as ^{55}Fe, Taylor et al. 2013). Another advantage of using DFB for Fe uptake experiments in situ is that because DFB is a strong Fe-binding ligand ($K_{Fe'L}^{cond} = 10^{16.5}$ M^{-1}) (Rue and Bruland 1995) the addition of FeDFB to seawater does not significantly increase the in situ Fe′ concentration over the course of the 24 h uptake assay. For example, if natural seawater has an [Fe′] of 1.5 * 10^{-12} M, and an Fe uptake experiment is performed with additions of 100 nM Fe and 105 nM DFB, the estimated [Fe′] resulting for the FeDFB addition is ~6.3 * 10^{-16} M, well below the in situ [Fe′]. This calculation was performed using $[Fe'] = [FeDFB] / \left([DFB'] * K_{Fe'L}^{cond} \right)$, where [DFB'] = $[DFB]_{free}$, and $K_{Fe'L}^{cond} = 10^{16.5}$ M^{-1}, (Rue and Bruland 1995).

To perform these Fe uptake experiments, before FeDFB is added it to the 1 L incubation bottles, the cold Fe and the ^{55}Fe (specific activity 740–1480 MBq mg^{-1}) must be complexed to DFB in small aliquots of Milli-Q water. All the manipulations must be performed inside a laminar flow hood. The pH of the ^{55}Fe stock solution (~0.11 mM ^{55}Fe in 0.005 M HCl) used to prepare the Fe complexes should be below 3, so that all of the Fe is truly dissolved. The DFB stock solution is prepared in sterile, Milli-Q water, and is kept refrigerated to prevent fungal growth. Complete complexation of the Fe to DFB is ensured by allowing the DFB to react with the Fe for 5–8 h at pH 3.3 (in 18.2 MΩ, MilliQ-H_2O with 0.0005 M HCl). At pH > 3, FeDFB is completely coordinated and very stable with respect to dissociation (Monzyk and Crumbliss 1982). Indeed, at pH 3.3 the concentration of DFB is 10^{13} times higher than that of $DFBH^+$, so most of the DFB is free and available to bind to Fe(III) (Maldonado and Price 2001). After FeDFB complexation has been achieved, the FeDFB can be added to triplicate 1 L polycarbonate bottles of seawater. The bottles are then capped and sealed with Parafilm, and then placed on-deck incubators at natural light levels and temperature. Every 4 h, samples are drawn from the incubation bottles, collected on polycarbonate filters of varying porosity and rinsed with Ti(III) citrate EDTA reagent (Hudson and Morel 1989) or oxalate wash (Tang and Morel 2006) to reductively dissolve iron hydroxides and surface adsorbed iron species. Control samples should be poisoned with glutaraldehyde (0.5 ml of 25 % glutaraldehyde L^{-1}) and then processed in an identical manner to that of the samples. These controls are used to correct for abiotic Fe uptake and ^{55}Fe adsorption by the filters, and normally represent <5 % of the total Fe uptake by the living cells.

Adding nM concentrations of Fe in the FeDFB uptake experiments (or FeEDTA, see below) allows Fe uptake rates to be calculated without a priori knowledge of the in situ Fe concentration, at least in most open ocean surface waters where $[Fe]_{diss}$ ~0.07–0.3 nM (Johnson et al. 1997). Volumetric ^{55}Fe uptake rates (mol L^{-1} h^{-1}) are then calculated from the linear regression of particulate ^{55}Fe concentration as a function of incubation time. These ^{55}Fe uptake rates are then converted to total Fe uptake rates using the ratio of dissolved concentrations of ^{55}Fe to cold Fe in the experimental Fe uptake bottles. In field Fe uptake studies, the volumetric Fe uptake rates can be normalized to phytoplankton biomass using chlorophyll as a proxy for biomass. These chl *a*-normalized rates can then be normalized to C using published C to chl-*a* ratios (Geider 1987). In laboratory Fe uptake experiments, the rates of Fe transport (ρFe) should be normalized to cellular surface area. Reporting Fe uptake rates as mol Fe μm^{-2} h^{-1} allows easy comparison between species of different sizes, or between Fe-limited growth treatments of the same species (given that a reduced cell size is a common acclimation to Fe limitation). In addition, Shaked and Lis (2012) suggested normalizing sub-saturating Fe uptake rates to the concentration of dissolved Fe, given that sub-saturating Fe uptake rates by a cell vary with Fe concentration. The resulting parameter is the uptake rate constant (k_{up} in Shaked and Lis 2012; or k_{in} in Lis et al. 2015), and has units of L $cell^{-1}$ h^{-1} or L μm^{-2} h^{-1}, depending on whether the ρFe are, in the first place, expressed as cellular rates or normalized to surface area, respectively. Therefore, k_{up} (or k_{in}) allow easy comparisons among multiple studies that used various sub-saturating dissolved Fe concentrations. Indeed, Lis et al. (2015) compared the uptake rate constants (k_{in}) of many Fe-limited phytoplankton and discovered a tight correlation and proportionality between their k_{in} and their respective surface areas, suggesting a physical constraint on the number of transporters at the cell surface allocated toward Fe uptake, as proposed earlier by Hudson and Morel (1993). Interestingly the k_{in} for Fe′ was 1000-fold higher than the k_{in} for strong organic complexes like FeDFB. Furthermore, many other Fe substrates have k_{in} between the values for Fe′ and FeDFB. This finding suggests that these two substrates (Fe′ and FeDFB) provide an empirical range for the bioavailability of all Fe substrates for uptake by phytoplankton.

Lis et al. (2015) argued that the ~1000-fold difference in the k_{in} between Fe′ and FeDFB is most likely due to large difference (100–1000-fold) between Fe reduction rates of Fe′ versus FeDFB. However, the limited data sets available suggest that biological reduction rates of inorganic and organic Fe complexes do not differ greatly (~2 fold; Weger 1999; Maldonado and Price 2001). So, the question of why there is a 1000-fold difference between the K_{in} for Fe′ and that of FeDFB, remains unresolved. It is possible that the difference reported in Lis et al. (2015) is an artifact of the way k_{in} was calculated for FeEDTA uptake experiments (ρFe/[Fe′]) versus FeDFB experiments (ρFe/[FeDFB]).

9.2.2 Determining Saturating Rates of Iron Uptake in the Presence of EDTA

Short-term Fe uptake rates from ^{55}FeEDTA (ρFeEDTA) can be determined to investigate saturating rates of Fe uptake, which are a direct function of the density of plasmalemma-bound Fe transporters, and are faster for Fe-limited phytoplankton (Harrison and Morel 1986). The experiments are performed in an identical manner to those of FeDFB, except that the Fe is added at saturating concentrations and is prechelated to EDTA before it is added to the water as a FeEDTA complex. We have used various concentrations of Fe:EDTA, from 20 nM Fe bound to 500 nM EDTA (5 % of the Fe as ^{55}Fe; Maldonado and Price 1999), to 100 nM Fe bound to 1 μM EDTA (1 % of the Fe as ^{55}Fe; Taylor et al. 2013). The high concentrations of EDTA in the Taylor et al. (2013) experiment were chosen so that most of Fe in the system was bound to EDTA and there was very little competitive binding of Fe by in situ natural ligands or DFB (in the induced Fe-limiting treatment bottles). As a general rule, the lower concentrations of EDTA used in Maldonado and Price (1999) are recommended to minimize EDTA chelation of other essential trace metals such as zinc.

10 Conclusions and Future Directions

Our knowledge of the important roles that iron plays within phytoplankton and the vast number of acquisition strategies invoked to cope with a limited Fe supply has increased substantially since the discovery of widespread Fe limitation in aquatic environments. Over the last century, we have gained an appreciation for the incredible amount of genetic, physiological and species diversity that exists within phytoplankton communities (Andersen 1992). Yet despite this diversity, much of our understanding of microalgal physiology is based on only a handful of model species, with only a subset of these having their full genomes sequenced. Over the next decade, we anticipate a substantial increase in the number of sequenced genomes of phytoplankton, covering all taxonomic groups, both prokaryotic and eukaryotic. This will certainly bring to light the complexity and variety that exists among phytoplankton with respect to the distribution of Fe-requiring proteins, uptake and storage strategies, and ways in which they have evolved to cope with Fe limitation. In addition, the number of studies implementing the 'omic' approaches (i.e., genomics, transcriptomics, proteomics and metabolomics) to examine both laboratory isolates and natural assemblages will continue to increase. Their findings will provide new insights into how phytoplankton interact with their biotic and abiotic environments and how Fe-related processes are affected by these interactions. Clearly, we are just at the beginning of a molecular era in phytoplankton research.

Another growing area of research is investigations into the degree to which microalgae experience co-limitation between Fe and other essential elements and compounds required for growth. Although it has been well acknowledged that Fe and light may be co-limiting for phytoplankton growth in their natural environment, other types of co-limitation exist (Saito et al. 2008) and may be widespread. For example, recent studies suggest that in certain HNLC regions, there is co-limitation of microalgal growth by Fe and vitamin B_{12}. Additions of both Fe and vitamin B_{12} have been shown to either further stimulate the growth of entire phytoplankton communities (Bertrand et al. 2007) or a subset of species, resulting in shifts in community composition when vitamins are added in addition to Fe (Koch et al. 2011). There have also been suggestions of co-limitation of phytoplankton by Fe and other nutrients: N (Behrenfeld et al. 2006; North et al. 2007; Taylor et al. 2013), P (Mills et al. 2004), Si (Marchetti et al. 2010; Brzezinski et al. 2011), as well as other essential trace elements including Cu (Guo et al. 2012), Co (Saito et al. 2005) and Zn (Crawford et al. 2003). Our current understanding of how such co-limitations will impact Fe-related phytoplankton physiology is still limited.

It has been over two decades since John Martin and others provided compelling evidence of widespread Fe limitation in the sea, indicating that Fe regulates the primary productivity of large areas of the ocean and suggesting that it has a profound effect on global climate. He will forever be remembered for proclaiming, "Give me a tanker of Fe and I'll give you an ice age". With the growing concerns over climate change, large-scale Fe fertilization continues to be considered as a geo-engineering strategy to reduce atmospheric CO_2 concentrations. To be successful, the efficiency in which an increased supply of Fe has the ability to result carbon transport to the deep ocean and thus long term carbon sequestration would need to be well constrained. However, an important lesson from the previous era of large-scale Fe fertilization experiments is that there is extensive biological, chemical and physical variability within the ocean and Fe-limited regions are no exception. With every Fe-stimulated

phytoplankton bloom, whether it be by natural or anthropogenic causes, the phytoplankton response and associated carbon export is influenced by the entire plankton community composition, their degree of Fe limitation, the ambient nutrient concentrations as well as a myriad of other variables; all of which can change on very small spatial and short temporal scales making the outcome somewhat unpredictable.

How future climate change will affect Fe-related processes in phytoplankton communities is presently uncertain. As the ocean environment responds to the increasing effects of ocean acidification and warming, there are many unknowns as to how phytoplankton will acclimate or adapt. If ocean acidification affects Fe distributions and speciation as predicted, Fe availability to phytoplankton will be affected. Indirectly, ocean acidification could also influence the distributions of other bioactive metals that interact with Fe nutrition. For example, it is predicted that Cu availability will increase as Fe availability decreases (Millero et al. 2009; Hoffmann et al. 2012). Depending on relative changers in Cu and Fe availability, this increase in Cu availability could be beneficial or detrimental. If the Fe concentration is very low and the Cu concentration is not toxic, higher Cu might enhance the activity of the high-affinity Fe transporters. However, if Fe concentration is very low but Cu concentration is at toxic levels, phytoplankton growth could be impaired. The availability of these metals may also affect the phytoplankton production of the climate-influencing osmolyte, dimethylsulfoniopropionate (DMSP) (Sunda et al. 2002). Recent evidence has also shown ocean acidification slows down N_2 fixation in the diazotroph *Trichodesmium* when they are Fe-limited (Shi et al. 2012). How all of these changes will alter phytoplankton Fe requirements and their elemental compositions as well as important cellular processes such as photosynthesis, respiration and the production of organic compounds such as DMSP is unclear. Most importantly, will all phytoplankton be affected in the same way and how will these changes influence the community composition throughout the different aquatic environments? Resolving the answers to these questions is critical given the significant roles phytoplankton play in aquatic food webs and global biogeochemical cycles.

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Selenium in Algae

Hiroya Araie and Yoshihiro Shiraiwa

1 Introduction

Selenium (Se) is a homologous element with oxygen and sulfur (S). In the environment, Se is present in the form of its elemental form (S^0), selenide ($Se(II)^{2+}$), selenite ($Se(IV)O_3^{2-}$), or selenate ($Se(VI)O_4^{2-}$). In vivo, Se acts as an analogous element to S and shows similar physical and chemical properties to sulfur. Therefore, Se is involved in some compounds as a substitute for S. For various organisms, Se is required as an essential trace element exhibiting biologically important functions, although it is very toxic at high concentrations. Many organisms, including microalgae, require Se for the synthesis of selenium containing proteins, called selenoproteins, which are thought to be the physiologically active and functional molecular form of Se in cells (Danbara and Shiraiwa 1999; Araie and Shiraiwa 2009).

2 Selenoproteins

The major biological form of Se is represented by selenocysteine (Sec), an amino acid analogue of cysteine (Cys) and sometimes referred to as the 21st amino acid (Turanov et al. 2011). It is known that the selenol in Sec is more highly reactive than the thiol in Cys. The most important biological function of Sec is as a component of selenoproteins. Sec possesses a greater ability to reduce peroxide than Cys because of its high nucleophilicity (Fox 1992). Due to the nature of Sec, most selenoproteins identified to date are categorized into oxidoreductases that require strong nucleophilicity of Sec for their activity (Stadtman 1996). Therefore, selenoproteins show a higher reducing capacity than other oxidoreductases without involving Sec. Such characteristics of selenoproteins are thought to play an essential role in maintaining cell viability (Brown and Arthur 2001; Kim et al. 2006). Glutathione peroxidase and thioredoxin reductase are well-known selenoproteins that catalyze the elimination of reactive oxygen species and the reduction reaction of thioredoxin, respectively.

The mechanism of selenoprotein biosynthesis is well-characterized in mammals. First, Sec is not synthesized in a single molecular form but synthesized as Sec-tRNA by binding of selenide onto Ser-tRNA, which is a very specific reaction to produce Sec (Tamura et al. 2004). Then, Sec is co-translationally incorporated into proteins at the site of the UGA codon, which usually acts as a stop codon, on mRNA through a specific Sec insertion mechanism. This mechanism includes a *cis*-acting mRNA structure known as the Sec insertion sequence (SECIS) that is located in the 3′-untranslated region (UTR) of selenoprotein mRNAs, and *trans*-acting factors consisting of Sec specific tRNA, a selenocysteyl-tRNA-specific elongation factor (EFSec), and an SECIS binding protein 2 (SBP2) (Hatfield and Gladyshev 2002) (Fig. 1). In eukaryotes, secondary structures of SECIS are categorized into forms 1 and 2, and the non-Watson–Crick quartet is highly conserved in both forms (Krol 2002). In this mechanism, the real stop codon is the UAA codon located downstream of the UGA codon on an mRNA strand.

More than 40 selenoprotein families have been identified in diverse organisms, including bacteria, archaea, and eukaryotes, by metabolic ^{75}Se-labeling experiments and bioinformatics approaches (Kryukov et al. 2003; Kryukov and Gladyshev 2004). Almost all selenoproteins are categorized into three groups: (1) Selenoproteins in which Sec is located in the N-terminal regions; these selenoproteins often exhibit thioredoxin or thioredoxin-like foldings. This group is most abundant; (2) Selenoproteins in which Sec is located in the C-terminal sequences; these selenoproteins have been described only in eukaryotes; (3) Selenoproteins which utilize Sec to coordinate redox metals (molybdenum, tungsten and nickel) in the active site of enzymes (Gladyshev et al. 1994;

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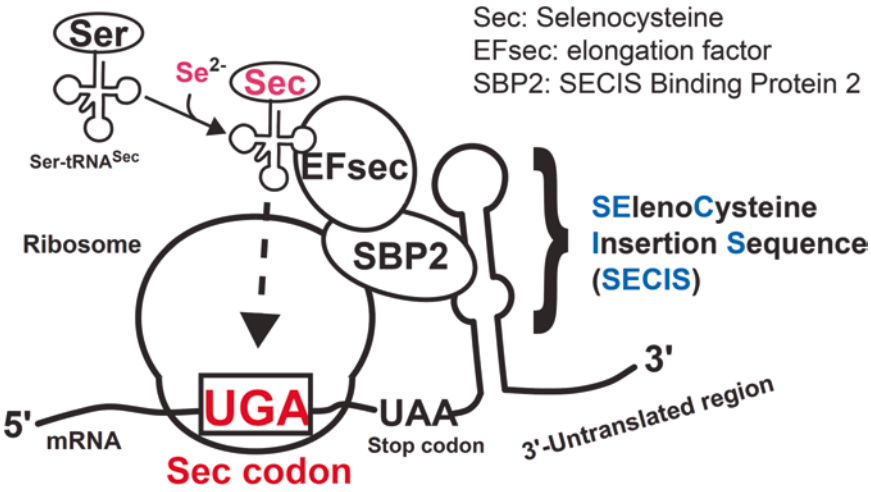

Fig. 1 Selenoprotein synthesis in mammals

Graentzdoerffer et al. 2003; Valente et al. 2005). This latter selenoprotein group is found only in prokaryotes. However, the function of many selenoproteins is still unknown.

Interestingly, neither selenoprotein genes nor any components of the Sec insertion mechanism have been found in the genomes of either the land plant *Arabidopsis thaliana* or the yeast *Saccharomyces cerevisiae* (Novoselov et al. 2002). There are no species of land plants that require Se. There is no information to explain the reason why/how yeasts and higher plants did not evolve Se utilization mechanisms during evolution.

3 Selenoproteins and Se Requirement in Microalgae

Previously, only little information has been available on selenoproteins in microalgae; namely, ten selenoproteins in the green alga *Chlamydomonas reinhardtii*, one in the diatom *Thalassiosira pseudonana*, and two in the haptophyte *Emiliania huxleyi*[1] (Price and Harrison 1988; Novoselov et al. 2002; Obata and Shiraiwa 2005; Araie et al. 2008). This was due to the difficulty of identification by physiological and biochemical analysis since the cellular content of selenoproteins is very low. Recently, many selenoproteins have been identified by using bioinformatic approaches in various photosynthetic organisms, such as the green algae *Ostreococcus tauri* and *Ostreococcus lucimarinus*, the pelagiophyte *Aureococcus anophagefferens*, and the coccolithophore, *Emiliania huxleyi* (Lobanov et al. 2007; Gobler et al. 2011; Read et al. 2013) (Table 1).

[1]Wherever possible the currently accepted names for species are used. The name used in the paper cited is also indicated. For details of names see chapter "Systematics, Taxonomy and Species Names: Do They Matter?" of this book (Borowitzka 2016).

Algae that required Se for their growth were found especially in secondary endosymbiotic algae derived from red algae (Table 2). The growth-stimulating effect of marine phytoplankton by the addition of Se was higher in the molecular form of selenite than the selenate ion (Hu et al. 1996), and this result was also confirmed in marine coccolithophores (Danbara and Shiraiwa 1999). The coccolithophore *Emiliania huxleyi* showed both an essential Se-requirement and selenoprotein biosynthetic ability. However, not all algae possessing an ability to synthesize selenoproteins show an essential requirement of Se for their growth. For example, *Chlamydomonas reinhardtii* shows a growth-stimulation effect of Se and possesses selenoprotein biosynthesis ability, but Se is not essentially required for its growth. When selenite is added to the culture medium, a Sec-containing glutathione peroxidase (selenoprotein) is synthesized *de novo*, while a Cys-containing form of the protein (cysteine protein) is generally synthesized (Yokota et al. 1988; Novoselov et al. 2002). The green alga *Tetraselmis* (*Platymonas*) *subcordiformis* also shows growth-stimulating effects by the addition of Se, although Se is not essentially required for its growth (Wheeler et al. 1982). Other green algae, such as *Ostreococcus* also produce selenoproteins, but there is no information on Se-essential requirement in the literature. In the red alga *Cyanidioschyzon merolae*, which is a primary symbiotic photosynthetic organism, selenoprotein genes have not been found in genome databases (Lobanov et al. 2007). Although there is still little information about selenoproteins and the requirement and essentiality of Se in algae, marine algae show a stronger trend to a preference for Se than freshwater algae do. In eukaryotes, it is suggested that aquatic organisms tend to possess more selenoproteins than terrestrial organisms (Lobanov et al. 2007). This may be due to the availability of Se in aquatic environments than to the terrestrial environments, since the area of Se-containing soils is limited to areas polluted by anthropogenic activity and near to copper mining. In the euphotic zone of the ocean, SeO_4^{2-},

Table 1 Selenoproteins identified by analysis of eukaryotic genomes[a]

Selenoprotein family	*Ostreococcus tauri*[b]	*Ostreococcus lucimarinus*[b]	*Mesostigma viride*[b]	*Volvox carteri*[b]	*Chlamydomonas reinhardtii*[b, c]
Selenoprotein K	+	+			+
Selenoprotein H	+	+		+	+
Selenophosphate synthetase					
Iodothyronine deiodinase					
Selenoprotein 15	+	+			
Membrane SelenoProtein	+	+	+	+	+
Phospholipid hydroperoxide glutathione peroxidase					++
Glutathione peroxidase	+++++	+++++		+	
Selenoprotein T	+	+		+	+
Thioredoxin reductase 1	+	+		+	+
Selenoprotein M	+	+	+	+	++
Selenoprotein U	+	+			+
Methionine sulfoxide reductase A	+	+		+	+
Methionine sulfoxide reductase B					
Protein disulfide isomerase	+++	+++			
Methyltransferase	+	+			
Peroxiredoxin	+	+++			
Thioredoxin-fold protein	+	+			
Selenoprotein O	+	+			
Selenoprotein W	+	++	+	+	++
Selenoprotein S	+	+			
Hypothetical protein	+++	+++			
Glutaredoxin					
Fe-S reductase					
Fe-S oxidoreductase					
Thiol:disulfide interchange protein					
UGSC-containing protein					
Dithiodisulfide oxidoreductase					
Rhodanase					
Gamma interferon inducible lysosomal thiol reductase					
Total number of genes identified	26	29	3	8	13

Selenoprotein family	*Pyropia (Porphyra) haitanensis*[b]	*Aureococcus anophagefferens*[d]	*Thalassiosira pseudonana*[b]	*Emiliania huxleyi*[e]
Selenoprotein K		+		
Selenoprotein H		+		++
Selenophosphate synthetase			+	
Iodothyronine deiodinase		+		++++
Selenoprotein 15		+		+
Membrane SelenoProtein		+		+
Phospholipid hydroperoxide glutathione peroxidase				+
Glutathione peroxidase		+++++	++	+++++
Selenoprotein T		+	+	++
Thioredoxin Reductase 1		+	+	+
Selenoprotein M		++	++	++++++
Selenoprotein U		++	++	+++
Methionine sulfoxide reductase A		++++	+	++
Methionine sulfoxide reductase B		++++		

(continued)

Table 1 (continued)

Selenoprotein family	*Pyropia (Porphyra) haitanensis*[b]	*Aureococcus anophagefferens*[d]	*Thalassiosira pseudonana*[b]	*Emiliania huxleyi*[e]
Protein disulfide isomerase		+++	++	+++
Methyltransferase		++	+	+
Peroxiredoxin		+++	++	
Thioredoxin-fold protein		+++++	+	+++
Selenoprotein O	+	+		++++
Selenoprotein W		+++++		+
Selenoprotein S				
Hypothetical protein		+++++++		
Glutaredoxin		+++		
Fe-S reductase		+		
Fe-S oxidoreductase		+		+
Thiol:disulfide interchange protein		+		+
UGSC-containing protein		+		
Dithiodisulfide oxidoreductase				+++++
Rhodanase		+		
Gamma interferon inducible lysosomal thiol reductase		+		++
Total	1	59	16	49

Number of selenoprotein-encoding genes identified as identical is expressed by number of '+'
[a]Modified from Lobanov et al. (2007)
[b]Lobanov et al. (2007)
[c]Novoselov et al. (2002)
[d]Gobler et al. (2013)
[e]Read et al. (2013)

Table 2 List of phytoplankton species showing growth-stimulatiion by Se and requiring Se essentially for their growth[a]

Class	Species	References
Bacillariophyceae (Diatoms)	*Chaetoceros debilis*	Harrison et al. (1988)
	Chaetoceros cf. *tenuissimus*[d]	Doblin et al. (1999)
	Chaetoceros pelagicus	Harrison et al. (1988)
	Chaetoceros vixvisibilis	Harrison et al. (1988)
	Coscinodiscus asteromphalus	Harrison et al. (1988)
	Corethron criophilum	Harrison et al. (1988)
	Ditylum brightwellii	Harrison et al. (1988)
	Skeletonema costatum (strain 18c NEPCC)	Harrison et al. (1988)
	Skeletonema costatum (strain 611 NEPCC)	Harrison et al. (1988)
	Skeletonema costatum (strain 616 NEPCC)	Harrison et al. (1988)
	Stephanopyxis palmeriana	Harrison et al. (1988)
	Stephanodiscus hantzschii var. *pusillus*	Lindström (1983)
	Thalassiophysa hyalina (as *Amphiprora hyalina*)	Harrison et al. (1988)
	Thalassiosira aestivalis	Harrison et al. (1988)
	Thalassiosira oceanica	Harrison et al. (1988)
	Thalassiosira pseudonana	Price et al. (1987) and Harrison et al. (1988)
	Thalassiosira rotula (= *T. gravida*)	Harrison et al. (1988)
Dinophyceae (Dinoflagellates)	*Alexandrium minutum*[b]	Doblin et al. (1999)
	Gymnodinium catenatum[b]	Doblin et al. (1999)
	Karenia mikimotoi (as *Gymnodinium nagasakiense*)[b]	Ishimaru et al. (1989)
	Peridinium cinctum f. *westii*	Lindström and Rodhe (1978)
	Pyrodinium bahamense[b]	Usup and Azanza (1998)

(continued)

Table 2 (continued)

Class	Species	References
Prymnesiophyceae (Haptophytes)	*Chrysochromulina breviturrita*	Wehr and Brown (1985)
	Chrysochromulina strobilus	Pintner and Provasoli (1986)
	Emiliania huxleyi	Danbara and Shiraiwa (1999)
	Gephyrocapsa oceanica	Edvardsen et al. (1990)
	Haptolina brevifila (as *Chrysochromulina brevefilum*)	Pintner and Provasoli (1986)
	Helladosphaera sp.	Edvardsen et al. (1990)
	Prymnesium kappa (as *Chrysochromulina kappa*)	Pintner and Provasoli (1986)
	Prymnesium polylepis (as *Chrysochromulina polylepis*)[b]	Edvardsen et al. (1990)
Raphidophyceae	*Pseudochattonella verruculosa* (as *Chattonella verruculosa*)[b]	Imai et al. (1996)
Pelagophyceae	*Aureococcus anophagefferens*[b]	Cosper et al. (1993)
Porphyridiophyceae	*Porphyridium cruentum*[d]	Wheeler et al. (1982)
Chlorophyceae	*Acutodesmus obliquus* (as *Scenedesmus obliquus*)	Abdel-Hamid and Skulberg (1995)
	Chlamydomonas reinhardtii[c]	Novoselov et al. (2002)
	Chlamydomonas sp.	Lindström (1983)
	Chlorella sp.	Lindström (1983)
	Chlorella sp.	Abdel-Hamid and Skulberg (1995)
	Chlorella sp.[d] (UTEX LB2068)	Wheeler et al. (1982)
	Dichtyosphaerium pulchellum	Lindström (1983)
	Dunaliella primolecta[c]	Wheeler et al. (1982)
	Dunaliella salina[c]	Reunova et al. (2007)
	Monoraphidium contortum	Abdel-Hamid and Skulberg (1995)
	Monoraphidium convolutum	Abdel-Hamid and Skulberg (1995)
	Monoraphidium griffithii	Abdel-Hamid and Skulberg (1995)
	Monoraphidium sp.	Lindström (1983)
	Pandorina morum	Lindström (1983)
	Scenedesmus quadricauda	Lindström (1983)
	Tetraëdron caudatum	Lindström (1983)
	Tetraselmis subcordiformis (as *Platymonas subcordiformis*)[c]	Wheeler et al. (1982)
	Tetraelmis sp. (as *Platymonas* sp.)[c]	Wheeler et al. (1982)

[a]Modified from a review by Araie and Shiraiwa (2009)
[b]Harmful algae
[c]Algal growth was stimulated, no essential requirement of Se for their growth
[d]No stimulation of algal growth by Se

SeO_3^{2-} and organic selenium are present in a ratio of about 3:1:1 (Cutter and Bruland 1984). Almost all Se is present in the form of selenate and the ratio of selenite decreases in species representing the gradual move to terrestrial environments from aquatic environments. Although the Se level is fairly constant in the ocean, there are some terrestrial regions where Se is only present at low consentrations or not at all.

4 Uptake of Selenium in Microalgae

Se shows high toxicity at high concentration, even though Se is an essential element for many organisms. On some wetlands, higher plants are sometimes damaged by Se-polluted water and soils, and therefore Se metabolism has been well-studied in land plants, especially in terms of its toxicity. Selenate is non-specifically absorbed via the sulfate transporter as a counterpart to sulfate transport in higher plants (Arvy 1993). The model of nutrient uptake simply shows Michaelis-Menten type kinetics as the same as that of enzyme kinetics. The value of $K_{m,}$ the Michaelis-Menten coefficient, can be calculated by using the Lineweaver-Burk plot analysis. There are a few recent reports that show the presence of a selenite transporter protein. In wheat and rice, selenite ion was found to be taken up via the phosphate transporter and the silicon transporter in a competitive manner to phosphate and silicate ions (Li et al. 2008; Zhao et al. 2010). In *C. reinhardtii*, a pH-dependent competition profile was observed between phosphate and selenite uptake (Riedel and Sanders 1996). In *E. huxleyi*, selenite is transported by an active transport system with a K_m of 40–142 nM (Obata et al. 2004; Araie et al. 2011). According to kinetic analysis, we

assume the induction of unknown selenite transporter possessing very high affinity under Se depleted conditions. The existence of such a high affinity transporter may be the reason why K_m values for the selenite active transport system show such a wide range. The active transport system is thought to be driven by a pH gradient energized by H^+-ATPase, as well as the sulfate and phosphate transporters (Hawkesford et al. 1993; Mitsukawa et al. 1997). The specificity of this transport system to selenite was higher than that of sulfite, selenate and phosphate (Araie et al. 2011). Although the K_m-value is very low, the value observed experimentally seems not to be sufficient to absorb selenite ion efficiently from seawater, since the concentration of selenite in the ocean is below 1 nM (Cutter and Bruland 1984; Cutter and Cutter 2001; Hattori et al. 2001). The yeast, *S. cerevisiae*, also shows selenite uptake by Jen1P, a proton-coupled monocarboxylate transporter, and via orthophosphate transporters with K_m values of 910 μM and 19 μM, respectively (Lazard et al. 2010; McDermott et al. 2010). To date, a selenite-specific active transport system has been found only in *E. huxleyi* (Araie et al. 2011).

5 Metabolism of Selenium in Microalgae

Generally, Se is detoxified to non-toxic, organic, compounds for accumulation or volatilization in land plants. A few land plants have been found to accumulate Se up to thousand-fold in their body as non-toxic organic compounds. For instance, Se-methylselenocysteine (MeSeCys), Se-methylselenomethionine (MeSeMet) and γ-glutamyl-Se-methylseleno-cysteine are well known as non-toxic organic seleno-compounds used to reduce the toxic effects of Se (Pickering et al. 2003). There are also some species that metabolize Se to dimethylselenide (DMSe) and dimethyldiselenide (DMDSe), which are volatile Se forms (Ellis and Salt 2003). There are only a few reports on Se metabolism in algae. Similar to land plants, the presence of non-toxic organic compounds of Se, such as MeSeCys and MeSeMet, have been identified in *Dunaliella primolecta*, *Chlorella* sp. and *Porphyridium cruentum* (Bottino et al. 1984). In *Chlorella* sp., volatile DMSe, DMDSe and dimethylselenenylsulfide were also detected (Fan et al. 1997). Furthermore, precursors of such volatile forms of Se-compounds, such as Se-allylselenocysteine, selenoethionine and dimethylselenonium propionate, have been detected in *Chlorella* sp. (Larsen et al. 2001). A study on ^{75}Se-labeled metabolites in *E. huxleyi* cells using a radio labeling technique with ^{75}Se-selenite proved useful for a understanding of algal Se metabolism (Obata et al. 2004). Cell components were separated into fractions of low-molecular weight compounds (LMCs), proteins, lipids, polysaccharides, and nucleic acids after a 16 h-incubation with ^{75}Se-selenite. *E. huxleyi* cells incorporated ca. 70 % of ^{75}Se into LMCs, and ca. 17 % was in the protein fraction. In thin-layer chromatography (TLC) analysis of LMCs, MeSeCys, which is known as a non-toxic organic Se compound, was detected. Interestingly, ^{75}Se pulse-chase labelling experiments clearly showed that ^{75}Se in LMCs was transferred into the protein fraction. These results suggest that *E. huxleyi* cells first accumulate Se into non-toxic organic compounds, and then use the compounds to supply Se for the synthesis of selenoproteins. *E. huxleyi* cells thus possess not only animal (selenoprotein) and land plant (non-toxic organic compound) pathways, but also a new bypass pathway connecting the animal and land plant pathways. This kind of Se metabolism is thought to be specific to algae.

6 Concluding Remarks

The utilization of selenium can be considered advantageous for many organisms to increase their viability by promoting the function of enzymes due to higher reactivity of selenium than sulfur, although selenium is generally very toxic at high concentration. Therefore, a high ability of Se-absorption may stimulate viability of organisms, but may sometimes damage organisms, depending on the property of organisms and environmental availability of selenium. Actually, the distribution of selenium is limited on land and therefore the property of an essential Se-requirement seems to become a negative factor for the wide distribution of organisms under such circumstances.

Interestingly, high activity of Se-uptake is observed even in organisms which have no essential Se-requirement for growth, such as land plants and green algae. Further, a part of such organisms is known to show detoxification of Se-toxicity by incorporating the Se into non-toxic organic and volatile Se-compounds. In marine ecosystems, nanomolar levels of selenium are dissolved in sea-surface water and its concentration becomes higher in the deep sea. As marine phytoplankton, such as haptophytes and diatoms, show Se-requirement for their growth, the supply of selenium may be one possible trigger for phytoplankton bloom formation in the ocean. The study of such regulation of bloom-producing phytoplankton growth by supplying selenium in the ocean will be important as a future research target to control biomass production and monitor global environment change. Collaboration between molecular biological studies on the function of selenium in marine organisms and studied of marine ecosystems will provide more information on the wide variation in Se-utilization strategies in living organisms.

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Silicification in the Microalgae

Zoe V. Finkel

1 Silicification in the Microalgae

Silicon (Si) is the second most common element in the Earth's crust (Williams 1981) and has been incorporated in species from most of the biological kingdoms (Knoll 2003). In this review I focus on what is known about: Si accumulation and the formation of siliceous structures in microalgae and some related non-photosynthetic groups, molecular and genetic mechanisms controlling silicification, and the potential costs and benefits associated with silicification in the microalgae. This chapter uses the terminology recommended by Simpson and Volcani (1981): Si refers to the element and when the form of siliceous compound is unknown, silicic acid, $Si(OH)_4$, refers to the dominant unionized form of Si in aqueous solution at pH 7–8, and amorphous hydrated polymerized Si is referred to as opal or silica.

2 Si Content across the Microalgae (and Some Related Groups)

Silicon has been found widely across the algae (Table 1). In some algal groups genetically controlled species-specific complex siliceous structures are formed (Fig. 1), while in other groups Si has been detected but has not been localized.

Silicon has been detected but poorly localized in the Cyanobacteria. Si has been detected both marine and freshwater genera of cyanobacteria, including species of *Synechococcus*, *Microcystis*, and *Spirulina/Arthrospira*, but the form of the Si and its location within the cell are unknown (El-Bestawy et al. 1996; Sigee and Levado 2000; Krivtsov et al. 2005; Baines et al. 2012). It has been hypothesized that, in some cases, the detected Si may be detrital and external to the cell wall, or Si may be bound to organic ligands associated with the glycocalyx, or that Si may accumulate in periplasmic spaces associated with the cell wall (Baines et al. 2012). In the case of field populations of marine *Synechococcus*, silicon to phosphorus ratios can approach values found in diatoms, and significant cellular concentrations of Si have been confirmed in some laboratory strains (Baines et al. 2012). The hypothesis that Si accumulates within the periplasmic space of the outer cell wall is supported by the observation that a silicon layer forms within invaginations of the cell membrane in *Bacillus cereus* spores (Hirota et al. 2010).

Significant quantities of Si, likely opal, have been detected in freshwater and marine green micro- and macro-algae (Fu et al. 2000), in particular the freshwater Hydrodictyaceae such as *Pediastrum* (Millington and Gawlik 1967; Sigee and Holland 1997), in some loricae of *Hemitoma* (Krienitz et al. 1990) and in the marine chlorophyte *Tetraselmis* (as *Platymonas*)[1] (Fuhrman et al. 1978). Si has been shown to be associated with the outer cell wall of *Pediastrum* and *Pedinomonas tuberculata* (Millington and Gawlik 1967; Preisig 1994). In the case of *P. tuberculata* Si is a component of the tubercular excrescence of the outer wall in the form of quartz (Manton and Parke 1960). Small amounts of Si have been detected widely across the Phaeophyceae (macroalgae) and in *Ptilonia okadai* (Rhodophyta) (Parker 1969; Fu et al. 2000; Mizuta and Yasui 2012). In *Saccharina japonica* Si is localized to the cuticle and mucilage caps of sori, in wounded tissues, and between the epidermal cells and outer cortical cells of sporophyte vegetative tissues (Mizuta and Yasui 2012). The Xanthophyceae may have silicified walls and cysts (Bold and Wynne 1978; Tappan 1980; Preisig 1994), but this should be confirmed (see Ariztia et al. 1991). Although rare, *Urceolus sabulosus* (Euglenophyta) is known

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[1]Wherever possible the currently accepted names for species are used. The name used in the paper cited is also indicated. For details of names see chapter "Systematics, Taxonomy and Species Names: Do They Matter?" of this book (Borowitzka 2016).

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Table 1 Microalgae and related groups that accumulate silicon

Kingdom	Phylum, Class, Order (common name)	Example genera	Form of Si	Marine or fresh
Bacteria	Cyanophyta (Cyanobacteria)	*Synechococcus*, *Microcystis, Arthrospira*	Amorphous opal? Not localized	Marine and fresh
Plantae	Chlorophyta, Chlorophyceae (Green algae)	*Tetraselmis Pediastrum*, *Hydrodictyon*	Si in cell wall	Marine and fresh
Chromista	Cercozoa, Imbricatea (Filoseans)	*Euglypha, Thaumatomastix*	External scales	Marine and fresh
Chromista	Dinophyta, Dinophyceae (Dinoflagellates)	1. *Actiniscus*	1. Internal skeletal elements	Marine and fresh
		2. *Ceratium, Peridinium*,	2. Si in outer layer of cyst wall,	
		3. *Eodinia*, *Jusella*	3. Fossil genera with silicified cysts or theca	
Chromista	Haptophyta, Coccolithophyceae (Coccolithophores)	*Prymnesium neolepis*	External scales	Marine
Chromista	Ochrophyta, Chrysophyceae	*Chrysosphaerella, Paraphysomonas, Spiniferomonas*	Silicified cysts and some species have ornamented external scales in motile stage	Fresh and marine
Chromista	Ochrophyta, Synurophyceae	*Synura, Mallomonas*	All species have external scales in motile stage	Fresh
Chromista	Ochrophyta, Dictyochophyceae (Silicoflagellates)	*Dictyocha*	Siliceous skeleton of hollow rods	Marine
Chromista	Ochrophyta, Phaeophyceae (Brown algae)	*Ectocarpus*, *Macrocystis*, *Pelagophycus*	Not localized	Marine
Chromista	Ochrophyta, Bolidophyceae, Parmales	*Triparma*, *Tetraparma*, *Pentalamina*	External Si cell wall of interlocking plates	Marine
Chromista	Ochrophyta, Bacillariophyceae, Coscinodiscophyceae, Fragilariophyceae (Diatoms)	*Coscinodiscus*, *Chaetoceros*, *Nitzschia*, *Thalassiosira*, etc.	External 2-part ornamented skeleton, termed a frustule	Marine and fresh
Chromista	Ochrophyta, Xanthophyceae	*Acanthochloris bacillifera*	Si in cell wall and/or cysts	Fresh
Protozoa	Protozoa incertae sedis, Ebriophyceae (Ebridians)	*Ebridia*, *Hermesinum* (*Synechococcus*-like symbionts)	Internal skeleton of rugose or spiny rods	Marine

AlgaeBase was used for taxonomic classification
Guiry and Guiry (2015)

to accrete siliceous particles on their cell surface (Preisig 1994). Although currently the general consensus is that silicon is not a required nutrient for the groups discussed above, there is some evidence that Si deficiency and germanium dioxide reduce growth in some species of Chlorophyceae and Phaeophyceae (Moore and Traquair 1976; Tatewaki and Mizuno 1979; Mizuta and Yasui 2012). It has been hypothesized that Si accumulation may improve the resistance of the cell wall to decay or grazing or parasites, help with cell wall wound recovery, or that removal of Si from the environment may provide them with a competitive advantage against Si-requiring organisms such as the diatoms (Millington and Gawlik 1967; Fuhrman et al. 1978; Mizuta and Yasui 2012). Si has been shown to be bound to polysaccharide matrices as part of certain glycosaminogylcans and polyuronides (Schwarz 1973), providing support to the hypothesis that Si may provide additional integrity to the cell wall, perhaps reducing decay and susceptibility to grazing and infection.

Complex siliceous structures, including intricate skeletons and scales, are formed by several groups within the Ochrophyta (Fig. 1a–h). The diatoms and the Parmales, both closely related to the naked flagellated Bolidophyceae (Daugbjerg and Guillou 2001; Lovejoy et al. 2006; Ichinomiya et al. 2011), produce external siliceous walls or plates that completely surround the plasmalemma. The diatoms produce a covering made of Si and tightly bound carbohydrates and proteins. This cell covering is composed of two siliceous valves (epi- and hypovalve) that fit together like a petri dish around the cell and are held together by overlapping siliceous girdle bands, and is termed a frustule (Fig. 1a). New daughter cells form within the parent frustule, causing mean population cell size to decrease with each round of asexual repro-

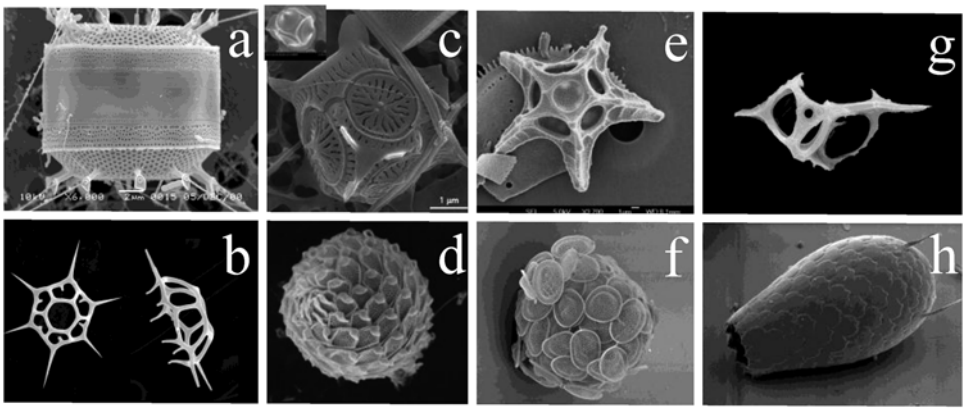

Fig. 1 Siliceous structures produced by microalgae and related groups. (**a**) Diatom frustule of *Thalassiosira* sp (Courtesy of Jim Ehrman and Mount Allison's Digital Microscopy Facility (MtA DMF)), (**b**) Silicoflagellate skeleton *Dictyocha* sp (courtesy of Jim Ehrman and MtA DMF), (**c**) Scales covering the Parmales *Tetraparma pelagica* (main panel, Courtesy of Drs. Susumu Konno and Richard W Jordan) and strain NIES 2565 (*inset*, Courtesy of Drs. Akira Kuwata and Mutsuo Ichinomiya), (**d**) Scale case of Synurophyta *Mallomonas* cf. *crassisiquama* (Courtesy of Astrid Saugestad), (**e**) Siliceous pentaster of the dinoflagellate *Actiniscus pentasterias* (Courtesy of Gloria Ernestina Sànchez with thanks to Drs. Diana Sarno and Marina Montressor), (**f**) Silicosphere of Haptophyte *Prymnesium/Hyalolithus neolithus* (Courtesy Dr. Masaki Yoshida), (**g**) Internal skeleton of Ebridian *Hermesinum adriaticum* (Courtesy of Dr. Paul Hargraves GSO-URI), (**h**) Scale test of Imbricatea *Euglypha* sp (By ja:User:NEON/commons:User:NEON_ja, Wikimedia Commons. Note: **a**–**f**, **h** are Chromista, **a**–**d** are Ochrophyta)

duction until a minimum threshold is met, triggering sexual reproduction. Maximum size is restored after sexual reproduction (Round et al. 1990). Valve shape, patterns of pores on the valve face, and structural features on the valves are used as taxonomic characters. At present the diatoms are the only known group with species with an absolute requirement for silicon, with the exception that *Phaeodactylum tricornutum* can grow without a silicified frustule, (Nelson et al. 1984) but always has lightly silicified girdle regions (Borowitzka and Volcani 1978). For most diatoms the cell division cycle becomes arrested at G1/S or G2/M without sufficient Si (Brzezinski et al. 1990; Brzezinski 1992).

The Parmales are pigmented, 2–5 µm diameter cells, surrounded by five or eight siliceous plates that fit together from edge to edge (Fig. 1c). The siliceous plates are ~80 nm thick and can have coarse or fine areolae, papillae, ornamented wings, keels and spines that can have very long projections (Booth and Marchant 1987; Ichinomiya et al. 2011; Konno and Jordan 2012). The cells are pigmented, contain chlorophylls *a* and *c*, have a large vacuole, and are not flagellated. Three genera and approximately 20 species have been described (Konno and Jordan 2012) with the number and characteristics of the plates used as taxonomic markers. At present silicification has been poorly characterized in the Parmales; only a single strain (NIES-2565) has been isolated and grown in culture and deposited in a culture collection (Ichinomiya et al. 2011). Although little is known about their biology, ecology and biogeographic distribution they are widespread and have been found in high numbers in polar and subpolar waters (Guillou 2011) and more rarely in tropical waters. More experimental work is required to determine if the Parmales have a strict growth requirement for Si and how they acquire Si and produce their siliceous plates.

There are two Phyla and three classes of microalgae that produce endogenous siliceous scales that surround the cell, the Chrysophyceae and the closely related Synurophyceae (Ochrophyta) and a few species within the Haptophyta. The Synurophyceae and Chrysophyceae include pigmented, chlorophylls *a* and *c*, fucoxanthin and violaxanthin, anthaxanthin and neoxanthin, solitary or colonial flagellated cells (Anderson 1987; Adl et al. 2012). All Synurophyceae and a number of species within the Chrysophyceae are covered in finely ornamented siliceous scales (Fig. 1d) and form siliceous cysts referred to as stomatocysts or statocysts (Sandgren et al. 1995). Some chrysophytes have siliceous loricae or outer coverings of basket-like siliceous rods (Tappan 1980). The siliceous scale-forming Chrysophyceae and Synurophyceae species typically have between one to four types of scales that are arranged on the outer surface of the protoplast. The scales can have complex ornamentation, including bristles and spines, and the total Si per cell can approach values reported for similarly sized diatoms (Leadbeater and Barker 1995; Sandgren et al. 1995). The Chrysophyceae and Synurophyceae examined do not have an absolute requirement for Si and can exist as naked (lacking siliceous scales) forms (Sandgren et al. 1996). When silicic acid is resupplied to cultures of cells without scales, growth rate is depressed until the siliceous scales are restored (Sandgren et al. 1996). Siliceous statocysts are found in freshwater and marine environments, are typically 3–35 µm in diameter, are flask or bottle shaped with a single pore with an organic plug that can be surrounded by a siliceous collar,

with a siliceous wall that can be smooth or reticulated and highly ornamented with spines, ridges, and punctae (Tappan 1980; Preisig 1994). Modern statocysts are common in freshwaters but fossil statocysts, sometimes referred to as Archeomonads, are more common in marine environments (Tappan 1980).

Most biomineralized scale-forming Haptophyta are calcareous but recently a marine haptophyte, *Hyalolithus neolepis* (=*Prymnesium neolepis)*, covered with siliceous scales was characterized (Yoshida et al. 2006). *Prymnesium neolepis* has several layers of oval and hat-shaped siliceous scales (termed liths), 4–6 μm wide by 5–7 μm long, with a hyaline brim, and an elevated region perforated by many pores (Fig. 1f). Other species of *Prymnesium* have been characterized with cysts with siliceous material on the distal surfaces of their outermost scales (Green et al. 1982). Siliceous microfossils, identified as *Pseudorocella barbadiensis* Deflandre, 1938 by Perch-Nielsen (1978) from the late Eocene to early Oligocene (Perch-Nielsen 1978, Plate 7) bear some resemblance to the liths formed by *Prymnesium neolepis*, suggesting these, or related genera, may have a fossil record. It is worth noting that several different types of siliceous fossils have been assigned to *Pseudorocella*, so most observations of *Pseudorocella* in the fossil record do not resemble a *Hyalolithus*-type lith. Silicification and the Si requirement of *Hyalolithus*/*Prymnesium neolepis* have yet to be fully characterized.

The silicoflagellates, members of the Ochrophyta, Dictyochophyceae, have an amorphous siliceous skeleton of fused, tubular/hollow rods (Lipps 1970). There has been some disagreement about whether the skeleton is fully internal or external to the cell but the work of Moestrup and Thomsen (1990) strongly indicate it is external with the flagellated cell often lying in the cavity of the one-sided skeleton, although there is microscopic evidence that the cell may sometimes cover the outside of the other side of the skeleton (Scott and Marchant 2005). The skeletons of the modern genera *Dictyocha* are star-shaped, characterized by two different hexagonal rings interconnected by six bars that attach to the corners of the smaller ring to the mid-points of the sides of the larger ring, with spines projecting from the apices of the outer hexagon (Fig. 1b). Silicoflagellate taxonomy uses skeletal morphology to differentiate amongst taxa, although the very limited experimental work indicates there is significant phenotypic plasticity in the skeletons produced within single species of *Dictyocha* (Van Valkenburg and Norris 1970). Silicoflagellates have life cycle stages that lack a siliceous skeleton and the National Center for Marine Algae and Microbiota (NCMA) maintains a naked strain of *Dictyocha speculum* in culture, indicating that silicon is not an absolute requirement for the growth of silicoflagellates.

There is evidence of silicification within several orders within the Dinophyta. Species within the class Actiniscaceae, a small, poorly studied group of heterotrophic Dinophyceae, are known to form internal biomineralized siliceous elements. Within *Actiniscus pentastarias* var. *arcticus*, 1–8 large and up to 14 smaller rudimentary star-shaped siliceous structures (pentasters) have been observed (Fig. 1e), although twin perinuclear penasters that surround the nucleus are most common (Bursa 1969; Hansen 1993; Preisig 1994). The form of Si is unknown although it has been assumed to be amorphous hydrated opal similar to what is formed by the diatoms. Bursa (1969) noted that for the Arctic lake species *A. pentastarias* v. *arcticus*, higher rates of silicification were associated with prolonged subzero temperatures and low light. A few other Dinophyta genera are reported to incorporate Si in their cell wall or cysts. High levels of Si have been detected in freshwater field populations of *Ceratium hirundinella*, Gonyaulacales (Sigee et al. 1999). Chapman et al. (1982) found that *C. hirundinella* produces two types of cysts: smooth-walled and granular walled cysts that are more resistant to decay. The young granular walled cysts contain vesicles filled with uniform electron dense granules ~60 nm in size (which could be silica nanospheres within a silicon deposition vesicle, see sections below) and an outer wall high in Si. There is also a report that *Peridinium cinctum* (Peridinales) may also form siliceous cysts (Eren 1969). A few siliceous fossil dinoflagellate theca and cysts have been described, *Eodinia* and *Lithodinia* with silicified walls from the Jurassic and siliceous cysts of *Lithoperidinium* from the Eocene and *Jusella* from the lower Oligocene (Loeblich and Loeblich 1984). There is some question about whether silicification in these rare specimens occurred before or after fossilization (Tappan 1980; Loeblich and Loeblich 1984). The Ebridophyceae, once thought to be Dinophyceae, but currently placed in their own class within the Protozoa *incertae sedis*, have an internal solid siliceous skeleton of rugose or spiny rods that are triaxially or tetraxially arranged (Fig. 1g). There are two extant species of Ebridians, *Ebridia tripartitia* and *Hermesinum adriaticum. Ebridia* is known to feed on diatoms and *Hermesinum* has *Synechococcus*-like endosymbiotic cyanobacteria (Hargraves 2002). Ebridians have never been cultured and little is known about how they acquire Si or form their siliceous skeletons. It would be interesting to determine how they acquire Si from the environment, if the cyanobacterial endosymbionts are involved, or if they recycle Si from diatom prey, or actively take up silicic acid from the environment.

Within the Cercozoa, the Imbricatea orders Euglyphida and Thaumatomonadida include heterotrophic species that form siliceous scales. In the past these groups have been referred to as Silicofilosea due to their external secreted siliceous scales and tubular cristae (Adl et al. 2012) but sequence analyses of rRNA and rDNA indicate they form separate groups within the Cercozoa (Wylezich et al. 2007; Ota et al. 2012). The Euglyphida and Thaumatomonadida have been

found in a wide variety of aquatic and marine habitats, but are common constituents of freshwater and estuarine benthic environments (Wylezich et al. 2007; Ota et al. 2012). Many species from these groups can produce solid or perforated external siliceous scales that surround the cell, with an opening for the cell to interact with the environment (Fig. 1h). The Euglyphida are testate filose amoebae (Ota et al. 2012). Many Euglyphida species have rounded to elliptical secreted siliceous plates that are bound by organic cement and when deprived of silicon in their media will produce deformed tests, cease to grow, and enter a quiescent state (Anderson 1990; Anderson and Cowling 1994). The Thaumatomonadida are gliding or swimming flagellated cells that can produce filopodia (Wylezich et al. 2007; Adl et al. 2012; Ota et al. 2012) and have oval or triangular siliceous scales often formed from the partial fusion of two plates that cover the body and sometimes the flagella. Siliceous fossil scales and body cases similar in morphology to the Euglyphida from the Lower Cambrian indicate that silicification in the Chromista may have very early origins (Allison 1981; Porter and Knoll 2009; Porter 2011).

3 Silicification: Molecular and Genetic Mechanisms

Most of what is known about silicification comes from work on diatoms, with some work on the Chrysophyceae and Synurophyceae and scattered observations on a few species from other classes of microalgae. There is very little information on how the other microalgae groups, even groups with complex siliceous structures, such as the silicoflagellates, Ebridia or Haptophyta, form their siliceous skeletons and scales.

3.1 Silicic Acid Transporters

Diatoms acquire silicic acid from the environment through diffusion and active uptake by silicic acid transporters (SITs) during cell wall formation in the synthesis phase of the cell division cycle (Hildebrand et al. 1997; Hildebrand 2003). The SITs belong to a unique gene family that differs fundamentally from silicon transporters identified from sponges and higher plants. To date SITs have been found in diatoms, Synurophyceae (*Synura petersenii*), Chrysophyceae (*Ochromonas ovalis*) and Choanoflagellates (Protozoa) with siliceous loricae (Hildebrand et al. 1997; Likhoshway et al. 2006; Sapriel et al. 2009; Marron et al. 2013). Currently it is unknown if SITs or other silicon specific transporters are present in other groups of silicifying micro- and macroalgae. Phylogenetic analysis indicates strong homology in the SITs analyzed from diatoms and the closely related Chrysophyceae and Synurophyceae; most of the charged lysines and arginines are in the same positions (Likhoshway et al. 2006; Marron et al. 2013). The choanoflagellate SITs form a separate monophyletic clade from the diatoms, Chrysophyceae and Synurophyceae. Marron et al. (2013) hypothesize that the SITs in the choanoflagellates may have originated from horizontal gene transfer from the Ochrophyta. Fossil evidence indicates silicification in the Chromista goes back to the Neoproterozoic with the Euglyphida (Porter et al. 2003; Porter and Knoll 2009; Porter 2011) and perhaps the Chrysophyceae (Allison 1981; Allison and Hilgert 1986; Knoll 1992), suggesting that a heterotrophic Chromist ancestor or photosynthetic Chrysophyte may be the source for SITs in the diatoms. A clearer picture of the evolution of SITs across the microalgae will develop as more sequence data becomes available representing more species from more taxonomic groups.

The SITs from diatoms have ~550 amino acids with ten trans-membrane alpha helices and a carboxy terminal. Many of the SIT sequences examined have a putative sodium binding site and conserved GXQ, MXD (Curnow et al. 2012) and CMLD/MMLD motifs (Grachev et al. 2005; Sherbakova et al. 2005). The GXQ and MXD motifs are hypothesized to be involved in the binding of silicic acid (Curnow et al. 2012) and the CMLD/MMLD domain has been hypothesized to act as a binding site that plays a role in the formation of a channel for silicic acid transport (Sherbakova et al. 2005; Annenkov et al. 2013). At present, SITs have been observed in the plasmalemma and associated with the membranes of small and large intracellular vesicles (Sapriel et al. 2009), likely silica deposition vesicles.

Many of the diatom species examined have more than one SIT gene that may have originated from duplication events; SITs within species usually cluster more closely together than SITs across species (Thamatrakoln and Hildebrand 2005; Sapriel et al. 2009)). In *Thalassiosira pseudonana*, Tp*SIT1* and Tp*SIT2* have 95 % similarity in amino acid sequence, but Tp*SIT3* is only 74 % and 76 % similar to Tp*SIT1* and Tp*SIT2*, respectively (Thamatrakoln and Hildebrand 2007) and may have originated prior to the origin of the Thalassiosirales (Alverson 2007). SIT concentration and expression is often not well correlated with mRNA levels (Thamatrakoln and Hildebrand 2007). Phylogenetic analyses indicate a sequence in SIT trans-membrane region seven to eight may separate pennate species from most of the centric species (Thamatrakoln et al. 2006; Alverson 2007; Sapriel et al. 2009). It has been hypothesized that sequence differences across SITs may reflect: (i) differences in function, some SITs may be higher or lower affinity or capacity or be targeted to function during different parts of the cell cycle, or act as sensors of silicic acid concentration, or (ii) different localizations within the cell such as the plasmalemma or intracellular vesicles such as the SDV or (iii) dif-

ferences associated with function in fresh versus marine waters (Thamatrakoln and Hildebrand 2005; Thamatrakoln et al. 2006; Alverson 2007). Much more work is required to quantify how different SITs, across different species, respond to different environmental conditions.

3.2 Silica Deposition Vesicle

Diatom valves, and siliceous structures formed by most of the microalgae examined, are formed within acidic membrane-bound organelles termed silica deposition vesicles (SDVs). SITs have been observed on cellular vesicles with high silicic acid content (Sapriel et al. 2009) but it has yet to be determined if SITs supply the majority of the silicic acid required to fuel frustule formation (Vrieling et al. 1999). To date our understanding of the role of the SDV in silicification has been hampered by the inability to isolate and study the SDV directly. Most evidence suggests that the SDV most likely originates from the Golgi/endoplasmic reticulum network (Li and Volcani 1984). Smaller transport vesicles may provide substrate to the SDV including silicic acid, plasma membrane, and silica-forming peptides (Li and Volcani 1984; Vrieling et al. 2007; Sapriel et al. 2009; Annenkov et al. 2013). In diatoms the SDV develops inside the plasma membrane of the newly formed daughter cells along the cleavage furrow (Li and Volcani 1984). A separate SDV may form for the valve and girdle bands (Li and Volcani 1984). During valve formation the SDV rapidly expands, its movement controlled by the cytoskeleton (Tesson and Hildebrand 2010). Microscopic evidence indicates close associations between the SDV and different organelles across the major microalgae groups. For example, siliceous scales form within vesicles associated with Golgi bodies within the cytoplasm in the Euglyphida while the scales of Thaumatomonadida form within vesicles closely associated with the mitochondria (Ota et al. 2012).

Many of the detailed molecular mechanisms that control the complex structures that characterize diatom frustules still remain a mystery. It is clear that the SDV plays a key role in silicification. Polymerization of silicic acid into nanometer-sized structures, typically silica spheres, occurs within the SDV. It has been hypothesized that in situ silica precipitating peptides and organic scaffolds within the SDV and positioning and movement of the SDV by the cytoskeleton may control the development of the three-dimensional siliceous structures (Hildebrand 2003; Tesson and Hildebrand 2010). Microtubules position and strengthen the SDV and actin filaments are intimately associated with the development of micro-scale siliceous structures (Tesson and Hildebrand 2010). Once a base layer is established, silica-associated and silica-forming peptides may catalyze self-assembly of three-dimensional siliceous structures (see below). A network of glucosamine has been found within the frustule wall of *T. pseudonana*, and chitin synthases are commonly present in diatoms, suggesting chitin may act as an organic scaffold for silicification or the strengthening of the frustule wall (Brunner et al. 2009; Durkin et al. 2009).

In the diatoms, Chrysophyceae, Synurophyceae, and Imbricatea, once siliceous structures are formed they are exocytosed (Ogden 1979; Li and Volcani 1984; Sandgren et al. 1996; Meisterfeld 2002). The siliceous scales of the Chrysophyceae, Synurophyceae, and Imbricatea are placed on the outside of the cell in species-specific arrangements. The formation of siliceous cysts within the Chrysophyceae and Synurophyceae is also associated with a silica deposition vesicle (Sandgren 1989). Patterns of silicification in the formation of the cyst wall varies across the species examined, but many species exhibit the formation of elongate finger-like Si projections, the production of irregular Si patches, or amorphous fine-grained Si accretion, followed by thickening and shaping of the cyst wall (Sandgren 1989). In the case of the internal siliceous pentasters of *Actiniscus pentasterias*, once the pentasters are formed the wall of the enclosing vesicle disintegrates (Bursa 1969).

3.3 Silica-Associated and Silica-Forming Peptides Found in the Diatom Frustule Wall

Several unique molecules have been isolated from diatom cell walls using anhydrous hydrofluoric acid or ammonium fluoride, including: frustulins, pleuralins, cingulins, silaffins, silacidins and long-chain polyamines. Frustulins are Ca^{2+} binding glycoproteins found on the outer protein coat of the frustule wall (Kröger et al. 1996), hypothesized to contribute to cell wall integrity (Poll et al. 1999). Pleuralins and cingulins are both localized in the girdle band region of the diatom frustule. The function of pleuralins is currently unknown but they are encoded by a small multi-gene family, are highly anionic, and tightly bound to diatom silica (Kröger and Wetherbee 2000; Zurzolo and Bowler 2001). Cingulins have been shown to be part of a chitin-independent organic matrix that becomes rapidly silicified in the presence of silicic acid (Scheffel et al. 2011) that are up regulated along with silaffin (*Tpsil1*) during cell wall and girdle band formation (Shrestha et al. 2012).

The most abundant molecules extracted from the diatom frustule are a diverse set of silaffins and long-chain polyamines that have the capacity to catalyze the precipitation of nanometer sized spheres of silica within minutes under physiologically relevant pH (Kröger et al. 1999, 2002). The silaffins, silacidins and long-chain polyamines form supramolecular assemblies with zwitterionic properties caused by the polyamine moieties and phosphate groups (Kröger et al. 1999, 2000, 2002; Wenzl et al. 2008; Tesson

and Hildebrand 2013). Silaffins are post-translationally modified polycationic phosphorylated peptides with a high proportion of hydroxyl amino acids such as serine and lysine residues that can form pentalysine clusters that can be linked by ε-amino groups to long-chain polyamines (Kröger et al. 2002; Poulsen et al. 2013). The long-chain polyamines (LCPAs) can have up to 20 repeated units and are the longest chains found in nature (Kröger et al. 2000). In *Cylindotheca fusiformis* the polyamines are attached to the polypeptide backbone of the silaffins, but in some other diatoms they may be linked to free amino acid derivatives (Kröger et al. 2000). Silacidins, are a class of acidic aspartate/glutamate and serine phosphate rich polypeptides formed from the post-translationally endoproteolytic processing of precursor polypeptides. Silacidins catalyze the precipitation of siliceous nanospheres in the presence of polyamines and silicic acid (Wenzl et al. 2008). Different combinations of silaffins and LCPAs under different combinations of pH and salinity have been shown to control the morphology of the nano-scale precipitated silica, forming blocks and spheres of different size, suggesting they may play a key role in guiding the formation of micro-scale structures associated with diatom frustule (Kröger et al. 2000). Hildebrand (2003) has hypothesized that the polypeptide backbone of silaffins may facilitate the formation of elongated structures while long chain polyamines may facilitate the formation of more complex molded structures. At present it is unclear whether the silica-precipitating peptides isolated from diatoms, such as the silaffins, silacidins and LCPAs, occur in other silicifying microalgal groups.

3.4 Transcriptomic Identification of Genes Associated with Silicification

Transcriptomic analyses of the differential expression of genes under Si-sufficient and low to limiting silicic acid conditions have identified a number of genes that may be involved in silicifiation in *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* (Mock et al. 2008; Sapriel et al. 2009; Shrestha et al. 2012). Mock et al. (2008) identified 159 up-regulated genes in *T. pseudonana* under low but not limiting silicic acid conditions. Seventy five of these genes were differentially expressed under the low silicic acid conditions but not under low iron, low nitrogen, low temperature or alkaline pH. Eighty four genes were differentially expressed under both low silicic acid and iron concentrations, suggesting there may be a molecular basis for elevated Si:C often observed in diatoms exposed to iron-limiting conditions. Many of the differentially expressed genes under low versus sufficient silicic acid conditions have no homology with known proteins and have trans-membrane spanning domains and/or secretory signals (Mock et al. 2008).

In a later study Shrestha et al. (2012) synchronized *T. pseudonona* cultures and analyzed differences in transcribed genes and expression levels under cell cycle arrest due to silicic acid limitation, during valve formation after silicic acid addition, and genes co-transcribed with silicic acid transporters *tpSIT1* and *tpSIT2*. Silicic acid starvation was associated with an over-representation of genes related to transcription and translation. The up-regulation of transcription and translation machinery in combination with high nitrogen stores maintained due to silicic acid stimulated cell cycle arrest may provide diatoms a competitive growth rate advantage over other phytoplankton groups once nutrient conditions improve (De La Rocha and Passow 2004; Shrestha et al. 2012). Frustule wall synthesis was associated with 485 differentially expressed genes, many putatively associated with cell signaling, protein degradation, extracellular proteins (including some with chitin binding domains), and genes whose products were potential precursors of silaffins, cingulins, SITs and choline and sugar transporters, and vesicle trafficking proteins that may be involved in the formation and function of the silica deposition vesicle. Twenty-four genes were differentially co-transcribed with *tpSIT1* and *tpSIT2*, many rich in repeated acidic amino sequences characteristic of silaffins and silacidins, and include a homolog of a silicon efflux transporter identified in higher plants (Shrestha et al. 2012).

The transcriptome of the fusiform morphotype of *P. tricornutum* was compared for cultures grown with (175 and 350 μM) and without silicic acid (Sapriel et al. 2009). The fusiform morphotype of *P. tricornutum* can incorporate Si fibers into the organic matrix of their cell wall but it does not strictly require silicic acid for growth and does not form a silicified frustule. The differential expression of 223 genes, 201 of which were up-regulated when silicic acid was present in the media were therefore assumed to be involved in the sensing, storage, and acquisition of silicic acid as opposed to frustule formation. Many of the putative genes that were significantly up-regulated by *P. tricornutum* in response to silicic acid were involved in pathways that can produce products known to be associated with silicification including: glycosylated trans-membrane proteins, silaffins and polyamines. Approximately a quarter of the over-expressed genes were expressed in clusters, including three of the four most highly over-expressed genes (16 to 330-fold) including two SITs, *ptSIT2-1* and *ptSIT2-2*. Sapriel et al. (2009) hypothesize that the ability to up-regulate genes in clusters may provide pennate diatoms with a selective advantage under temporally variable environmental conditions.

The transcriptomic analyses of Mock et al. (2008), Shrestha et al. (2012), and Sapriel et al. (2009) have identified many putative genes that may be important in silicification, many are newly discovered and need to be studied in more detail. Many of the genes identified show evidence of

translational and post-translational control, and vary considerably across the three studies, highlighting that the transcribed genome may be extremely sensitive to differences in experimental conditions.

4 Why Microalgae Silicify: The Costs and Benefits of Silicification

The construction and maintenance of complex siliceous structures, such as the diatom frustule, silicoflagellate skeleton, or scales covering chrysophytes and synurophytes requires a metabolic investment in silicic acid acquisition and establishment of the silicic acid gradient between the dilute external environment and the cell and SDV. Further costs are incurred in the construction and maintenance of silica transportation and deposition vesicles. In diatoms, and perhaps many other silicifying microalgae, there may be investment in silica-forming and silica-associated polypeptides and long-chain polyamines. A quantification of the costs associated with silicification is hampered by a fundamental lack of knowledge of the basic mechanisms responsible for silicification in most of the microalgal groups. In addition to direct metabolic and capital investment, the increased density of siliceous structures may increase sinking rate for phytoplankton. High sinking rates may reduce time in the upper sunlit water column and may reduce photosynthetic rate. Given these costs it seems logical there must be benefits associated with silicification (Knoll 2003).

Benefits attributed to silicified structures, specifically the diatom frustule, have been divided into three major categories (Finkel and Kotrc 2010): (I) siliceous structures may impede grazing, parasitoid or viral attack, (II) siliceous structures may positively promote the acquisition of growth-limiting resources or protect the cell from excess photon flux or UV or toxic metals, and (III) siliceous structures may alter sinking rate and this increased sinking rate may have a net positive effect on the availability of resources and interactions with predators, parasitoids and viruses. Specifically it has been argued that sinking may increase access to the nutrient-rich deep waters and shrink the nutrient-poor boundary layer around the cell, may facilitate sexual reproduction or resting stage formation, and may selectively remove cells infected by viruses or parasitoids (Smetacek 1985; Raven and Waite 2004). The balance of costs and benefits will be influenced by environmental and biotic conditions such as the availability of silicic acid concentrations in the environment and the degree of grazing pressure, that vary over space and time (Racki and Cordey 2000; Knoll 2003; Finkel et al. 2010; van Tol et al. 2012).

Biomineralized plankton have radiated over the last ~65 million years (Falkowski et al. 2004; Katz et al. 2004) and some silicified plankton (silicoflagellates, radiolarians, and perhaps the diatoms) have become less heavily silicified in response to declining surface silicic acid concentrations and increasingly armored (spines) in response to increased grazing pressure (Harper and Knoll 1975; Racki and Cordey 2000; Lazarus et al. 2009; Finkel et al. 2010; Finkel and Kotrc 2010; van Tol et al. 2012). It is commonly assumed that biomineralized skeletons provide protection against parasitoids and viruses and predators but quantitative measures are scarce (Hamm and Smetacek 2007). There is some evidence that microalgae covered by siliceous plates, such as diatoms or chrysophytes, synurophytes or the Parmales may be provided with some protection against digestion. External siliceous coverings, such as the diatom frustule or chrysophyte scale coverings, are not fully impervious to viruses or parasitoids, but may reduce the target area available for a viable attack and infection. The siliceous spines of diatoms and silicoflagellates and the spiny siliceous internal elements in Ebridia and dinoflagellates may have the potential to injure the digestive tract or feeding apparatus of grazers (Bell 1961; Thomsen and Moestrup 1985) and/or increase grazer handing time and the external skeleton may increase the effective size of some species removing them from the size range of some grazers. The phenotypic induction of increased silicification in diatoms when exposed to grazers is perhaps one of the strongest lines of evidence that diatom silicification may have evolved in response to grazing pressure (Pondaven et al. 2007). The origin of silicification may trace back to the Neoproterozoic vase-shaped microfossil *Melicerion poikilon*, possibly related to the Euglyphid amoeba (Porter et al. 2003; Porter 2011). These biomineralized eukaryotes appear as oxygen concentration and nitrogen availability and prey populations were on the rise, likely stimulating increased predation pressure and the evolution of defensive strategies (Knoll et al. 2007; Porter 2011).

The physical structure and chemical properties of the diatom frustule can influence resource acquisition and cell physiology. The silica frustule of diatoms alters both the scattering and absorption of light; silica frustules absorb UV and the micro-scale patterning of the frustule may alter the pattern of light within the cell (Kitchen and Zaneveld 1992; Davidson et al. 1994; Fuhrmann et al. 2004; De Stefano et al. 2007). The micro-scale structure of the diatom frustule has also been shown to influence the size sorting of particles at the frustule surface and nutrient diffusion rates (Hale and Mitchell 2001, 2002; Mitchell et al. 2013). In addition diatom silica may act as a pH buffer increasing the reaction rate of carbonic anhydrase (Milligan and Morel 2002) and frustulins in diatom silica may externally bind cadmium, suggesting silica may have the capacity to reduce the bioavailability of potentially toxic metals (Santos et al. 2013). The quantitative effect of these phenomena has not been clearly determined and it is unclear if these benefits apply to siliceous structures produced by groups of silicifying algae other than the diatoms.

5 Conclusions

Many taxonomically diverse organisms acquire Si and produce complex siliceous structures. Due to a lack of data it is unclear if there are significant differences in the genetic, molecular, and physiological mechanisms that allow the different taxonomic groups of microalgae to acquire, store and form siliceous structures. A comparative analysis of physiological responses to silicic acid concentrations and molecular and genetic mechanisms that control silicification across the Chromista would be expected to yield insight into the diversity of biological mechanisms that allow organisms to exploit Si and the evolutionary history of silicification.

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Calcification

Alison R. Taylor and Colin Brownlee

1 Introduction, Overview of Microalgal Calcification

The production of calcium carbonate by the process of biogenic calcification occurs widely throughout the tree of life. On a global scale, photosynthetic organisms, both prokaryote and eukaryote, may contribute up to 25 % of biogenic calcification, while coral reefs and free-living foraminifera generate much of the rest. However, even in corals and foraminifera, photosynthetic symbiotic algae play an indirect, though essential, role in facilitating calcification.

Calcification occurs amongst all algal lineages (Fig. 1), including red, green and brown macroalgae and the diverse microalgal phyla (Borowitzka 1982, 1984, 1987; Faber and Preisig 1994). The formation of calcite can occur by a wide range of mechanisms with both intra- and extracellular production being widespread. Here we will focus on the direct production of calcite by microalgae, considering both prokaryote and eukaryote systems.

Calcifying microalgae, and the coccolithophores in particular, play important ecological and geological roles, influencing the carbon cycle in complex and incompletely understood ways (Rost and Riebesell 2004; Ziveri et al. 2007; Shutler et al. 2013). Through photosynthesis they bring about direct drawdown of atmospheric CO_2. In contrast, the calcification process leads to reduced alkalinity and opposes the photosynthetic reduction of partial pressure of CO_2 (pCO_2) with a consequent effect of reducing the drawdown of atmospheric CO_2. Coccolithophores also influence the drawdown of atmospheric CO_2 through the carbonate counter pump whereby the sinking of $CaCO_3$ enhances the removal of particulate organic carbon from the ocean surface, also known as the ballasting effect (Ziveri et al. 2007). This reduces the recycling of organic carbon to CO_2 thereby contributing to the maintenance of the ocean-atmosphere CO_2 gradient.

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2 Evolution of Calcification

2.1 Cyanobacterial Calcification-Biofilms

The oceans and hypersaline lakes are supersaturated with Ca^{2+} and CO_3^{2-} ions, yet spontaneous abiotic inorganic mineralisation of $CaCO_3$ is rare due to presence of ions and organic compounds that cause the precipitation of carbonates to be kinetically unfavourable. Formation of crystalline carbonates in the ocean is therefore almost invariably a biologically mediated process. Prokaryote algal-mediated calcification emerged early in life's history (~3.5 billion years ago), (Altermann et al. 2006), and cyanobacterial microbial mats have subsequently played a significant role in precipitation of $CaCO_3$ from the oceans and production of major geological formations. Mineral formation by bacteria is generally not under direct biological control, although an early branching microbiolite cyanobacterium has recently been reported to precipitate amorphous carbonates within intracellular inclusions (Couradeau et al. 2012), suggesting a novel cellular mechanism of biomineralisation. Nevertheless, in the vast majority of calcifying cyanobacteria, precipitation of calcite is regarded as either biologically influenced or biologically induced, together being considered organomineralisation (Dupraz et al. 2009). In essence, this involves negatively charged organic extracellular structures of bacteria that provide a potentially large surface area for initial binding of metal ions, formation of nucleation sites and subsequent precipitation with counter ions, dependant on the prevailing geochemistry (i.e. alkalinity) of the adjacent fluid (Douglas and Beveridge 1998; Dupraz et al. 2009).

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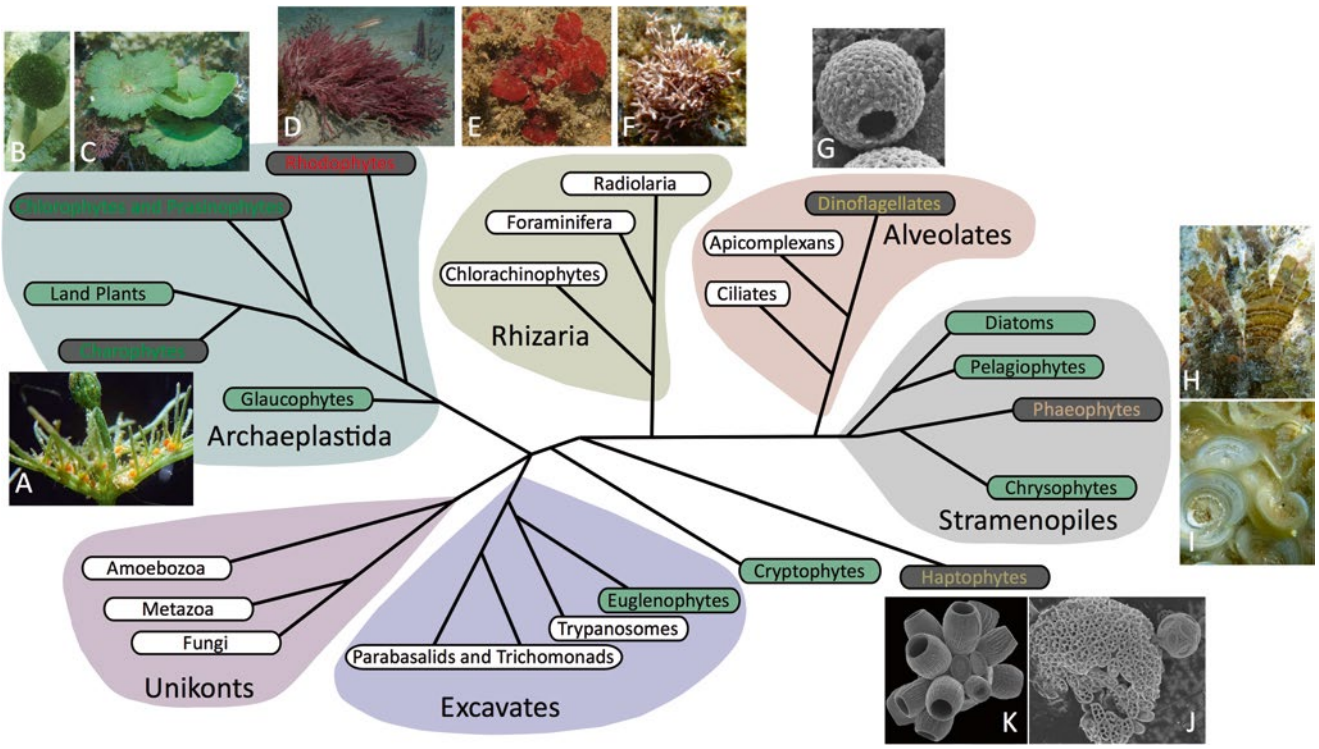

Fig. 1 Major eukaryote groups with calcifying algae. Eukaryote tree is redrawn with reference to Keeling (2004) and Not et al. (2012), photosynthetic lineages represented by green labels and those with calcifying taxa are represented by dark grey labels. Examples of calcifying algae from each of the major lineages are given which are, clockwise: (**a**) *Chara vulgaris* credit: Alberto Garcia (CC 2.0 license, Flickr); (**b**) *Penicillus capitatus*; (**c**) *Udotea cyathiformis* credit: Wilson Freshwater; (**d**) *Dichotomaria obtusata* credit: Wilson Freshwater; (**e**) *Peyssonnelia* sp. credit: Wilson Freshwater; (**f**) *Amphiroa rigida*; (**g**) *Thoracosphaera gondii*, credit: Hannes Grobe (CC 3.0 License, Wikimedia); (**h**) *Stypopodium zonale*.; (**i**) *Padina gymnospora*; (**j**) *Emiliania huxleyi* (*top right*) and holococcolith stage of *Helicosphaera carteri* from English Channel bloom; (**k**) *Scyphosphaera apsteinii* (Photo credits: Alison Taylor if not otherwise stated)

Cyanobacterial microbialites are well represented in the geological record (Riding 2000) and continue to form in warm shallow marine environments, where fine grained calcite and aragonite particles are precipitated and trapped within the extracellular matrix of the stromatolite biofilm community (Douglas and Beveridge 1998; Riding 2000). Blooms of planktic cyanobacteria are also thought to be a significant driver of rapid, large-scale precipitations of $CaCO_3$ known as whitings that periodically occur in surface waters of calcareous lakes (Thompson et al. 1997) and tropical oceans (Robbins et al. 1997), although the relative contribution of biologically mediated vs abiotic precipitation stimulated by re-suspension of seed crystals from underlying marine aragonite sediments continues to be debated (Broecker et al. 2000; Morse et al. 2003; Bustos-Serrano et al. 2009). In the case of microbial mats, biologically-mediated calcification is well established (Fig. 2) whereby inorganic carbon uptake through the carbon-concentrating mechanism (CCM) associated with photosynthetic cyanobacteria raises the alkalinity at the cell surface and promotes localised carbonate super-saturation needed for extracellular calcification (Riding 2000; Jansson and Northen 2010). Moreover, a close relationship with sulphate reducing heterotrophic bacteria is thought to be important in removal of SO_4^- ions that inhibit carbonate deposition. Thus, although the calcification process is essentially extracellular and passive, it can be considered a genuine biologically-induced mineralisation (Dupraz et al. 2009) as there are physiological and metabolic determinants on calcification (photosynthesis and CCM), resulting in modification of boundary layer alkalinity with the cyanobacterial extracellular organic matrix that provides an organo-chemical control on calcification via binding of metals and provision of nucleation sites (Dupraz and Visscher 2005; Dupraz et al. 2009). The role of cyanobacterial CCM in promotion of extracellular calcification has recently been further demonstrated by induced up-regulation of components of the CCM by deletion of Ca^{2+}/H^+ exchanger genes in *Synechocystis* sp. (Jiang et al. 2013). The mutants showed increased expression and activity of HCO_3^- transporters and periplasmic carbonic anhydrase (CA) that resulted in higher rates of photosynthesis, more alkalinised cell surface, and increased calcification (Jiang et al. 2013).

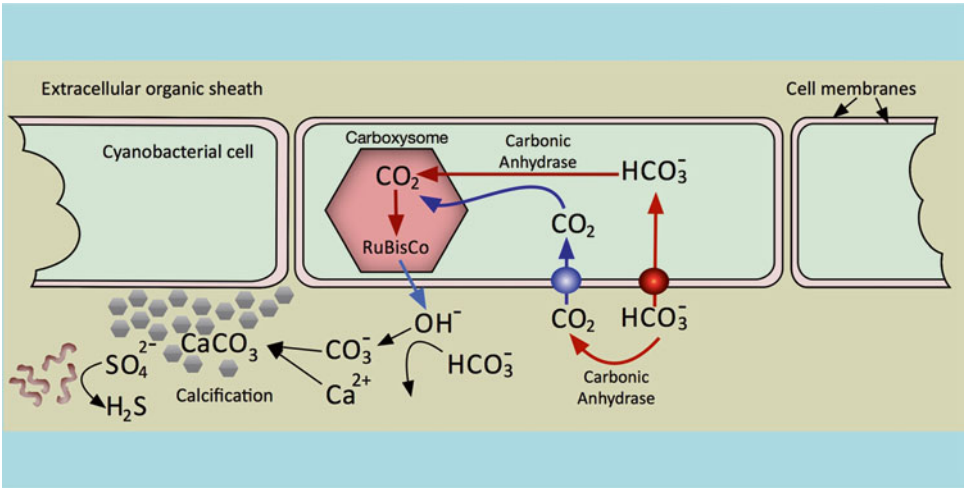

Fig. 2 Model of cyanobacterial calcification. Diagram redrawn with reference to Riding (2006). Biologically induced extracellular calcification in the sheath of cyanobacterial filaments is driven by CCM-enhanced photosynthesis in which bicarbonate ions are actively transported into the cell and converted via carbonic anhydrase to CO_2. The resultant hydroxyl ions that are liberated cause a localized increase in extracellular pH facilitating the production of carbonate ions, increasing the saturation state for $CaCO_3$ and promoting $CaCO_3$ precipitation that is nucleated by organic components of the sheath. Presence of sulphate-reducing bacteria may also enhance calcification by lowering inhibitory SO_4^{2-} ions in the vicinity of calcification

2.2 Eukaryote Algal Calcification

Although the basal phylogenetic relationships among the eukaryotes remain poorly resolved, they are commonly considered to comprise six supergroups; Opisthokonta, Amoebozoa, Archaeplastida, Excavata, Rhizaria, and Chromalveolata (Burki et al. 2007; Parfrey et al. 2011; Pawlowski et al. 2012). Microalgal groups with green and red plastids derived from primary endosymbiosis reside within the Archaeoplastida, with the Chlorarachinophytes (Rhizaria) and Euglenid algae (Excavates) arising from secondary endosymbiosis of a green alga (Falkowski et al. 2004; Stoecker et al. 2009). Calcification evolved in multicellular algae (see Fig. 1) within the Archaeplastida including coralline red algae (Rhodophyta; Corallinaceae) and the green algae (Chlorophyta; Ulvophyceae and Bryosidophyceae, Charophyceae) with the heterokont multicellular brown algae also possessing taxa capable of secreting calcite (Ochrophyta and Dictyotales). Eukaryote microalgal calcification appears to have evolved only within the Chromalveolata (see Fig. 1). The chromalveolates are a much debated assemblage of six eukaryote groups (stramenopiles, haptophytes, cryptophytes, dinoflagellates, apicomplexans and ciliates) possessing plastids putatively derived from a single common ancestral secondary endosymbiosis of a red alga (Keeling 2004; Yoon et al. 2004), although alternative models of red plastid acquisition via tertiary endosymbiosis among subgroups have been proposed (Stiller et al. 2009; Baurain et al. 2010; Dorrell and Smith 2011). Recent nuclear gene based phylogenetic analysis support a monophyletic group comprising the stramenopiles, alveolates and rhizaria (the SAR group (Burki et al. 2007)) with haptophytes and cryptophytes forming a weakly supported sister group to SAR (Harper et al. 2005; Hackett et al. 2007; Hampl et al. 2009; Burki et al. 2012). Calcifying taxa of haptophytes and dinoflagellates have been well described and are the focus of the remainder of this review.

Divergence of haptophytes from stramenopiles is estimated to have occurred between ~1100 and 820 Ma (Medlin et al. 2008). Molecular phylogenetics support the hypothesis that intracellular calcification evolved only once within the haptophytes (Edvardsen et al. 2000; Medlin et al. 2008; Liu et al. 2010) ~310 Ma, possibly before the divergence of the Calcihaptophycidae and Prymnesiales (Liu et al. 2010) and was subsequently lost in the Prymnesiales and branches of the Isochrysidales (de Vargas et al. 2007).

Calcareous dinoflagellate cysts appear in the fossil record at least 160 Ma with molecular clock estimates for the initial divergence at this time, which is consistent with the diversification of dinoflagellates overall. The emergence of the major extant clades of the monophyletic Thoracosphaeraceae appears to have occurred in the late Cretaceous (~70 Ma) (Gottschling et al. 2008).

2.3 Dinoflagellate Calcification: Cysts and Vegetative Cells

Dinoflagellates exhibit complex life cycles in which the dominant stage is typically motile, haploid, and covered in cellulosic plates formed in vesicles known as amphiesma. They can also form non-motile coccoid haploid vegetative

cells and diploid hypnozygote cysts (Meier et al. 2007; Elbrachter et al. 2008). Both laboratory studies on selected species (Karwath et al. 2000) and sampling of preserved of dinoflagellate cysts in surface sediments (Vink 2004; Zonneveld et al. 2013) demonstrate that temperature, salinity, N and P availability are environmental variables that most strongly correlate with species distribution and yields of cysts in field samples. Encystment may therefore be a strategy that enables dinoflagellates to survive long periods (seasonal- multi-year) of environmentally unfavourable conditions, and could also serve to resist infection by parasites (Chambouvet et al. 2011).

Members of the monophyletic Thoracosphaeraceae (Peridiniales) comprise 3 clades with ~30 known extant species that are characterised by their ability to produce calcified walls of their coccoid cysts (Meier et al. 2007; Elbrachter et al. 2008; Gottschling et al. 2008, 2012). Interestingly, some members of the Thoracosphaeraceae do not calcify at all, while others are proposed to have lost the ability to produce calcite structures in the diploid phase and regained the ability to calcify in the haploid vegetative phase (Gottschling et al. 2005; Meier et al. 2007; Zinssmeister et al. 2013). Notwithstanding the predominance of organic walled dinoflagellates and their extensive use in paleoenvironmental investigations (Zonneveld et al. 2013) and references therein), the well preserved calcareous taxa have also been used in several paleoecological studies (Vink et al. 2000; Wendler et al. 2002; Meier and Willems 2003; Kohn and Zonneveld 2010) and have received increasing attention due to the potential utilization of their calcite as additional geochemical paleoindicators (Vink 2004; Zonneveld 2004; Zonneveld et al. 2007; Gussone et al. 2010). However, little is known about the mechanism of calcification, which results in cubic or needle-like calcite crystals embedded within the extracellular wall matrix (Janofske 2000). This is perhaps one of the most important processes to establish in order to progress mineral-based paleoecological studies and to evaluate potential impacts of future ocean carbonate chemistry on calcareous dinoflagellates. Recent ultrastructural investigations of immature encysting coccoid cells of cultivated strains belonging to two calcifying clades (the Scrippsiella and T/Pf (Meier et al. 2007)) confirm the presence crystal-like particles within intracellular vesicles that are transported to the surface of the cell, where they are presumably released and incorporated into the developing cyst wall (Zinssmeister et al. 2013). Calcite production between the outer and middle membrane of encysting cells has also been described in *Scrippsiella minima*[1] (Gao et al. 1989), and calcification associated with amphesemal sacs has also been proposed (Zinssmeister et al. 2013). In spite of limited information on the precise mechanisms, it is apparent that calcification in dinoflagellates is, as with their coccolithophore counterparts, under strong biological control. Polarised light crystallography of *Scrippsiella* supports this contention, with crystal growth promoted along the a-axis with suppression of c-axis crystal growth that would normally be characteristic of abiotic deposition of calcite (Montresor et al. 1997; Janofske 2000).

2.4 Other Minor Groups-Reports of Calcification

Calcified structures associated with extracellular mucilage and EPS have been described for several eukaryotic microalgae including those within chrysophytes, non-coccolithophore prymnesiophytes as well as some members of the chlorophytes (Faber and Preisig 1994).

3 Coccolithophore Calcification: Mechanisms, Physiology and Roles

3.1 Life History and Calcification

Coccolithophores are calcifying microalgae belonging to the Haptophyceae can account globally for ~50 % of $CaCO_3$ burial flux from the surface oceans (Broecker and Clark 2009). With the sole exception of *Hymenomonas roseola*, extant coccolithophores are marine species, with several forming blooms that can regionally contribute up to 80–90 % of burial flux of $CaCO_3$ to carbonate deposits on the ocean floor (Ziveri et al. 2007). Thus, coccolithophores play an important role in marine biogeochemical cycles including the marine carbonate system (Rost and Riebesell 2004) by contributing to the organic carbon pump via photosynthesis and the carbonate counter pump through precipitation and subsequent sedimentation of calcite (de Vargas et al. 2007) and see Sect. 5.1). Coccolithophores first appeared in the fossil record ~220 Ma (Fig. 3) although recent molecular clock approaches estimate the first calcihaptophytes originating ~330 Ma (Liu et al. 2010).

A variety of heteromorphic life histories in the haptophytes have been described, with all those studied in detail revealing haploid and diploid phases that can reproduce asexually. In the case of coccolithophores, two distinct coccolith producing life history phases are known to occur and have been validated in most extant taxa in both cultured strains (Houdan et al. 2004; Nöel et al. 2004) and in natural field samples (Young et al. 2005; Frada et al. 2009). Diploid phase cells bear heterococcoliths with complex radial calcite

[1]Wherever possible the currently accepted names for species are used. The name used in the paper cited is also indicated. For details of names see chapter "Systematics, Taxonomy and Species Names: Do They Matter?" of this book (Borowitzka 2016).

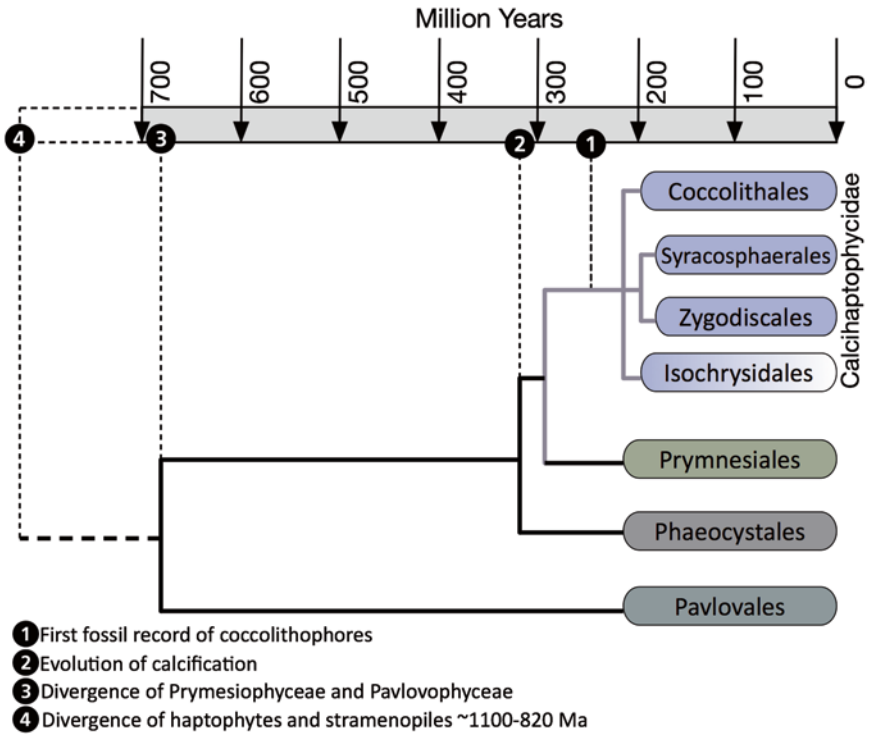

Fig. 3 Evolution of coccolithophores and calcification (Redrawn with reference to deVargas 2007, see text for details)

crystal arrangements forming elaborate coccolith structures. The alternate haploid phase cells generally produce holococcoliths comprising small cuboid crystallites (Young et al. 1999; Geisen et al. 2002). Syngamy to produce heterococcolith bearing diploid cells has been directly observed in a few cases (Houdan et al. 2004). In some coccolithophores (e.g. members of the Pleurochrysidaceae and Hymenomonadacae) the heterococcolith phase may alternate with a non-calcifying haploid phase that has been described as benthic (Fresnel 1994; Nöel et al. 2004). Studies of species that posses the alternate calcified forms have been hampered because phase transitions are infrequently observed in culture (although see below); while both phases reproduce asexually, the dominant phase tends to be the diploid heterococcolith producing. However, the presence of transitional coccospheres composed of both holo and heterococcoliths have been well documented in field samples (see (Cros et al. 2000) and references therein), and these so called 'species associations' have subsequently led to important revisions of the earlier coccolith morphology-based taxonomy (Cros et al. 2000; Geisen et al. 2002; Young et al. 2005).

The ecological significance of the alternating haploid-diploid life cycle of coccolithophores is unclear although diploid to haploid transitions potentially represent a response to the prevailing nutrient conditions; with motile haploid cells favoured in nutrient poor pelagic waters and diploid cells better adapted for warmer, nutrient rich coastal waters. Such alternation of life phases in response to changing environmental conditions is supported in a culture study of the oceanic holococcolith bearing *Calyptrosphaera sphaeroidea* (= *Holococcolithophora sphaeroidea*) where increased trace metals and vitamins induced the transition from haploid holococcolith motile phase to diploid heterococcolith non-motile phase (Nöel et al. 2004). Reversal of the culturing conditions, including a brief cold temperature treatment reliably induced the diploid-haploid switch (Nöel et al. 2004). Consistent with this, the diploid phases of temperate species *Coccolithus braarudii* and *Calcidiscus leptoporus*, sustain higher growth rates than the haploid motile phase in high nutrients (Houdan et al. 2006). Moreover, growth of haploid cells of these two species is stimulated by addition of an organic C-source and they can actively phagocytose bacteria (Houdan et al. 2006). Motile haploid *E. huxleyi*, unlike their diploid calcified counterparts, experience significant photoinhibition in high light, suggesting they are not as well adapted for summer stratified waters or turbulent conditions (Houdan et al. 2005). Sensitivity to turbulence has been directly demonstrated in haploid cells of *Coccolithus braarudii* where after just a few hours of turbulent conditions, haploid cell growth is inhibited and phase transitions to the diploid non-motile phase are evident (Houdan et al. 2006). Interestingly, stationary phase cultures of diploid calcifying *E. huxleyi* appear to have functional phagocytosis machinery, suggesting partial transition to mixotrophy when conditions are unfavourable (Rokitta et al. 2011).

Such life cycle transitions likely enable coccolithophores to occupy a wider range of ecological and nutritional niches,

with mixotrophic haploid cells competing in stable oligotrophic waters and diploid cells being best adapted for nutrient rich and turbulent conditions. A recent field study of the NW Mediterranean suggests niche separation may also correlate with depth, as holococcolithophores predominate in the upper oligiotrophic layer and heterococcolithophores are associated with deeper nutrient rich waters (Cros and Estrada 2013). A further advantage of life phase transitions, as has been demonstrated in *Emiliania huxleyi*, may be to escape catastrophic pathogen infection by induction of the (non-calcified) haploid phase that is resistant to viral infection (Frada et al. 2008) – a hypothesis supported by mesocosm blooms where changes in abundance of calcified cells, haploid specific transcripts and *Emiliania huxleyi* virus (EhV) indicate a significant increase of haploid cells during the viral burst that accompanies bloom collapse (Frada et al. 2012). Switching to the haploid, and presumably mixotrophic phase, may also represent a nutritional strategy to overcome inorganic nutrient limitation and capitalise on the increased DOC and bacterial abundance that arises with bloom termination.

3.2 Cell Ultrastructure Associated with Calcification

Cell ultrastructure studies on a wide range of heterococcolith-bearing species demonstrate commonality in structures and processes involved in heterococcolith production (Young and Henriksen 2003). Coccolith ontogeny has been previously well described and the reader is referred to a number of excellent reviews (Westbroek et al. 1984, 1989; Young et al. 1999; Young and Henriksen 2003). Briefly, with reference to heterococcolith bearing *E. huxleyi* (Isochrysidales) and *Coccolithus pelagicus* (Coccolithales), an organic baseplate is laid down in a Golgi-derived vesicle onto which crystal nucleation is initiated in a peripheral protococcolith ring. Crystal growth progresses with R and V crystal units extending radially (Young and Henriksen 2003) and upon maturation the coccolith vesicle fuses with the plasma-membrane and the entire structure is secreted and secured onto the surface. The functional coccolith vesicle is considered a single membrane bound compartment because it originates from a trans-Golgi cisterna, although recent work (Drescher et al. 2012) on *Scyphosphaera apsteinii* (Zygodiscales) suggests a much more complex layer of membranes may surround the vesicle as it matures. An association of tubules of endoplasmic reticulum with the coccolith compartment occurs in most coccolithophore species examined to date, and may facilitate transport of ions and organic components needed for calcification (Taylor et al. 2007). Some species, e.g. *E. huxleyi* (Westbroek et al. 1984), *C. pelagicus* (*C. braarudii*) (Taylor et al. 2007), and *S. apsteinii* (Drescher et al. 2012), exhibit a more distinctive reticular mass of membranous tubules in close association with the distal face of the coccolith within its vesicle. This implies a very specific functional role, and in the case of *S. apsteinii* it has been demonstrated that tubular projections from this reticular body to the baseplate scale during coccolith development are responsible for selectively inhibiting calcification and forming regular fine scale pores in the coccoliths (Drescher et al. 2012). This observation lends weight to the argument that coccolith morphology is controlled by the shape of the membrane bound compartment, although how that shape is determined is not known. Obvious candidates include cytoskeletal microfilaments and microtubules and the production of malformed coccoliths by *E. huxleyi* cells treated with cytochalasin B and colchicine respectively (Langer et al. 2010), would support the contention that mechanical forces generated by the cytoskeleton may shape the vesicle and exert mechanical control over coccolith morphology, although vesicle transport to the coccolith compartment would also presumably be affected and therefore disrupt normal calcification.

Very little is known about the mechanism of holococcolith production and associated cellular ultrastructure. This is partly due to unpredictable and infrequent observations of life history transitions from heterococcolith-holococcolith bearing cells in laboratory cultures. Examination of *Coccolithus pelagicus* and its holococcolith bearing motile stage, previously referred to as *Crystallolithus hyalinus* (Parke and Adams 1960) provide one of the few examples of an ultrastructural analysis of holococcolith production in which the authors conclude calcification of the small holococcolith crystallites occurs external to the plasmalemma but underneath an organic layer or 'skin' (Rowson et al. 1986). The argument for extracellular calcification is based on examination of multiple thin sections in which intracellular calcite was never observed. More recent observations of life history transitions of *Calyptrosphaera sphaeroidea* (= *Holococcolithophora sphaeroidea*) also report an 'external membrane' under which holococcoliths are secreted, although TEM analyses were not undertaken to confirm whether or not this represents a true membrane or an organic layer (Nöel et al. 2004). Interestingly, this organic layer is not frequently observed in holococcolith bearing species collected in the field samples (e.g. Geisen et al. 2002) suggesting it is highly labile/ephemeral, calling into question its role as an obligate feature of extracellular crystallite deposition during holococcolith production. Nevertheless, the extracellular organic layer (test) of foraminifera appears to play a predominant role in $CaCO_3$ precipitation in both hyaline (completely extracellular) and miliolid (intracellular production of calcite needles) deposition (de Nooijer et al. 2009). To what degree the extracellular matrix determines holococcolithophore calcification in coccolithophores certainly requires further study.

3.3 Chemistry of Calcification in Coccolithophores

Heterococcolith-bearing coccolithophores precipitate calcite in membrane-bound intracellular compartments derived from the Golgi apparatus (Fig. 4). This coccolith-forming compartment, often referred to as the coccolith vesicle provides a restricted space potentially affording fine control of chemical composition allowing for the precipitation of calcite from Ca^{2+} and CO_3^{2-}

$$Ca^{2+} + CO_3^{2-} \leftrightarrow CaCO_3 \quad (1)$$

A large number of physiological studies suggest that HCO_3^- is the species of carbon that enters the cell to provide the inorganic carbon substrate for calcification (e.g. Berry et al. 2002; Bach et al. 2013). At a cellular level, the calcification reaction can be described as:

$$HCO_3^- + Ca^{2+} \leftrightarrow CaCO_3 + H^+ \quad (2)$$

Potentially the H^+ produced can in turn react with HCO_3^- to produce CO_2

$$H^+ + HCO_3^- \leftrightarrow H_2O + CO_2 \quad (3)$$

Combining reactions 2 and 3 gives an overall reaction for coccolithophore calcification:

$$2\,HCO_3^- + Ca^{2+} \leftrightarrow CaCO_3 + CO_2 + H_2O \quad (4)$$

Calcite precipitation requires that the concentrations Ca^{2+} and CO_3^{2-} exceed the solubility product of calcite:

$$[Ca^{2+}][CO_3^-] / Ksp = \Omega$$

Where K*sp* and Ω are, respectively, the solubility product and saturation coefficient for calcite. Calcite precipitation may be achieved by raising the concentration of either Ca^{2+} or CO_3^{2-} above that required to reach Ω >1.0 Since the concentration of CO_3^{2-} in the calcifying compartment will be inversely related to $[H^+]$, H^+ will need to be removed from this compartment to allow calcification to occur unless Ca^{2+} is elevated to very high levels (Mackinder et al. 2010).

3.4 Molecular Mechanisms, Ion Transport and pH Regulation

The precipitation of calcite within an intracellular compartment poses a number of problems relating to transport of the substrates to, and removal of the ionic products from, the site of calcification (Fig. 4). Estimates of the rate of calcification in an actively calcifying cell of *E. huxleyi* indicate that the net trans-cellular flux of Ca^{2+} required to sustain calcification is amongst the highest encountered by any cell type (Brownlee and Taylor 2004). Delivery of Ca^{2+} to the site of calcification poses particular problems because, as with all eukaryotic cells, coccolithophores need to maintain a very

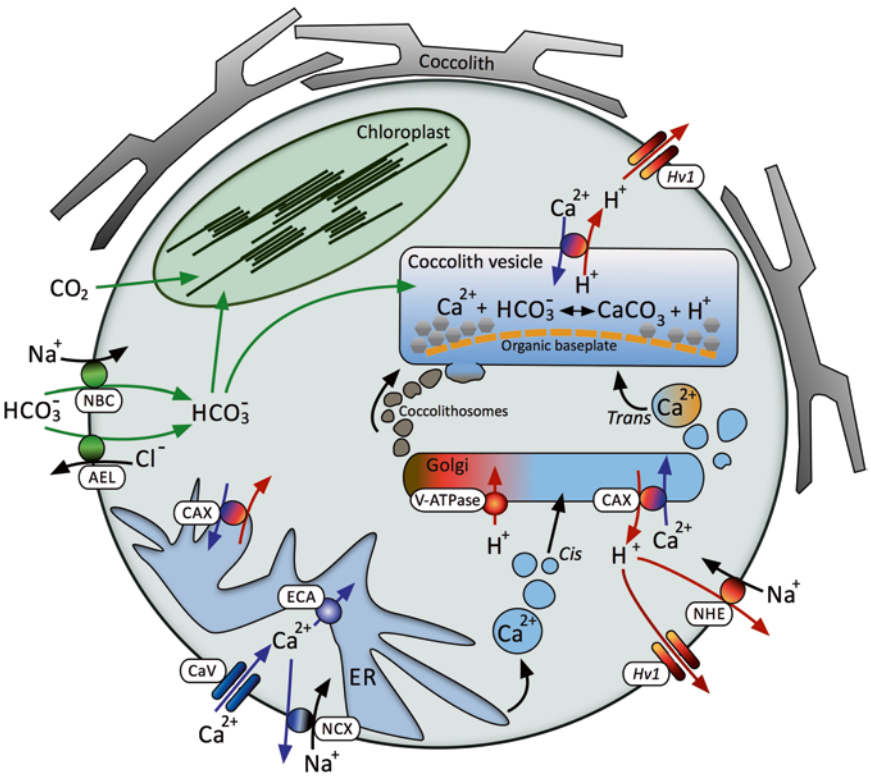

Fig. 4 Major ion transport pathways for coccolithophore calcification. Transport components are based on electrophysiological studies for ion channels and gene expression studies for pumps (V-ATPase, ECA), exchangers and co-transporters (CAX, NCX and AEL). The model proposes accumulation of Ca^{2+} via the endomembrane system into a Golgi-derived precursor compartment prior to precipitation of calcite and the competition for HCO_3^- from calcification and photosynthesis. Details of the pathways for ionic substrates (Ca^{2+}, HCO_3^-) and products (H^+) are provided in the text

low (around 0.1 μM) resting [Ca^{2+}] in the cytosol. This places constraints on the rate at which Ca^{2+} ions can be moved across the cytosol to the calcifying compartment. Moreover, a number of studies, have shown that delivery of Ca^{2+} to the calcifying compartment cannot be accounted for by an endocytotic mechanism (Berry et al. 2002; Holtz et al. 2013) whereby vesicles carrying a cargo of Ca^{2+} may bud off from the plasma membrane and subsequently fuse with the coccolith-forming compartment membrane. For such a mechanism to deliver Ca^{2+} to the calcifying compartment would require unrealistically high rates of vesicle transport and recycling (Holtz et al. 2013). The transport of Ca^{2+} into the cell through plasma membrane channels and the isolation of the calcification process from the bulk medium chemistry is also supported by evidence from isotope fractionation studies (Gussone et al. 2006).

A further transport challenge during intracellular calcification is the production of H^+ in the coccolith compartment (Eq. 2) that must handled by the cellular pH homeostasis machinery. Cell physiological experiments (Suffrian et al. 2011; Taylor et al. 2011) in which intracellular pH was monitored in response to external pH loads, showed that that *C. pelagicus* and *E. huxleyi* cells had high permeability to H^+. Taylor et al. (2011) went further to show, using a combination of intracellular pH monitoring and patch clamp electrophysiology, that *C. pelagicus* uses a voltage-regulated H^+ channel, also present in *E. huxleyi*, in its plasma membrane to mediate H^+ efflux from the cell. They showed that this potentially provided a rapid membrane-voltage dependent cellular pH regulatory mechanism. Moreover, this pathway was shown to be essential for calcification to proceed.

Molecular characterisation of the transport pathways associated with calcification have benefitted from the availability of the whole genome sequence of *E. huxleyi* (Read et al. 2013) and associated genome and transcriptome resources. Comparative genomics and proteomics have been used to examine regulation of calcification, life cycle and viral infection, and responses to climate change (Wahlund et al. 2004; Nguyen et al. 2005; Fujiwara et al. 2007; Pagarete et al. 2009; Richier et al. 2009, 2011; Mackinder et al. 2011; Rokitta et al. 2011; Vardi et al. 2012; Benner et al. 2013). For example, expression profiling of calcified and non-calcified cells of *Pleurochrysis haptonemofera* (= *Chrysotila haptonemofera*) yielded 54 calcifying cell specific genes that were significantly up-regulated (>threefold) and of those, six were expressed tenfold. Putative genes involved in carbohydrate and lipid metabolism were identified (Fujiwara et al. 2007) as well as phosphate permease, and carbonic anhydrase homologues. A similar approach compared haploid (non calcifying) and diploid (calcifying) cells of *E. huxleyi* (von Dassow et al. 2009). A more recent comparative study with calcifying and non-calcifying (but diploid) strains of *E. huxleyi* identified some likely candidate membrane transporters that may facilitate the calcification process (Mackinder et al. 2011). Genes that show significant positive correlation in their expression with calcification include: a member of the anion exchange-like (AEL) family AEL-1, which likely plays a role in uptake of HCO_3^- from the external medium (Parker and Boron 2013); a gene encoding a putative Na^+/Ca^{2+} exchanger that may be involved in maintaining Ca^{2+} homoeostasis; a vacuolar (V-type) H^+-ATPase and a CAX-like H^+/Ca^{2+} exchanger (CAX3). CAX-like transporters in plants are tonoplast-localised and play important roles in removing Ca^{2+} from the cytosol in exchange for vacuolar H^+ (Shigaki and Hirschi 2006). The up-regulation of these genes led Mackinder et al. (2010) to propose a model of Ca^{2+} loading into a precursor coccolith compartment driven by acidification of the lumen that in turn drives the uptake of Ca^{2+} via exchange with H^+. This could potentially allow Ca^{2+} to be accumulated to high levels into an acidic precursor compartment. Subsequent alkalinisation of this compartment during coccolith vesicle maturation would allow precipitation of calcite in a controlled manner. This is consistent with theoretical considerations of Ca^{2+} delivery to the site of calcification that also propose the feasibility of a Ca^{2+}/H^+ exchange mechanism for Ca^{2+} delivery to the coccolith vesicle (Holtz et al. 2013).

Contrary to this, an RNA seq transcriptomic study of *E. huxleyi* (CCMP 371) showed that while calcification appeared to increase in cells grown at elevated temperature and pCO_2 only 121 transcripts were differently expressed of which just 2 appeared to represent genes with a possible role in calcification (Benner et al. 2013). For genes most likely involved in calcification including CAX, NCKX, Ca^{2+} channels, CA isoforms, pH homeostasis and dissolved inorganic carbon (DIC) transport, RNA seq demonstrated most were abundantly expressed in cells grown in both 400 ppm/20 °C and 900 ppm/24 °C conditions, and there was no significant response among the treatments (Benner et al. 2013). Further, Q-PCR using primers for a subset of these calcification related genes (AEL1, V-ATPase, CAX3 and γ-CA (Benner et al. 2013) showed a higher number of transcripts that were differently expressed between replicates within treatments (229) than between present and future ocean treatments (121). Overall, it appears that genomic and transcriptomic interrogations of *E. huxleyi* calcification and its responses to environmental perturbations may be confounded by rapid adaptation and strain-specific responses and are dependent on the approaches used (elevated pCO_2, ion limitation, life history phase) that give rise to changes in calcification.

3.5 Regulation of Calcification- Organic Components

A number of studies have examined the organic components associated with coccolithophore calcification. Earlier work with *E. huxleyi* revealed a specific Ca^{2+}-binding acidic

polysaccharide to be associated with coccoliths. This polysaccharide was also shown to inhibit calcification *in vitro* and was proposed to be a regulator of calcite precipitation (Dejong et al. 1976; Borman et al. 1982; de Vrind-de Jong et al. 1994). Complexation between cations, coccolith-associated polysaccharide, and calcite in the regulation of coccolithophore calcification is also likely (Henriksen and Stipp 2009). Interestingly, the polysaccharides isolated from different *E. huxleyi* coccolith morphotypes were shown to differ in their monosaccharide composition and electrophoretic mobility (Borman et al. 1987). It is also clear that different coccolithophore species produce different types of calcification-associated polysaccharide. Marsh (2003) used ^{14}C to trace the production of coccolith-associated polysaccharide in *Pleurochrysis carterae (= Chrysotila carterae)*, showing that all of the coccolith-associated polysaccharide was secreted and not recycled. However, the coccolith-associated polysaccharides PS1 and PS2 described in *C. carterae* have not been identified in *E. huxleyi*, and to date there is no reliable quantitative data on the amount of coccolith-associated polysaccharide produced by different coccolithophore species. Given that polysaccharide production is likely to be the major carbon sink in calcifying coccolithophores (Brownlee and Taylor 2004), there is an urgent need to better understand and quantify calcification-associated polysaccharides.

To date, surprisingly little information exists on coccolith-associated proteins for any species. An exception is the protein GPA (glutamine, proline, alanine-rich protein) first isolated from *E. huxleyi* and described by Corstjens et al. (1998) in an immunological study of coccolith-associated polysaccharides. Subsequently, molecular analysis (Schroeder et al. 2005) showed that the sequence of a non-coding region of the GPA gene, termed the coccolith morphology motif (CMM) correlates with A or B morphotypes of *E. huxleyi* coccoliths. Interestingly, expression of the GPA gene relates inversely to the rate of calcification (Mackinder et al. 2011). This may suggest that GPA plays a negative regulatory role in calcification. However, further studies of GPA localisation and its chemical properties are required to further elucidate its role.

4 Functional Roles of Calcification

The sophistication of the calcification machinery and widespread occurrence in the haptophytes implies a functional role that has positive fitness benefits. Amongst the variety of functional roles that have been proposed for calcification in coccolithophores are protection, improved tolerance to high light, supply of CO_2 to photosynthesis and improvement of nutrient acquisition, for example by modulation of sinking speeds. Here we discuss those functions that have received most attention in more recent studies.

4.1 Protection

Many phytoplankton species have evolved protective coats, such as the silica frustules of diatoms and cellulosic cell walls of dinoflagellates, to defend against pathogens and grazers. Coccolithophores are potentially subject to attack from viruses, bacteria, other protists and copepods. The most obvious function of the coccosphere is that it affords protection from various forms of attack. While there is insufficient data to make conclusions about the role of the coccosphere in protection against algicidal bacteria, *E. huxleyi* is known to be susceptible to infection by giant phycodnaviruses. Indeed viral infection has been proposed as a major factor regulating *E. huxleyi* bloom dynamics (Frada et al. 2008). Direct observations of viral attack showed that an intact coccosphere caused virus particles to detach from *E. huxleyi* cells (Mackinder et al. 2009). This study also showed higher rates of viral infection during cell division when the coccosphere is incomplete. Paradoxically, the apparent resistance of haploid, non-calcifying cells to viral attack (see Sect. 3.1) suggests that viral defense mechanisms do not rely solely on mechanical repulsion by the coccoliths.

Microarray based transcriptional analysis of *E. huxleyi* and its lytic DNA virus during infection of mesocosm blooms demonstrate massive virally induced shift in gene expression profiles of the host accompanied by up-regulation of 20 % of the estimated 470 gene viral genome itself (Pagarete et al. 2011). Up-regulated host genes include those required for translation, ribosomal structure and biogenesis, energy production and conversion, lipid transport and metabolism. There has been much interest in the lipid metabolism genes specifically because of the presence of a near complete host-derived sphingolipid synthesis pathway in the virus genome which is expressed during infection in both laboratory cultures and mesocosm blooms (Pagarete et al. 2009), causing trans modification of lipid synthesis in the host, accumulation of viral glycosphingolipids and induction of caspase mediated programmed cell death that likely facilitates the viral lytic phase (Vardi et al. 2009, 2012). Interestingly, no obvious infection-induced transcriptional response of genes associated with the calcification machinery has been reported, suggesting there is no dependence on, or interaction with calcification in spite of the fact that non-calcifying cells are resistant to calcification.

Calcifying coccolithophore cells are readily ingested by predators such as copepods (Harris 1994), although in both laboratory (Kolb and Strom 2013) and field studies (see Schmoker et al. 2013 and references therein) microzooplankton grazing rates on *E. huxleyi* can be much lower than on other non-calcifying phytoplankton. Nevertheless, there is little direct evidence to suggest that the presence of the coccosphere specifically affects ingestion, although this has been a proposed role for the large articulated calcite appendages

that can apparently be deployed by some species of Syrachosphaeraceae (Young et al. 2009). Interestingly, haploid but non-calcifying cells of *E. huxleyi* are readily grazed and appear to induce a chemical defense against grazing within 24 h of exposure to ciliate predators (Kolb and Strom 2013), whereas ciliate grazing on diploid calcified cells did not induce a chemical defense response, yet ingestion rates rapidly declined after ~0.5 h of feeding. This suggests that the diploid calcified cell may interfere with digestion efficiency in microzooplankton grazers, lowering ingestion rates, slowing their population growth and potentially leading to reduced grazing pressure. The same argument can be made for metazoan zooplankton as *E. huxleyi* has been shown to be a sub-optimal food for copepod grazers compared with other phytoplankton species (Nejstgaard et al. 1995), though this study did not show any significant effects of coccolith coverage on grazing rates. Direct measurements of copepod gut pH have shown significantly increased gut pH in copepods grazing exclusively on *E. huxleyi* compared with diatoms (Pond et al. 1995). The effects of this alteration in gut pH on digestion and grazing rates remain to be determined.

4.2 Metabolic Roles

Knowledge of the costs of calcification in terms of energy or nutrient requirements can shed light on its functions. Clearly, if calcification requires significant re-allocation of resources away from other essential metabolic processes, then it is likely that it fulfils an essential role leading to increased fitness at the population level. The direct nutrient requirements for production of $CaCO_3$ coccoliths and associated polysaccharides are low. However, it will be important to quantify the indirect nutrient and energy costs of transport and metabolic processes. Earlier estimates of the energetic transport costs for delivery of substrates to, and removal of H^+ from the site of calcification suggest that production of calcite incurs a significant though smaller direct energy cost than fixation of the equivalent amount of organic carbon through photosynthesis (Anning et al. 1996). While the energy requirements for transport and polysaccharide synthesis could be met through photosynthetic ATP production under sufficiently high irradiance, the related nutrient costs, in terms of P requirement of ATP synthesis and N requirement for transport proteins are more difficult to quantify.

The use of HCO_3^- as the substrate for calcification results in the production of H^+ within the cell. In principle, H^+ could be used to produce CO_2 for photosynthesis from HCO_3^- within the cell (see Berry et al. (2002) for a critical review). However the bulk of evidence, including the lack of any significant effect on photosynthesis at a range of Ca^{2+} concentrations that reduce or inhibit calcification (Herfort et al. 2002; Trimborn et al. 2007; Leonardos et al. 2009) suggests that coccolithophores do not utilise this potential pathway. Furthermore, Bach et al. (2013) provided strong evidence, based on detailed analysis of the effects of precise manipulation of inorganic carbon speciation that calcification and photosynthesis may in fact compete for HCO_3^- as a substrate under low DIC conditions.

Emiliania huxleyi is able to tolerate very high irradiance without showing significant photoinhibition (e.g. Tyrrell et al. 1999). Observations such as this have led to the view that calcification may provide a sink for excess energy associated with high light levels. This is consistent with experiments comparing photoinhibition in haploid, non-calcifying and diploid, calcifying cells (Houdan et al. 2005). More recently Xu and Gao (2012) showed that when calcification of *E. huxleyi* was inhibited by reducing external Ca^{2+}, cells contained lower photoprotective carotenoid pigments and reduced non-photochemical quenching (NPQ). Moreover, it has been shown that rapid increases in irradiance from 50 to 800 μmol photons m^{-2} s^{-1} cause strong enhancement of calcification (Ramos et al. 2012), suggesting that calcification may play a more direct role in photoprotection, acting as an alternative energy sink in response to a sudden increases in light intensity.

Certain species may also use their coccoliths to modify the light field within the cell to enhance photosynthesis at limiting irradiances. *Florisphaera profunda*, a deep water coccolithophore (Quinn et al. 2005) arranges its coccoliths into a dish-shaped configuration that may act to reflect light into the cell. Difficulties in culturing this species and other coccolithophores with unusual arrangements of coccoliths would need to be overcome in order to test hypotheses around non-typical roles of coccoliths.

5 Calcification, Climate and Future Oceans

5.1 Impacts of Changing Ocean Chemistry on Coccolithophore Calcification

Raven and Crawfurd (2012) have comprehensively discussed the changing environmental factors associated with climate change that need to be considered for a deeper understanding of coccolithophore responses in a changing ocean. These include, increased CO_2, altered availability of N and P due to increased temperature resulting in increased stratification along with changing ocean currents and associated patterns of nutrient availability. The impacts of altered nutrient availability on coccolithophore calcification are likely to be complex and a range of experimental studies show a variety of responses. For example, it has been proposed that phosphate deprivation is a trigger for calcification and that a rather long induction period is needed for calcification

compared to the increase in alkaline phosphatase activity (Satoh et al. 2009). Moreover, while calcification was greatly stimulated by cold stress, other cellular activities such as growth, phosphate utilization, and the induction of alkaline phosphatase activity were suppressed. A significant increase in calcification rate in response to P limitation has also been demonstrated in cells of *Calcidiscus leptoporus* (Langer et al. 2012), although no evidence for altered coccolith morphology in response to P or N limitation was observed. Interestingly Kaffes et al. (2010) showed that while growth rates and C to N ratios of *E. huxleyi* were not altered by NO_3^- availability, organic C and N as well as inorganic C quotas were reduced under lower NO_3^- accompanied by a dramatic increase in the number of incomplete or malformed coccoliths. Clearly, environmental responses of calcification need to be considered in the context of multiple chemical and physical factors.

5.2 Ocean Acidification: Effects of Dissolved CO_2

Since Riebesell et al. (2000) reported a decrease in calcification in *E. huxleyi*, under elevated pCO_2 conditions, similar trends have been reported from a range of subsequent laboratory (Sciandra et al. 2003; Feng et al. 2008) and mesocosm studies (Delille et al. 2005; Engel et al. 2005) and in the closely related species, *Gephyrocapsa oceanica* (Zondervan et al. 2001). However, Langer et al. (2006) showed species-specific differences in calcification responses to elevated CO_2 while Iglesias-Rodriguez et al. (2008) and Shi et al. (2009) showed an increase, rather than decrease, in calcification response with elevated pCO_2, contradicting previous experiments. Nevertheless, these examples illustrate significant differences between experiments. A number of meta-analyses of these datasets point to the strong likelihood that strain and species differences underlie most of the variability in coccolithophore calcification response to elevated CO_2 (e.g. Ridgwell et al. 2009). Moreover, it is increasingly recognised that establishing responses to a single environmental change factor (i.e. pCO_2) is unlikely to yield robust predictive capability to determine coccolithophore assemblage responses to future ocean conditions. This is due to physiological plasticity and genetic diversity among and within species of coccolithophores, and the interactions of pCO_2, light, nutrients and temperature (Gao et al. 2012; Lefebvre et al. 2012; Jin et al. 2013b; Sett et al. 2014; Arnold et al. 2013).

Most laboratory studies to date have involved coccolithophores exposed to high pCO_2 for a relatively small number of generations (Riebesell et al. 2000; Zondervan et al. 2002; Langer et al. 2006; Feng et al. 2008). Even after *E. huxleyi* and *C. braarudii* were grown over 152 and 65 generations exposed to high pCO_2 respectively, they failed to show an adaptive response (Muller et al. 2010). Very recently, Lohbeck et al. (2012) showed that after exposure to elevated pCO_2 conditions for 500 asexual generations, calcification rates in both multi-clone and single-clone *E. huxleyi* populations were partly restored, with rates up to 50 % higher in adapted compared with non-adapted cultures. This suggests the possibility of genotypic selection as a mechanism of population-adaptation, as well as the emergence and partial fixation of advantageous new mutations. It also suggests epigenetic changes; heritable changes in regulation of gene expression without altering the underlying DNA sequence. Despite this however, the study only reports the potential for adaptation; such laboratory findings should be examined alongside field observations. Importantly, experimental evolution in response to pCO_2 and temperature should continue to be conducted among a range of genetic strains under dynamic mixed treatments of light and nutrient availability in order to distinguish physiological plasticity, intraspecific diversity and evolution (Collins et al. 2014).

5.3 Calcification, Geochemical Indicators and Paleoproxies- Implications for Reconstructing Past and Future Oceans

Coccolithophores have been the focus of numerous paleoceanographic studies due to their wide distribution in past and present surface oceans and presence of well-preserved calcite plates in marine sediments. However, coccolith nanofossils cannot be used reliably to estimate coccolithophore paleodiversity because of pseudocryptic speciation and biased preservation and sampling (Young et al. 2005). Nevertheless, the potential use of coccolith calcite to develop geochemical paleoproxies for reconstructing physiochemical properties of surface oceans, understanding physiological responses to past and future climate change, the consequences for the rain ratio (relative export of particulate inorganic and organic carbon (PIC:POC)) and thus ocean carbon cycle has been the subject of several studies. For example, metal cation ratios such as Sr/Ca of polyspecific coccoliths in core top sediments have been proposed as an indicator of growth and calcification rates and thus a potential proxy for variations in primary productivity in surface communities (Stoll and Schrag 2000; Stoll et al. 2002). Subsequent laboratory calibrations of coccolith Sr/Ca ratio and growth rates in a range of extant species confirmed a positive correlation of Sr/Ca ratio with temperature and growth rates (Rickaby et al. 2002; Stoll et al. 2002). However, when growth rate is controlled by light levels and not temperature, changes in Sr/Ca are small and not always correlated to growth rate. This is further confounded by inter- and intraspecific variations in the correlation between Sr/Ca ratio and calcification

rates (Stoll et al. 2002). Interestingly, Sr/Ca ratios in field samples across upwelling transects can vary more widely (20–50 %) compared to those observed across a range of growth rates in culture (~10 %), suggesting factors other than growth rates are important determinants of geochemistry of the calcite. Strontium, incorporation also appears to be dependent on crystal growth orientation, suggesting a mechanism for interspecies differences in metal ion ratios due to the diversity of coccolith morphologies (Payne et al. 2008).

Other geochemical analyses have focussed on understanding the physiology of calcification. For example the B/Ca ratio of coccolith calcite has been proposed as a potential indicator of coccolith compartment pH (Stoll et al. 2012). Calcium isotope fractionation into coccolith calcite in *E. huxleyi* (Gussone et al. 2006) shows a pattern of incorporation that is consistent with a general model of trans-cellular transport discussed above (Fig. 4). The partitioning of Ba, Sr and Mg have also been examined as a means to interpret the likely transport processes that contribute to biological fractionation (Langer et al. 2009). Finally the $\delta^{18}O$ in coccolith calcite has been found to co-vary as a function of external carbonate chemistry which is assumed to influence the relative contribution of cellular CO_3^{2-} and HCO_3^- pools to calcification (Ziveri et al. 2012).

Although progress has been made in defining potentially useful paleoproxies from coccoliths, there remain several uncertainties regarding the range of vital effects that influence calcite elemental or isotopic composition. A more detailed understanding of the mechanism of ion transport and the selectivity/permeability of the associated pathways will be essential to understand the precise geochemical make up of calcite produced within the coccolith vesicle (see Sect. 3.4). Moreover, the role of the organic components (see Sect. 3.5) of the coccolith mineral likely influence calcite composition and it has been shown that organic components of coccoliths can significantly alter rates of digenesis (Hassenkam et al. 2011) that could differently effect composition of sedimentary coccoliths. Finally, intraspecific physiological plasticity and genetic diversity may result in altered ion partitioning in response to different environmental growth conditions (see below).

5.4 Biogeography and Evolution/Genetic Variation

Intraspecific genotypic diversity of *E. huxleyi* has been demonstrated by microsatellite polymorphisms (Iglesias-Rodriguez et al. 2006) and recent sequencing efforts (Read et al. 2013) together with comparative genome hybridization (Kegel et al. 2013) highlight the extensive variability of *E. huxleyi* genome with respect to size and gene complement among isolates. The *E. huxleyi* genome is dominated by repetitive elements which account for 64 % of the sequence with 22 % of the DNA coding for ~30,500 predicted genes. Surprisingly, 17 % of these genes in the reference genome of CCMP1516 were absent in one or more of the genomes of three other deeply sequenced *E. huxleyi* isolates (92A, EH2 and Van556) although a core set of eukaryote genes were highly conserved among all isolates (Read et al. 2013). Further analysis of the coccolithophore pan-genome retrieved by high throughput sequencing of 13 isolates, revealed significant differences in gene copies for nutrient acquisition present in the core genome (e.g. N-related transport and metabolism) and striking differences in number of genes related to nutrient and trace metal utilisation in the variable genome (Read et al. 2013) among the strains. The potential for metabolic variability represented in the *E. huxleyi* pan-genome most likely underlies biogeographical distributions and the variable phenotypic responses to nutrient regime and carbonate chemistry observed among strains of this species complex.

Emiliania huxleyi is a cosmopolitan coccolithophore, although whether it can be considered a single species has clearly been brought into question by studies revealing distinctive morphotypes based on coccolith structure (Young et al. 2003) that correlate with genetic differences (Young and Westbroek 1991; Geisen et al. 2004) and biogeography. Mitochondrial gene sequencing grouped strains into two genetic clades that correlate with annual mean temperature of the biogeographical regions from which they were isolated (Hagino et al. 2011). Intraspecific genetic diversity based on microsatellite markers of clonal isolates from a coastal Fjord and NE Atlantic demonstrate the high degree of intraspecific genetic diversity that may reflect functional specialisation of the community to these ecologically distinct regions (Iglesias-Rodriguez et al. 2006; Cook et al. 2013).

Given the genetic diversity within *E. huxleyi* species reflected in the corresponding pan-genome along with relatively stable transcriptome and proteomes described (see above), different metabolic capabilities/sensitivities would be expected to be genetically selected once the acclimation capacity for any given genotype is exceeded. Better understanding of how such selection will determine adaptation and the nature of future populations is required, for example through well controlled microalgal evolution experiments (Reusch and Boyd 2013). Adaptive evolution within any given strain might also be critical in determining the response of the *E. huxleyi* species responses as a whole to future ocean conditions. This would be reflected in genetic or pleiotropic changes, resulting in phenotypic divergence as a strain adapts to altered physiochemical properties of seawater. Such an adaptive evolutionary response of *E. huxleyi* to high CO_2 spanning 1000 generations has recently been described for both single and multi-clone experiments (Lohbeck et al. 2013). Adaptive evolution in *Gephyrocapsa oceanica* is also reported using reciprocal transfer experiments among cells

grown for >600 generations in high and low pCO_2 conditions (Jin et al. 2013a).

The relative contribution of coccolithophores to phytoplankton community composition is critical in determining the overall efficiency of the ocean biological pump. Increased thermal stratification and decrease of nutrient supply to the euphotic zone will likely favour coccolithophores over diatoms, leading to increased activity of the carbonate pump and thus decreasing net drawdown of atmospheric CO_2 by surface oceans in these regions. The ratio of coccolithophores to diatoms (C/D) has been explored along the Atlantic Meridonal Transect (AMT) and shown to be high in regions of lower primary productivity where thermal stratification and deepening of the nutricline predominate (Cermeno et al. 2008). Positive feedback on atmospheric pCO_2 by decreased drawdown by phytoplankton communities (increased C/D) is predicted regardless of whether coccolithophores increased or decrease calcification in response to increased pCO_2 (Cermeno et al. 2008). This is supported by estimates of 55 % reduction of average monthly air-sea flux of atmospheric CO_2 in the North Atlantic in regions dominated by *E. huxleyi* blooms (Shutler et al. 2013).

Sensitivity of *E. huxleyi* to salinity and temperature over ocean carbonate chemistry may be critical factors that drive pole-ward expansion of coccolithophores as has been indicated by satellite composites of coccolithophore blooms spanning a 30 year period (Winter et al. 2014).

5.5 Effects on Other Microalgae-Calcareous Dinoflagellates

Given the importance of calcified dinoflagellates in paleoecological studies, and as contributors to ocean carbonate fluxes, it is remarkable that only one study to date has examined their response to ocean acidification (Van de Waal et al. 2013). A strong effect of increased pCO_2 was observed in dilute batch cultures of *Thoracosphaera heimii* with 50 % decline in growth rate and a 60 % decrease in PIC/POC ratio driven by both an increase in POC and significant decrease in PIC at 1400 µatm compared with 150 µatm. Decreased calcification was manifest by the observation that only 20 % of *T. heimii* calcified cysts sampled from the highest CO_2 treatments were complete. Effects of elevated pCO_2 were also observed on stable isotope fractionation with $\delta^{18}O$ and $\delta^{13}C$ in calcite demonstrating relatively high sensitivity to changes in prevailing carbonate chemistry compared to coccolithophores. Expression of genes likely to be important in DIC transport, calcification and pH regulation were strongly regulated by the treatments. Specifically, Ca^{2+}/Na^{+} exchangers (NCX1) and vacuolar ATPase, CA related genes and aquaporins were down regulated in response to high pCO_2, demonstrating that *T. heimii*, as with coccolithophores, adjusts its calcification machinery in response to changes in CO_2 availability. Although in the case of *E. huxleyi* the gene expression response is only seen in reduced pCO_2 treatments that correspond with the induction of a CCM (Bach et al. 2013).

6 Concluding Remarks

We have seen that calcification occurs in phylogenetically diverse microalgal taxa and has important geological and ecological roles. Calcification evolved early in the prokaryotes but is restricted to the chromalveolates in the eukaryotes. Coccolithophores dominate the modern calcifying microalgae in terms of abundance and number of calcifying species. Between taxa, calcification occurs through fundamentally different cellular mechanisms of which the coccolithophores are the best studied. While great progress has been made in understanding the mechanism of coccolithophore calcification, future progress will depend significantly on the development of new genetic, molecular and cell physiological tools. It is also becoming clear that the fundamental calcification process in coccolithophores has evolved to carry out a range of functions, including grazing and pathogen protection, metabolic protection against high light and, likely, other more specific functions of highly evolved coccoliths. However, a role for calcification in provision of CO_2 for photosynthesis under limiting availability of CO_2 does not appear to be borne out by recent experimental studies.

Much current research on coccolithophores concerns their responses to changes in the oceans associated with climate change. These include changing temperature, light, nutrients and acidification. Extensive experimental studies of the impacts of ocean acidification on calcification are revealing a spectrum of responses with both positive and negative effects on calcification even within the *E. huxleyi* species complex. Understanding this variability in relation to genetic differences, geographical location and population level will be a major challenge into the future.

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Part IV

Algae Interactions with Environment

Chemically-Mediated Interactions in Microalgae

Michael A. Borowitzka

1 Introduction

Chemically-mediated interactions between algae and algae and other organisms are widespread in nature and their roles in structuring algal communities, bloom formation, algal development, and their effects on the productivity of algae are being understood better and appreciated (Pohnert 2010; Ianora et al. 2011; Roy et al. 2013). However, the study of the chemical ecology of microalgae is still very much at an early stage.

The most studied type of chemical interaction between algae is *allelopathy* and the action of *allelochemicals*. Several related terms are used for other types of specific chemical interactions between organisms. For example *kairomones* are chemicals produced by an organism which mediate interspecific interactions which benefit individuals of another species without benefitting the emitter. *Allomones* on the other hand, benefit the produced but harm the emitter, whereas *synomones* benefit both parties. *Pheromones* are compounds involved in mediating sexual reproduction. These can all be considered specialized forms of allelochemicals. More recently, it has been found that the 'quorum sensing' signaling molecules produced by bacteria can affect not only cyanobacteria, but also eukaryotic algae, and that in turn, the eukaryotic algae can also interfere with chemical signaling of bacteria.

The study of chemically-mediated interactions is confounded by several issues. For example, the role bacteria commonly associated with microalgae is largely unknown. Although researchers often try to work with axenic cultures, in nature bacteria are a normal part of the phycosphere, the boundary layer immediately surrounding all algal cells (Bell and Mitchell 1972; Jasti et al. 2005; Sapp et al. 2007). Bacteria potentially can metabolise and modify molecules in the vicinity of the algal cells, either potentiating the effects of metabolites or deactivating them. They can also affect the release of metabolites by the algal cell, and can also cause algal cell lysis. The recent review by Amin et al. (2012) describes and evaluates what is known about the interactions between diatoms and bacteria.

Another way microalgae can affect the growth of other algae is by the release of nutrients. For example, Carey and coworkers (Carey and Rengefors 2010; Carey et al. 2014) have found that the cyanobacterium *Gloeotrichia echinulata*[1] stimulates the growth of other phytoplankters, and that this stimulation is likely to be due to release/leaking of nitrogen and phosphorus from the cyanobacterial cell.

In this chapter I review the range of chemically–mediated interactions between microalgae, focusing on the major types of interactions described in the literature. One difficulty encountered is the fact that there is no consistent terminology used for the types of interactions, and it is quite clear that at times the terminology used is biased by the preconceived views of the researcher. I have largely used the same terminology and categories as used in the papers cited, however it must be recognized that this can also create conceptual 'compartments' which in reality do not exist, resulting in a narrow view of the type of interaction occurring. Overall the field of chemical ecology in marine and freshwater environments is still a young field, despite 70+ years of research, and it is only in recent years that new tools and methodologies have become available allowing more detailed studies which are providing much greater and more detailed insights into this complex and exciting field.

2 Pheromones and Hormones

2.1 Pheromones

Pheromones are chemical signals that affect individuals of the same species (Karlson and Lüscher 1959). The most

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[1]Wherever possible the currently accepted names for species are used. The name used in the paper cited is also indicated. For details of names see chapter "Systematics, Taxonomy and Species Names: Do They Matter?" of this book (Borowitzka 2016).

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studied pheromones are those involved in the control of life cycles and sexual reproduction. A knowledge and understanding of this kind of intraspecific signaling not only provides fundamental insight into life cycle regulation and physiology, but may also lead to the ability to control sexual reproduction in breeding programs to improve strains and to prevent reduced productivity resulting from sexual reproduction in algal production facilities. Alternatively, understanding the role of pheromones can potentially also be useful in controlling the adhesion of microalgae to surfaces (i.e. biofouling). A range of pheromones have been identified and characterized for several species of microalgae (see Frenkel et al. 2014 for a recent review).

In the green algae sexual reproduction and associated pheromone signaling has been best studied in the unicellular *Chlamydomonas reinhardtii* and the colonial *Volvox carteri* (Hallmann 2011). In the green algae sexual reproduction is often triggered by unfavorable environmental conditions such as reduced N availability or non-optimal temperatures (Coleman and Pröschold 2005). In *C. reinhardtii* reduced N triggers the induction of gametogenesis (Starr et al. 1995), leading to the synthesis of specific glycoproteins (agglutinins) located on the flagella (Ferris et al. 2005). Pairing of compatible gametes requires that the flagellae of these gametes come into physical contact initiating the production of an enzyme that results in the shedding of the cell wall and the flagellar collar, ultimately leading to total gamete fusion and the eventual formation of the non-motile zygote. *C. reinhardtii* shows no chemotactic behavior, relying wholly on successful random encounters between compatible cells. On the other hand, the motile MT^- gametes of *Chlamydomonas allensworthii* produce a plastoquinone-related attraction pheromone, lurlenic acid (Fig. 1), which attracts the motile MT^+ gametes at concentrations as low as 10^{-12} M (Jaenicke and Marner 1995; Starr et al. 1995). Other isolates of this morphospecies utilise lurlenol (Fig. 1), a derivative of lurlenic acid, as the attraction pheromone (Jaenicke and Starr 1996). Phylogenetic analysis of the ITS 1 and 2 regions has shown that there are at least two cryptic lineages within this species, corresponding to the two sexually incompatible pheromone types (Coleman et al. 2001).

In the heterothallic species *Volvox carteri* f. *nagariensis*, heat shock leads to the production of an inducer molecule by male clones that initiates gametogenesis in both male and female colonies (Starr and Jaenicke 1974; Kirk and Kirk 1986). This pheromone inducer is a large-molecular-weight glycoprotein which is produced by the somatic cells and acts at concentrations around 10^{-16} M (Sumper et al. 1993). Subsequently, the sperm cells also produce this molecule which now acts as a pheromone synchronizing all individuals in the population for the mating process. This system is extremely sensitive, and a single sperm cell produces enough pheromone to convert all other individuals in a 1 liter culture (Hallmann et al. 1998). One of the first effects of this sex-inducing pheromone is to cause changes in the hydroxyproline extracellular matrix by which the cells making up the *Volvox* colony are held together; additional proteins, called pherophorins, are produced (Sumper et al. 1993; Godl et al. 1995). These proteins have a structure similar to that of the pheromone and it has been suggested that they act as a mechanism of signal magnification. The somatic cells also start to incorporate sulphated glycoproteins in the matrix and these might act to collect and transport the positively charged sex-inducing pheromone to the gonidia (Wenzl and Sumper 1986). Furthermore, the pheromone also induces an extracellular glycoprotein consisting almost exclusively of hydroxyproline (Ender et al. 1999). The identical protein is also induced by wounding of *Volvox*, suggesting a molecular link between environmental stress, wound-healing, and sexual reproduction (Amon et al. 1998). On the basis of the response of stress genes Nedelcu (2005) has proposed that this induced sexual response has evolved as a response to oxidative stress induced by unfavorable environmental conditions and wounding.

In a zygnematophycean alga from the *Closterium peracerosum-strigosum-littorale* complex it has been shown that the MT^- cells secrete a glycoprotein pheromone that induces another glycoprotein in the MT^+ gametes (Tsuchikane et al. 2005; Sekimoto et al. 2012). This protoplast-release-induction protein subsequently induces the release of protoplasts in the MT^- gametes prior to cell fusion. A structurally similar sex pheromone with a similar function has also been found in *Closterium ehrenbergii* (Fukumoto et al. 2002).

As in the green algae, nutrient stress (at least in a few species) has been reported to trigger sexual reproduction in diatoms (Chepurnov et al. 2004). However, the evidence for the role of pheromones in sexual reproduction in diatoms is still extremely limited, although Sato et al. (2011) provide evidence of sex pheromones in the araphid pennate diatom *Pseudostaurosira trainorii*. They detected the action of a 'female' sex pheromone secreted by vegetative cells below the sexual size threshold (the cell size at which sexual reproduction is initiated in diatoms), which induces meiosis and thus sexualization of male vegetative cells. Subsequently, these male gametangial cells and/or the gametes produced within them release a different pheromone, which stimulates sexualization of the females. Finally, when a male gamete comes (randomly) within close range of a female gamete, it becomes amoeboid and moves directly towards the female suggesting the action of a possible third pheromone which directs movement of male gametes and serves as an attractant.

Recently the first structure of a diatom pheromone, a cyclic dipeptide (Fig. 1), was identified with the aid of metabolomics in the pennate *Seminavis robusta* (Gillard et al. 2013). This diatom also employs an elaborate, multi-

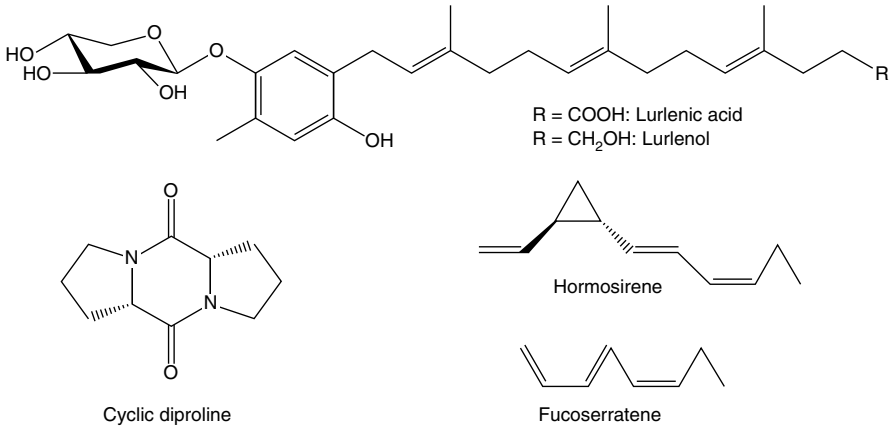


Fig. 1 Structure of compounds with have been suggested to act as pheromones in microalgae

stepped sequence of chemical signaling involved in the different phases of the auxosporulation process. However, a key difference to *P. trainorii* is that in *S. robusta*, meiosis and gamete formation only take place following successful pairing of the gametangia of the opposing sexes. Furthermore, interaction between the gametangia is strictly light dependent. In cells below the sexual size threshold the attracting mating type MT^- secretes a sex-inducing pheromone that induces a putative pheromone receptor in the attracted mating type MT^+. MT^+ itself also secretes a sex-inducing pheromone which triggers MT^- to produce an attractant that finally guides MT^+ to MT^- cells.

Compounds such as hormosirene and fucoserratine (Fig. 1), which are identical to the pheromones in brown algae, have also been found in the related heterokont diatoms (Pohnert and Boland 1996, 2001; Hombeck and Boland 1998). The biosynthetic pathways forming these hydrocarbon pheromones from fatty acids have striking similarities to the pathways in brown algae; however, as pointed out by Frenkel et al. (2014), the function of these metabolites in diatoms has never been connected to sexual reproduction, but has been linked to chemical defense (see later).

2.2 Hormones

Higher plant hormones, especially the auxin indole-3-acetic acid (IAA, Fig. 2), have been found in the cyanobacteria, the Chlorophyta, Charophyta, the Rhodophyta and the Phaeophyce (Tarakhovskaya et al. 2007; Lau et al. 2009; Hashtroudi et al. 2013), including many microalgal species (Stirk et al. 2013; Lu et al. 2014). The formation of these hormones under various culture conditions has been studied in detail in *Chlorella minutissima* (Stirk et al. 2014). Release of IAA to the growth medium has been reported by Mazur et al. (2001) in axenic cultures of *Chlorella pyrenoidosa* and *Scenedesmus armatus*, and by Prieto et al. (2011) in cultures of *Acutudesmus* (*Scenedesmus*) *obliquus* and, therefore,

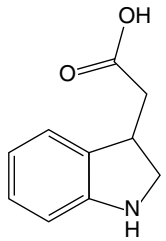


Fig. 2 Indole-3-acetic acid

there is the possibility that IAA may also function as an allelochemical. Uptake of IAA by *Chlorella vulgaris* via pH-sensitive diffusion was demonstrated by Dibb-Fuller and Morris (1992). The IAA taken-up was subsequently degraded by the cells. Over 60 years ago auxin was shown to affect growth, cell division and cell size in *Chlorella* (Yin 1937; Pratt 1938), and later also cell division in *Micrasterias* (Wood and Berliner 1979). However, Lien et al. (1971) could not observe any effect of IAA or gibberellin on the growth of *Scenedesmus fuscus* (as *Chlorella fusca*). Exogenously supplied IAA has been shown to induce morphological changes, changes in cell composition and in the excretion of metabolites in some algae. IAA increases chain length in the diatom *Skeletonema costatum* (Bentley-Mowat 1967) and, at concentrations between 28 and 114 µM, stimulates the growth and formation of four-celled rather than two-celled colonies in the green alga *Acutodesmus obliquus* (Prasad 1982). Recently, Salama et al. (2014) observed that IAA increased both growth and lipid content in *A. obliquus*. Exogenously supplied IAA at a concentration of 57 µM also enhanced cell division in *Chlorella* (Vance 1987), and between 50 and 100 µM enhanced the content of chlorophylls, proteins and saccharides in C. *pyrenoidosa* (Czerpak et al. 1999; Piotrowska-Niczyporuk and Bajguz 2014). IAA also enhanced the excretion of proteins and saccharides (Czerpak and Bajguz 1993). Furthermore, auxins and brassinosteroids were observed to act synergistically in enhancing growth in

Chlorella vulgaris (Bajguz and Piotrowska-Niczyporuk 2013). Exogenous IAA also enhanced the growth of *Dunaliella salina* and *Porphyridium cruentum*, but not *Chaetoceros muelleri* (Li et al. 2007).

In studies of multicellular algae from the Chlorophyta, Phaeophyceae and Rhodophyta, the observed spectrum of biological activities of algal phytohormones is similar to that of higher plant phytohormones, however the auxin signaling pathways found in higher plants do not seem to be present, although there is some evidence for alternative pathways which are yet to be elucidated (Lau et al. 2009). Many of the observed effects of IAA on microalgae can be related to the H^+-ATPase stimulating activity of IAA (Napier and Venis 1995). However, there is no evidence so far that IAA acts as an allelochemical in microalgae in nature.

3 Quorum Sensing Signal Compounds

Bacteria show a form of chemical communication by secreting small signal molecules into the environment that induce gene expression in themselves and neighbouring cells once the concentration in the environment reaches a threshold value (Waters and Bassler 2005). This process is called 'quorum sensing (QS)' as the effect occurs mainly when the bacterial population reaches a critical size (quorum). The best-studied quorum sensing signal molecules are the *N*-acylhomoserine lactones (AHLs), secreted by diverse Gram-negative proteobacteria belonging to α, β and γ subdivisions (Williams et al. 2007).

Evidence for QS in cyanobacteria is limited. The AHL, N-octanoyl-homoserine lactone (C8-AHL), has been found in the epilithic colonial cyanobacterium *Gloeothece* PCC6909 (Sharif et al. 2008). Accumulation of C8-AHL in the culture medium of laboratory cultures of *Gloeothece* followed a pattern characteristic of the phenomenon of autoinduction, a common feature of functional AHL-based quorum-sensing systems. In response to treatment with C8-AHL, early growth-stage cells of *Gloeothece* showed changes in expression of 43 proteins compared with untreated cells. Among the 15 proteins that showed a more than two-fold change in expression were RuBisCo, glutamate synthase, chorismate synthase, a member of the LysR family of transcriptional regulators (all upregulated), and enolase and aldolase, both of which were downregulated. Several of the expressed proteins are associated with CO_2 fixation. As the *Gloeothece* mats become thicker CO_2 supply is expected to be reduced due to diffusional limitation and the upregulation of these genes may help to overcome this limitation. For example ribulose bisphosphate carboxylase (RuBisCo), the key enzyme of CO_2 fixation, is upregulated three-fold. Similarly a LysR-type transcriptional regulator is also upregulated 2.4-fold, and these are transcriptional regulators of RuBisCo in most microorganisms (Shively et al. 1998). Similarly the changes expression of an ABC-type amino acid transporter and glutamate synthethase may help overcome possible N-limitation in the cyanobacterial mat and affect amino acid metabolism. There is also preliminary evidence for a possible role of quorum sensing in *Microcystis* colony formation (Zhai et al. 2012), but this remains to be confirmed.

Quorum sensing also has been implicated in the production of bioactive compounds in cyanobacterial blooms. Using cultures of the cyanobacteria *Microcystis panniformis* Mp9, *M. aeruginosa* Ma26, and *Radiocystis fernandoi* R28, Pereira and Giani (2014) observed that most of the 17 peptides detected, including microcystins, cyanopeptilins and aeruginosins, had significantly higher cell quotas in high density cultures, suggesting a possible role for QS in triggering the production of these peptides.

AHLs have been shown to inhibit nitrogen fixation in *Anabaena* sp. PCC7120 (Romero et al. 2011). The inhibition seems to take place at post-transcriptional level, as no effect on heterocyst differentiation or on the expression of nitrogenase was observed. *Anabaena* PCC7120 produces an acylase which is capable of inactivating long-chain AHLs, apparently as a defence mechanism against these molecules (Romero et al. 2008). In the colonial filamentous planktonic cyanobacterium *Trichodesmium*, AHLs produced by associated bacteria stimulate the activity of alkaline phosphatases (Van Mooy et al. 2012). By contrast, addition of (*S*)-4,5-dihydroxy-2,3-pentanedione (DPD) – the precursor to the autoinducer-2 (AI-2) family of universal interspecies signalling molecules – led to the attenuation of alkaline phosphatase activity. In field collected colonies of *Trichodesmium* both AHLs and AI-2 have been found. Both types of molecules turned over rapidly, indicating a complex chemical interplay among epibionts using AHLs and AI-2 to control access to phosphate in dissolved-organic phosphorus.

Although quorum sensing was originally thought to occur only between bacterial species, there is growing evidence that bacterial QS systems are involved in cross-kingdom signalling with eukaryotic organisms and that eukaryotes are capable of actively responding to bacteria in their environment by detecting and acting upon the presence of these signalling molecules. Likewise, eukaryotes produce compounds that can interfere with QS systems in bacteria by acting as agonists or antagonists. Some microalgae produce compounds that mimic the bacterial AHL quorum-sensing signals or they can degrade or inhibit the action of the quorum-sensing signals, presumably as a kind of defence mechanism. For example, the green alga *C. reinhardtii* has been shown to secrete compounds that mimic the bacterial AHL quorum-sensing signals and alter quorum sensing-regulated gene expression in the associated bacteria (Teplitski et al. 2004). Inhibition of the production of these mimics allowed the

AHL-producing isolate of the bacterium *Aeromonas veronii* to colonize the transgenic algal cultures and form biofilms more readily than the parental algal cultures, indicating that production of these mimics by the alga can significantly affect its interactions with bacteria it encounters in natural environments (Rajamani et al. 2011). The diatom *Chaetoceros didymus* is resistant to cell lysis by bacterial proteases unlike some other diatom species. Paul and Pohnert (2013) have found that this resistance apparently is due to algal proteases which are induced by a >30 kD fraction of bacterial exudates. It is hypothesised that this fraction includes bacterial quorum sensing compounds. Another diatom, the benthic *Nitzschia* cf. *pellucida* inhibits QS by the deactivation of β-keto-AHLs by the action of a H_2O_2 mediated haloperoxidase (Syrpas et al. 2014). Similarly, malyngolide produced by *Lyngbya* spp. has also been shown to disrupt QS in bacteria (Dobretsov et al. 2010). A number of other microalgae species, including *Chlamydomonas saccharophila* and *Tetraselmis* spp., also inhibit quorum sensing signals (Natrah et al. 2011). Furthermore, interference with QS systems has been shown in a number of seaweeds. The best studied example of this is the production of halogenated furanones by the red alga *Delisea pulchra* (Manefield et al. 1999, 2002). Other examples are the red seaweed *Asparagopsis taxiformis* (Jha et al. 2013), and members of the Caulerpaceae, Rhodomelaceae and Galaxauraceae (Skindersoe et al. 2008).

Another, well studied example of the interaction of quorum sensing compound producing bacteria and algae, is the settling of *Ulva* zoospores on a substratum. The flagellated zoospores of *Ulva*, when released, are negatively phototactic and swim away from the water surface. Their settling and attachment to a substratum is also not random, but is influenced by a number of substratum characteristics, including surface chemistry and roughness. In particular, bacterial biofilms can influence *Ulva* zoospore settling and attachment, and bacteria which either stimulate or inhibit zoospore settlement have been isolated from marine surfaces colonized by *Ulva* (Thomas and Allsopp 1983; Patel et al. 2003). Where the bacteria have a positive effect on zoospore settling, there exists a positive relationship between zoospore settlement and bacterial density (Joint et al. 2000). Using the bacterium *Vibrio anguillarum* as a model bacterium with a sophisticated QS regulatory system, it was shown that the zoospores settling was dependent on bacteria that produced AHLs (Tait et al. 2005). The mechanism of zoospore attraction does not appear to involve chemotaxis toward AHLs, but rather a chemokinesis in which the swimming speed of the spores decreases in the presence of AHLs so that the zoospores accumulate at the AHL source (Wheeler et al. 2006). The presence of AHL causes a rapid influx of Ca^{2+} which results in a reduction in swimming speed via a calcium-dependent modulation of the flagella beat pattern. A detailed analysis of the effect of AHLs and their mode of action can be found in the papers by Joint et al. (2007) and Mieszkin et al. (2013). The QS molecules of the *Ulva*-associated bacteria can also affect germination and growth of *Ulva* zoospores (Twigg et al. 2014).

4 Allelopathy

Allelopathy is the best known type of chemically-mediated interaction between organisms. The term allelopathy derives from the Greek *allelon* (ἀλλήλων) meaning mutual and *pathos* (πάφοζ) meaning 'suffering' or 'experience'. The term was first used by Molisch (1937) to describe a process by which one plant affects the growth of another by biochemical means, although the concept of allelopathic interactions has a much older history (see Willis 2007). Rice (1984) expanded the term to include microorganisms such as bacteria, fungi and microalgae, and included both negative and positive effects on the target organism, and this is the current widely-used definition. The biochemical compounds which exert this effect are secondary metabolites and are known as allelochemicals. Some biologists restrict the definition of allelopathy only to direct biochemical interactions (Lampert and Sommer 1997; Lambers et al. 1998) while others include predator-prey interactions (Rizvi and Rizvi 1992; Wolfe 2000). In this chapter I focus mainly on algae-algae interactions between microalgae, both positive and negative.

Although putative allelopathic interactions between microalgae have been widely described in the literature (see Table 1 for a listing of these reports), a definitive proof for allelopathic interactions in aquatic systems is very difficult to almost impossible. Willis (1985) listed six requirements that must be met (note that he limited his definition of allelopathy only to negative interactions). These requirements have been expanded and adapted slightly so as to correspond to the broader definition of allelopathy used here in this chapter. The requirements are:

1. A pattern of inhibition or stimulation of one species or plant by another must be shown;
2. The putative 'donor' plant must produce a toxin or a growth stimulator;
3. There must be a mode for release of this compound to the environment;
4. There must be a mode of transport and/or accumulation of this compound or its breakdown product in the environment;
5. The compound must be shown to be active at the concentrations found in the environment
6. The target organism must have some means of uptake of this compound;
7. The observed pattern of inhibition or stimulation cannot be explained solely by physical or other biotic factors.

Table 1 Reported apparent allelopathic effects either under co-culture, agar diffusion cultures, cultures where the algae are separated by membranes or filters, or due to addition of spent medium in which the donor species had been grown

Donor species	Target species	Effect	References	Note
Cyanobacteria				
Many cyanobacterial strains of the genera *Fischerella*, *Calothrix* and *Nostoc*	*Coelastrum microporum* *Monoraphidium convolutum* *Scenedesmus acutus* (=*Acutodesmus obliquus*) *Trichormus* (*Anabaena*) *dolioum* *Anabaena circinalis* (=*Dolichospermum sigmoideum*) *Microcystis aeruginosa* *Nodularia spumigena*	Growth inhibition	Schlegel et al. (1998)	A total of 198 cyanobacterial strains were screened. Only the genera with strains which showed activity are listed here. The effect of proteinase K was also tested
Several heterocystous species (*Anabaena* spp., *Nostoc* spp. *Cylindrospermum licheniforme*)	Range of cyanobacteria species	Cell death	Flores and Wolk (1986)	
Anabaena sp. (cf. *lemmermannii*) KAC 16 *Aphanizomenon* sp. Tr183 *Nodularia spumigena* KAC13	Natural phytoplankton sample	Culture filtrates of *Anabaena* and *Nodularia* inhibited cryptophytes, but filtrates of all 3 species stimulated growth of *Snowella* spp., *Pseudonanabaena* spp., *Amphidinium* sp. *Nodularia* filtrate also stimulated growth of *Anabaena* spp., *N. spumigena*, and *Oocystis* sp. Diatoms, other dinoflagellate spp. and *Binuclearia* (*Planctonema*) *lauterbornii* were not affected	Suikkanen et al. (2005)	The *N. spumigena* filtrate contained 27.8 ng nodularin equivalents (μg Chl *a*)$^{-1}$
Anabaena flos-aquae	*Chlamydomonas reinhardtii*	Growth inhibition and induction of settling	Kearns and Hunter (2000, 2001)	High concentrations of *C. reinhardtii* extracellular products inhibit toxin production in *A. flos-aquae*
Anabaena flos-aquae	*Conticribra* (*Thalassiosira*) *weissflogii* *Rhodomonas* sp. *Prymnesium parvum*	Growth inhibition	Suikkanen et al. (2004)	*Conticribra* recovers rapidly from the inhibition
Anabaena torulosa	*Acutodesmus obliquus* *Pandorina morum* *Staurastrum crenulatum* *Anabaena cylindrica* *Fragilaria* sp.	Growth inhibition	Schagerl et al. (2002)	Little or no effect on other species
Cylindrospermopsis raciborskii LS124	*Coelastrum sphaericum* *Monoraphidium contortum* *Microcystis wesenbergii*	Inhibits photosynthesis as measured by the rETR	Figueredo et al. (2007)	No effect on *Navicula* sp.
Cylindrospermopsis raciborskii CYRF-1	*Microcystis aeruginosa*	Growth inhibition when exposed to medium from mixed cultures only; induction of colony formation	Mello et al. (2012)	

(continued)

Table 1 (continued)

Donor species	Target species	Effect	References	Note
Cylindrospermopsis raciborskii	*Microcystis aeruginosa*	Growth inhibition, inhibition of microcystin-LR production, and up-regulation of alkaline phosphatase activity	Rzymski et al. (2014)	This strain of *C. raciborskii* does not produce cylindrospermopsin
Dolichspermum (*Anabaena*) *lemmermannii*	*Rhodomonas* sp. *Prymnesium parvum*	Growth inhibition	Suikkanen et al. (2004)	
Microcystis aeruginosa	*Chlorella vulgaris*	Growth inhibition	Żak and Kosakowska (2014)	
Microcystis aeruginosa	*Microcystis wesenbergii*	Growth inhibition	Yang et al. (2014)	
Microcystis aeruginosa	*Oocystis marssonii*	Growth inhibition	Dunker et al. (2013)	No growth inhibition of *Acutodesmus* (*Scenedesmus*) *obliquus* was observed
Microcystis sp.	*Peridinium gatunense*	Growth inhibition. Inhibition of carbonic anhydrase	Sukenik et al. (2002)	
Microcystis ichthyoblabe *Microcystis aeruginosa*	*Aphanizomenon flos-aquae*	Growth inhibition	Ma et al. (2015)	Microcystin-LR is not the active compound
Nodularia spumigena	*Conticribra* (*Thalassiosira*) *weissflogii* *Rhodomonas* sp. *Prymnesium parvum*	Growth inhibition	Suikkanen et al. (2004)	The effect is not due to nodularin
Nodularia spumigena	*Skeletonema marinoi*	Growth inhibition	Śliwińska and Latala (2012)	
Nostoc ASW01020 and ASW01010	*Acutodesmus obliquus* *Staurastrum crenulatum* *Anabaena cylindrica* *Microcystis flos-aquae* *Fragilaria* sp.	Growth inhibition	Schagerl et al. (2002)	Little or no effect on other species
Planktothrix agardhii	*Microcystis aeruginosa*	Stimulation of Microcystin-LR synthesis	Engelke et al. (2003)	
Synechocystis aquatilis	*Microcystis aeruginosa* *Pseudoanabaena* (*Oscillatoria*) *limnetica* *Chlorella vulgaris* *Ulothrix* sp.	Growth inhibition	Mohamed (2013a)	Active compound is norharmane
Trichormus doliolum	*Trichormus* (*Anabaena*) *variabilis* *Synechococcus* sp. PCC6911 *Synechocystis* sp. CB3 *Nannochloris* sp. SAG 55-81 *Stigeoclonium helveticum*	Growth inhibition	Von Elert and Jüttner (1996)	Several other species showed slight growth inhibition
Tychonema bourrellyi	*Microcystis aeruginosa*	Growth inhibition	Shao et al. (2013)	β-ionone?
Haptophyta				
Prymnesium parvum	Natural Phytoplankton community	Growth inhibition and cell death. Different taxa show different sensitivities	Fistarol et al. (2003)	
Prymnesium parvum	*Conticribra* (*Thalassiosira*) *weissflogii* *Prorocentrum minutum* *Rhodomonas* cf. *baltica* *Prymnesium parvum* f. *patelliferum*	Growth inhibition (no effect on *P. parvum* f. *patelliferum*)	Granéli and Johansson (2003)	

(continued)

Table 1 (continued)

Donor species	Target species	Effect	References	Note
Prymnesium parvum	*Rhodomonas salina*	Growth inhibition and cell lysis	Barreiro et al. (2005) and Uronen et al. (2007)	
Prymnesium parvum	*Heterocapsa rotundata*	Inhibition of motility, cell lysis	Skovgaard and Hansen (2003)	
Prymnesium polylepis	*Scrippsiella trochoidea*	Growth inhibition (cell death) and induction of cyst formation	Fistarol et al. (2004a)	
Prymnesium polylepis	*Heterocapsa triquetra* +6 other dinoflagellate species	Loss of motility (no effect on *Karenia* (*Gymnodinium*) *mikimotoi*) and cell death	Schmidt and Hansen (2001)	Effect dependent on cell density of *P. polylepis*
Prymnesium polylepis	*Eutreptiella gymnastica* *Heterosigma akashiwo* *Rhodomonas marina* *Skeletonema costatum* *Pyramimonas propulsa* *Dictyocha speculum*	Growth inhibition	Schmidt and Hansen (2001)	Growth inhibition observed after 2–6 days of co-culture
Heterokontophyta				
Chaetoceros neogracilis	*Diacronema* (*Pavlova*) *lutheri*	Growth promotion	Yamasaki and Hikida (2013)	
Cylindrotheca fusiformis	*Skeletonema costatum* *Nitzschia longissima* *Phaeodactylum tricornutum* *Heterocapsa triquetra* *Amphidinium carterae*	Growth inhibition	Chan et al. (1980)	No effect on *Scrippsiella sweeneyae. Prorocentrum micans*, *Tetraselmis* sp., and an unidentified cyanobacterium
Heterosigma akashiwo	*Skeletonema costatum* *Chaetoceros muelleri*	Growth inhibition	Yamasaki et al. (2007)	
Nitzschia cf *pellucida*	*Navicula arenaria* *Ceratoneis* (*Cylindrotheca*) *closterium* *Entomoneis paludosa* *Stauronella* sp.	Inhibition and cell death	Vanelslander et al. (2012)	*Stauronella* is more resistant than other species
Nitzschia palea	*Asterionella formosa*	Growth inhibition	Jøgensen (1956)	
Ochromonas sp.	*Chlamydomonas reinhardtii* *Cryptomonas pyrenoidifera* (*as Cryptomonas ozolini*)	Growth inhibition	Hiltunen et al. (2012)	
Phaeodactylum tricornutum	*Thalassiosira pseudonana*	Growth inhibition	Sharp et al. (1979)	
Pseudo-nitzschia multiseries *Pseudo-nitzschia pungens*	*Akashiwo sanguinea* *Rhodomonas salina* *Chattonella marina*	Growth inhibition	Xu et al. (2015)	No effect on *Prorocentrum minimum* or *Phaeocystis globosa*
Skeletonema costatum	*Conticribra* (*Thalassiosira*) *weissflogii*	Growth promotion	Paul et al. (2009)	
Skeletonema costatum	*Heterosigma akashiwo* *Chaeotoceros muelleri*	Growth inhibition	Yamasaki et al. (2007)	
Skeletonema costatum	*Heterosigma akashiwo*	Growth inhibition	Yamasaki et al. (2012)	
Dinophyta				
Alexandrium fundyense	*Thalassiosira* cf. *gravida*	Growth inhibition	Lyczkowski and Karp-Boss (2014)	Effect depends on cells size of the diatom
Alexandrium minutum	*Chaetoceros neogracile*	Reduction in cell size and inhibition of photosynthesis	Lelong et al. (2011)	

(continued)

Table 1 (continued)

Donor species	Target species	Effect	References	Note
Alexandrium ostenfeldii	*Kryptoperidinium foliaceum* *Levanderina fissa* *Heterocapsa triquetra*	Cyst formation Loss of cell shape and lysis Shedding of thecae	Hakanen et al. (2014)	Effects are temporary. After 24 h *K. foliaceum* returned to vegetative cells, and the number of immotile cells of the other 2 species had significantly decreased
Alexandrium sp.	*Prorocentrum donghaiense*	Growth inhibition	Yang et al. (2010)	
Alexandrium tamarense	Range of phytoplankton spp. from several phyla	Cell death/cell lysis	Fistarol et al. (2004b) and Ma et al. (2009, 2011b)	Probable allelopathic compound(s) are large non-proteinaceous and probably non-polysaccharide compounds between 7 and 15 kDa
Alexandrium tamarense	*Prorocentrum donghaiense*	Cell death	Wang et al. (2006)	Co-culture studies show a complex, density-dependent interaction between these two species
Alexandrium tamarense *Karenia mikimotoi*	*Scrippsiella trochoides*	Growth inhibition (cell death) and induction of cyst formation	Fistarol et al. (2004a)	
Amphidinium klebsii	*Ostreopsis lenticularis* *Ganbierdiscus toxicus*	Growth inhibition	Sugg and VanDolah (1999)	Only small effect on *Coolia monotis* and *Prorocentrum lima*
Biecheleria baltica *Gymnodinium corollarium* *Scrippsiella hangoei*	*Melosira arctica* *Skeletonema maironoi* *Thalassiosira baltica*	Growth inhibition	Suikkanen et al. (2011)	
Biecheleria baltica *Gymnodinium corollarium* *Scrippsiella hangoei*	*Rhodomonas* sp.	Growth stimulation	Suikkanen et al. (2011)	
Cochlodinium polykrikoides	Wide range of phytoplankton species from several Phyla	Loss of motility, distortion of cell shape, cell death	Tang and Gobler (2010)	
Coolia monotis	*Gambierdiscus toxicus* *Ostreopsis lenticularis* *Prorocentrum lima*	Slight growth inhibition	Sugg and VanDolah (1999)	No effect on *Alexandrium klebsii*
Gambierdiscus toxicus	*Ostreopsis lenticularis*	Growth inhibition	Sugg and VanDolah (1999)	No effect on *Amphidinium klebsii*, and only small effect on *Coolia monotis* and *Prorocentrum lima*
Heterosigma akashiwo	*Skeletomena costatum*	Growth inhibition	Yamasaki et al. (2009)	Active compound(s) are large (>10^6 Da) protein-polysaccharide complexes
Karenia brevis	*Amphora* sp. *Asterionellopsis glacialis* *Prorocentrum mexicanum* *Rhizosolenia* sp. *Skeletonema costatum* *Thalassiosira pseudonana*	Growth inhibition	Kubanek et al. (2005), Prince et al. (2008, 2010), Poulson et al. (2010), and Poulson-Ellestad et al. (2014b)	Filtrates not active against a number of other species. The brevetoxins are not the allelopathic compound(s)
Karenia (*Gymnodinium*) *mikimotoi*	*Heterocapsa circularisquama*	Growth inhibition and induction of cyst formation	Uchida et al. (1999)	

(continued)

Table 1 (continued)

Donor species	Target species	Effect	References	Note
Ostreopsis lenticularis	*Gambierdiscus toxicus*	Growth inhibition	Sugg and VanDolah (1999)	No effect on *Amphidinium klebsii* and only small effect on *Coolia monotis* and *Prorocentrum lima*
Peridinium aciculiferum	*Rhodomonas lacustris* *Cryptomonas* sp. *Cyclotella* sp. *Synura petersenii* *Parvodinium* (*Peridinium*) *inconspicuum*	Growth inhibition and cell lysis	Rengefors and Legrand (2007)	No effect on *Chlamydomonas reinhardtii*
Prorocentrum cordatum (as *Prorocentrum minimum*)	*Skeletonema costatum*	Growth inhibition	Tameishi et al. (2009)	
Prorocentrum donghaiense	*Scrippsiella trochoidea*	Growth inhibition	Wang and Tang (2008)	
Prorocentrum donghaiense	*Alexandrium tamarense*	Cell filtrate from 1×10^4 cells mL^{-1} culture stimulated growth, but filtrate from 1×10^5 cells mL^{-1} depressed growth	Wang et al. (2006)	
Prorocentrum lima	*Coolia monotis* *Gambierdiscus toxicus* *Ostreopsis lenticularis*	Growth inhibition	Sugg and VanDolah (1999)	No effect on *Amphidinium klebsii*. Okadaic acid is not the active compound
Prorocentrum micans	*Skeletonema costatum* *Chaetoceros didymus*	Growth inhibition	Uchida (1977)	
Chlorophyta				
Several species of volvocine algae	Several species of volvocine algae	Growth inhibition (variable between different species combinations)	Harris (1971)	
Botryococcus braunii	Range of chlorophytes, diatoms and cyanobacteria	Growth inhibition	Chiang et al. (2004)	Note that Song et al. (2013) found no inhibitory effect of *B. braunii* on *C. vulgaris* or *C. reinhardtii*
Chlamydomonas reinhardtii	*Haematococcus pluvialis*	Growth inhibition	Proctor (1957)	
Chlamydomonas reinhardtii	*Cryptomonas pyrenoidifera* (*Cryptomonas ozolinii*)	Growth inhibition	Barreiro and Hairston (2013)	Only cell-free filtrate of N or P limited cultures had an effect. No effect on other species (*Microcystis aeruginosa*, *Ochromonas danica*)
Chlorella vulgaris *Chlamydomonas reinhardtii*	*Botryococcus braunii*	Cell-free medium from low density cultures stimulates growth, from high density cultures growth is inhibited	Song et al. (2013)	
Chlorococcum ellipsoideum	*Chlamydomonas globosa*	Growth inhibition	Kroes (1971)	
Picochlorum (*Nannochloris*) *eukaryotum*	*Karenia brevis* (as *Gymnodinium breve*)	Cell lysis	Pérez et al. (2001)	
Scenedesmus sp.	*Microcystis* spp.	Cell lysis	Harel et al. (2013)	
Uronema conferficola	*Scenedesmus* (*Desmodesmus*) *quadrispina*	Induces colony formation	Leflaive et al. (2008)	
Volvulina pringsheimii	Range of chlorophytes	Growth inhibition	(Harris 1971)	*P. pringsheimii* also shows autoinhibition

Most of the studies here are laboratory based, but some have also been carried out in micro- and mesocosms

Confirmation of the above points does not prove that allelopathy is operative, only that allelopathy offers the most reasonable explanation of the observed pattern of interactions between organisms (Willis 1985). Very few studies of algal allelopathy have considered all of these criteria and it is extremely difficult to devise experiments to evaluate all of them. Clear and unambiguous identification of specific allelopathic compounds and determination of their mode of action are further complicated by the fact that algae often produce a range of compounds, several of which can have biological activities including allelopathic activities, and separation of these compounds is made difficult by their often very low concentrations. Furthermore, the released exometabolites may undergo chemical modification through oxidation, the action of extracellular enzymes and/or bacterial action and this may affect their activity.

Historically, cross culturing is the most commonly used method to detect and study allelopathic interactions between two algae species. Here the target species is grown in a medium enriched with cell-free filtrate of the medium in which the donor species had been cultured (Arzul et al. 1999; Pérez et al. 2001). It is important to ensure that the nutrient content of the culture is maintained to avoid the possibility of any observed effects being due to nutrient depletion. Alternative methods used to demonstrate possible allelopathic interactions commonly used include (i) culture on solid media (Chan et al. 1980; Mason et al. 1982; Flores and Wolk 1986), (ii) a culture system where the two species are separated by a fine filter, dialysis membrane or other type of barrier, which only allows exchange of soluble chemicals of a particular molecular size range (Yamasaki et al. 2007), or (iii) co-culture of the donor and host species (Sharp et al. 1979; Tameishi et al. 2009). Less frequently the allelopathic effects of a species are studied in more complex (but more environmentally relevant) systems such as meso- and microcosms where the donor species is grown together with a natural planktonic community (Mohamed 2013b), or where the cell-free filtrate is added to this natural planktonic community (Fistarol et al. 2003; Hattenrath-Lehmann and Gobler 2011). In all these experimental methods care must be taken to ensure that the results obtained are due to allelochemicals and not due to different competitive adaptations of the algae to abiotic factors such as changes in pH and nutrients or possibly irradiance in denser cultures.

In this chapter the focus is on microalgae/microalgae allelopathic interactions. However, it must be remembered that the key target(s) of the metabolites produced may not be other algae, but rather other organisms such as bacteria or zooplankton, and that the effects the metabolites have on other algae may be only coincidental secondary effects.

The study of allelopathic interactions in microalgae is a component of the broader field of chemical ecology (Pohnert 2010) and is of importance both to the understanding of the ecology of microalgae and also for the optimisation of dense large-scale algal cultures for commercial applications. Most studies so far have focused on the natural phytoplankton and the role of allelopathy in species succession and bloom development and demise (Cembella 2003; Granéli et al. 2008; Leão et al. 2009b; Roy et al. 2013). There are far fewer studies on allelopathic interactions in benthic microalgae and algal mats (e.g., Vanelslander et al. 2012) and almost none on allelopathic interactions in terrestrial microalgae (Safonova and Reisser 2005). Allelopathic effects (usually autoinhibition) also have been observed to limit productivity in some very high density unialgal cultures (Javanmardian and Palsson 1991; Zou et al. 2000), especially in photobioreactors, however this aspect of allelopathy still remains little researched. More recently there has been some interest in using mixed species algal cultures (polycultures) for potential commercial applications (Hong and Xu 2013; Liu 2014) and, as this field develops further, it is very likely that an understanding of potential allelopathic interactions will be very important in order to achieve stable, high productivity microalgal cultures.

The actual allelochemical(s) causing the allelopathic effects are generally not known, however the number of identified allelochemicals is gradually increasing. Table 2 summarises reports of identified allelochemicals.

4.1 Autoinhibition

Autoinhibition is where an alga inhibits itself. Autoinhibition was first demonstrated by Harder (1917) in old cultures of *Nostoc*. Potential inhibitory effects of algae in natural populations, which we would now call allelopathic effects, were first postulated by Akehurst (1931) in his study of algal succession in a number of freshwater ponds. Later, Pratt and Fong (1940) showed that the medium of old cultures of *Chlorella vulgaris* contained a substance which inhibited the growth of *Chlorella*, other algae and bacteria. This substance was called chlorellin (Pratt et al. 1944); subsequent analysis has shown that the 'chlorellin' is a mixture of fatty acids and hydrocarbons (Spoehr et al. 1949; DellaGreca et al. 2010). The chlorophyte *Platydorina caudata* also appears to produce an autoinhibitor (Harris 1970). This autoinhibitor is very specific and apparently does not affect the growth of the closely related algae *Volvox carteri*, *Pleodorina californica*,

Table 2 Allelopathic effects and the allelochemicals causing them

Organism	Marine/freshwater	Culture/field	Compound(s)	Activity	Target species	Reference
Anabaena flos-aquae	FW	C	Anatoxin-a Microcystin-LR	Inhibits motility and causes faster settling	*Chlamydomonas reinhardtii*	Kearns and Hunter (2001)
Aphanizomenon ovalisporum	FW	C	Cylindrospermopsin	Induces extracellular alkaline phosphatase activity in target spp.	*Chlamydomonas reinhardtii* *Debarya* sp.	Bar-Yosef et al. (2010)
Microcystis sp.	FW	C	Microcarbonin A	Inhibition of photosynthesis and eventual cell death. Inhibits internal carbonic anhydrase. Pure compound only partially active	*Peridinium gatunense*	Beresovsky et al. (2006)
Oscillatoria sp. LEGE05292	FW	C	Cyclic peptides - Portoamide A and B	Growth inhibition (mechanism unknown). Compounds act synergistically and not individually. Optimum ratio of portoamide A:portoamide B = 4.4:1	*Ankistrodesmus falcatus* *Chlamydomonas reinhardtii* *Cylindrospermopsis raciborskii* Inactive against: *Cyclotella meneghiniana* *Anabaena* sp. *Aphanizomenon* sp. *Microcystis aeruginosa*	Leão et al. (2010)
Chlorella vulgaris	FW	C	Chlorellin (mixture of mainly C18 free fatty acids)	Low concentration stimulate growth, higher concentrations inhibit growth	*Raphidocelis* (*Pseudokirchneriella*) *subcapitata*	Fergola et al. (2007) and DellaGreca et al. (2010)
Closterium aciculare	FW	C	Siderophore-like substance	In iron-limiting growth conditions: Stimulates growth Inhibits growth	*Closterium aciculare* *Monactinus* (*Pediastrum*) *simplex* *Staurastrum paradoxum* *Aulacoseira granulata* *Cosmocladium constrictum*	Naito et al. (2006)

Nannochloropsis oculata	FW	C	Methyl stearate and methyl palmitate	Cell lysis	*Gymnodinium breve*	Perez et al. (1997) and Pérez and Martin (2001)
Quadrigula chodatii	FW	C	Dibutyl phthalate and β-sitosterol?	Growth inhibition	*Microcystis aeruginosa*	(Zhang et al. 2013)
Isochrysis galbana	MAR	C	Stearic acid (C18:0) or oleic acid (C18:1)	Growth inhibition	A range of microalgae species	Sun et al. (2012)
Heterosigma akashiwo	MAR	C/F	Polysaccharide-protein complexes	Growth inhibition	*Skeletonema costatum*	Yamasaki et al. (2009)
Karenina brevis	MAR	C	Unstable, polar molecules with mw between 100 and 1000 Da (n.b. brevetoxin is not allelopathic)	Growth inhibition	*Asterionellopsis gracilis*	Prince et al. (2010)
Haslea ostrearia	MAR	C	Polyphenolic pigment 'marennine'	Growth inhibition (due to the combination of an allelopathic effect and shading by the blue-green coloured mareninne)	*Skeletonema costatum* *Ceratoneis* (*Nitzschia*) *closterium* *Haslea crucigera*	Pouvreau et al. (2007)
Nitzschia cf. *pellucida*	MAR	C	BrCN	Inhibition and death	*Entomoneis paludosa*	Vanelslander et al. (2012)

Eudorina elegans or *Pandorina charkowiensis*. In contrast, culture medium from *Volvulina pringsheimii* caused not only autoinhibition, but also inhibited the growth of a number of related green algae (Harris 1971). When the medium was autoclaved no inhibition was observed. Autoinhibition also has been observed in the prymnesiophyte *Prymnesium parvum* (Olli and Trunov 2007).

Autoinhibition in diatoms was first observed by von Denffer (1948) in cultures of *Nitzschia palea* and he observed that the cells were arrested in the late anaphase/telophase stage of mitosis. Autoinhibition also has been shown in the diatom *Skeletonema costatum* (Imada et al. 1991, 1992; Yamasaki et al. 2012; Wang et al. 2013), with the autoinhibitor identified as *15*(*S*)-hydroxy-eicosapentaenoic acid (Fig. 3). However, autoinhibition in *S. costatum* apparently only occurs under nutrient-limiting growth conditions, especially P-limited conditions (Wang et al. 2013). Imada et al. (1991) found that the autoinhibitor was produced gradually during the exponential growth phase of *S. costatum* and mostly accumulated in the cells up to the stationary phase. However, the inhibitor in the dying or dead cells was rapidly released into the medium, resulting in the increase in the concentration of the inhibitor in the medium. After almost all the cells died, the largest portion of the inhibitor produced by *S. costatum* was found in the medium.

The apparent production of autoinhibitors also has been observed in very dense cultures of *Nannochloropsis* sp. in photobioreactors (Richmond and Zou 1999; Zou et al. 2000), although Rodolfi et al. (2003) concluded that at least part of this inhibition is due to 'old' cell walls shed by the cells. How the 'old' cell walls would inhibit algal growth is unclear, but the effect may simply be a physical one due to an increase in turbidity which would reduce light availability, as well as possible changes in the viscosity of the medium. The autoinhibition observed in dense cultures of *Monodus subterraneus* in flat panel photobioreactors seems to be due to free fatty acids (Bosma et al. 2008), similar to what is observed in *Chlorella*. Sun et al. (2001) also have reported autoinhibition in cultures of *Haematococcus pluvialis*.

Free fatty acids have also been found to act as autoinhibitors in *Synechococcus* spp. which have been metabolically engineered to overproduce free fatty acids (Ruffing and Jones 2012).

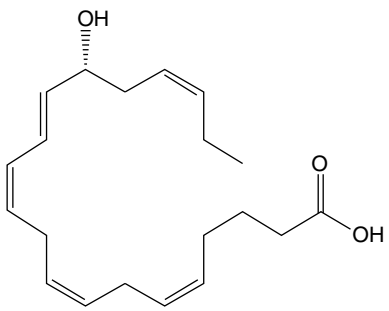


Fig. 3 *15*(*S*) hydroxy-eicosapentaenoic acid

4.2 Cyanobacteria

Cyanobacteria are well known for their production of a wide range of toxins and other biologically active compounds, many of which play a role in the ecology of these organisms (Leão et al. 2012a). Some of these have been shown to have allelopathic activities. In his classical studies in the 1970s, Keating (1977, 1978) showed that cell-free filtrates of dominant cyanobacteria in a lake, as well as the lake water, induced either positive or negative effects on the growth of other cyanobacteria and diatoms from the same lake. In a large-scale screening study Schlegel et al. (1998) found that out of 198 cyanobacterial strains, 20 strains showed bioactivity against green algae, and most of these strains also showed activity against other cyanobacteria; whether these activities can be considered as allelopathic remains to be determined. The active strains were all from the genera *Fischerella*, *Calothrix* and *Nostoc*.

The first allelopathic compound isolated from a cyanobacterium was the chlorine-containing γ-lactone, cyanobacterin (Fig. 4), which was released by the freshwater cyanobacterium *Scytonema hofmannii* and which inhibited the growth of a range of algae (Mason et al. 1982; Pignatello et al. 1983; Gleason and Baxa 1986). Cyanobacterin acts by damaging thylakoid membranes and inhibiting electron transport in photosystem II (Gleason and Paulson 1984; Gleason 1990). Based on the observation that *Anabaena* spp. were inhibited by extracellular compound(s) of the cyanobacterium *Hapalosiphon*, Moore et al. (1984) isolated the allelochemical hapaloindole A (Fig. 4) from a terrestrial *Hapalosiphon fontinalis*. Another member of the hapalindole family of compounds, 12-*epi*-hapaloindole E isonitrile (Fig. 4) which also shows allelopathic activity against cyanobacteria and green algae has been isolated from *Fischerella muscicola* (Schwartz et al. 1987; Schlegel et al. 1998; Doan et al. 2000). This compound has been shown to interact directly with RNA polymerase, preventing RNA elongation in *Bacillus subtilis* (Doan et al. 2001). The related metabolite 12-*epi*-hapaloindole F isothianate (Fig. 4) inhibits the growth of cyanobacteria of the genera *Microcystis* and *Synechococcus* (Etchegaray et al. 2004).

Screening of cell-free filtrates and cyanobacteria cells for their ability to clear lawns of cyanobacteria on solid media found that the cyanobacterium *Fischerella muscicola* UTEX1829 was active towards all 30 strains tested (Flores and Wolk 1986). This led to the isolation of fischerellin A (Fig. 4) which is a potent photosystem II inhibitor (Gross et al. 1991; Hagmann and Jüttner 1996; Srivastava et al.

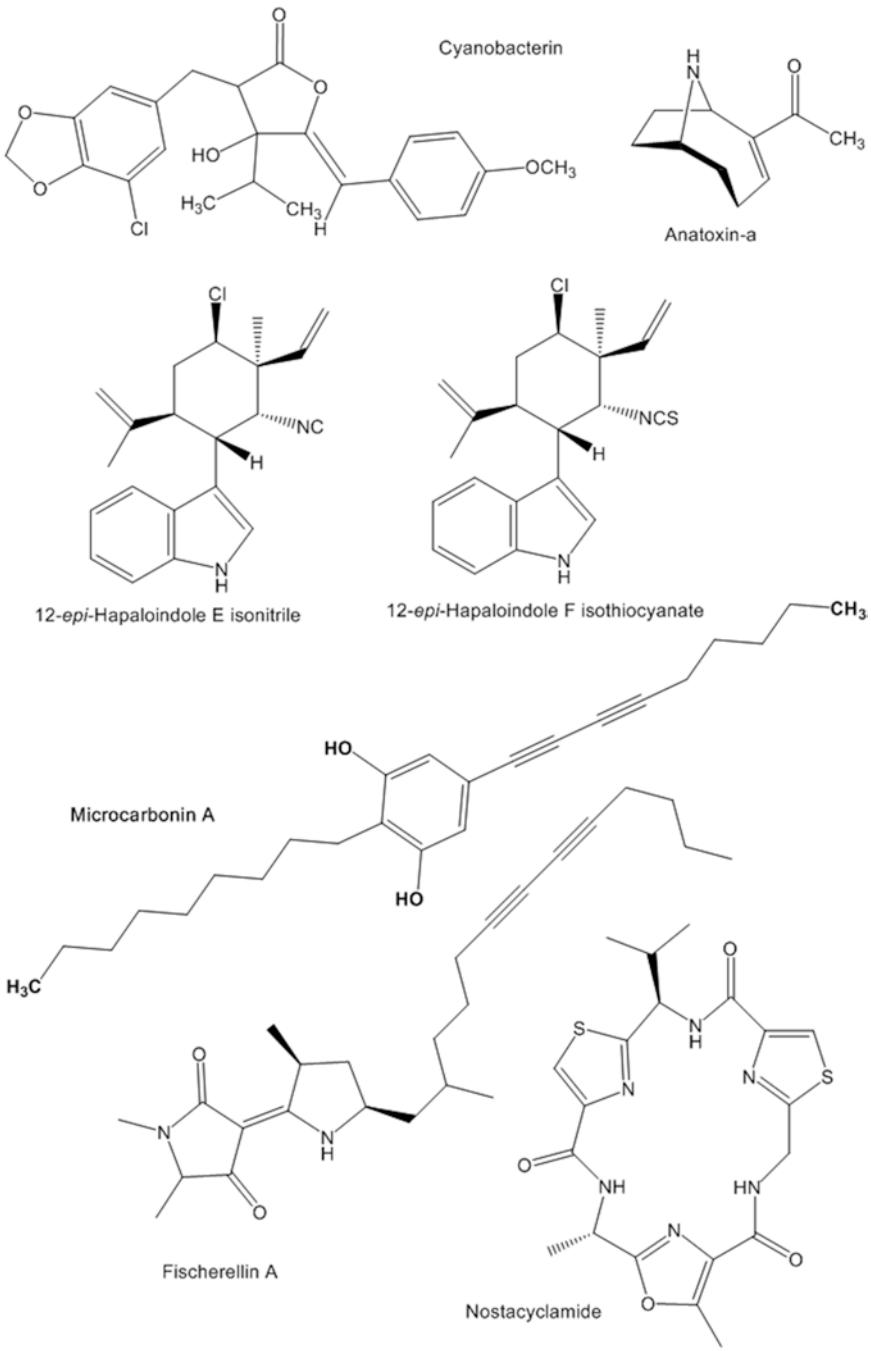
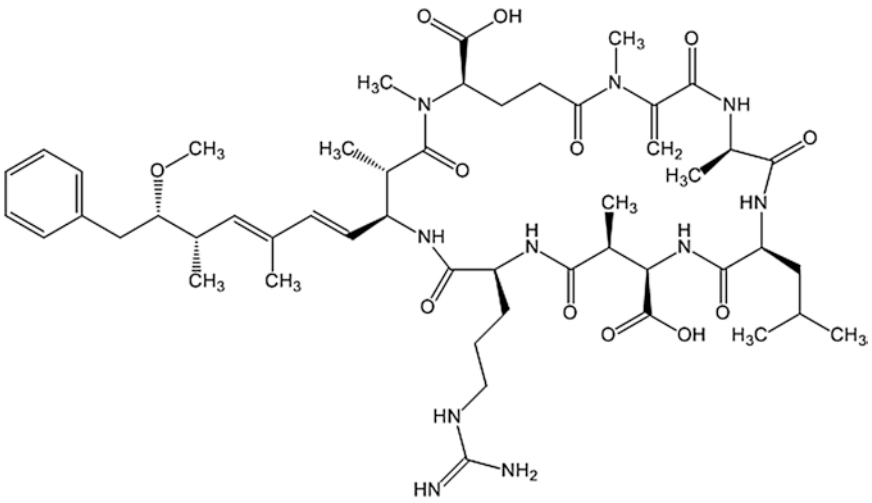


Fig. 4 Structures of allelochemical compounds produced by cyanobacteria

1998). Fischerellin A is active against cyanobacteria, green microalgae and higher plants, but does not inhibit photosynthesis in the purple bacterium *Rhodospirillum rubrum* (Srivastava et al. 1998) nor the growth of heterotrophic bacteria (Gross et al. 1991). The same screening study also found that the cyanobacterium *Nostoc* sp. 31 inhibited 29 of the 30 cyanobacterial strains. Using a bioassay guided isolation procedure it was found that the cyclic peptide nostocyclamide (Fig. 4) was responsible for the majority of the inhibitory activity (Todorova and Jüttner 1995). Further bioassays showed that nostocyclamide inhibited the growth of cyanobacteria, green algae and diatoms (Todorova and Jüttner 1996).

Kearns and Hunter (2001) found that the toxins anatoxin-a (100 ng mL^{-1}) (Fig. 4) and microcystin-LR (10 ng mL^{-1}) (Fig. 5) which are produced by the cyanobacterium *Anabaena flos-aquae*, paralyzed the green alga *C. reinhardtii*, leading to settling of this alga. A similar effect was observed when

Fig. 5 Microcystin-LR



these two algae were co-cultured. The action of these toxins is unknown, but anatoxin-a activates calcium ion channels in mammals (Ruge Holte et al. 1998) causing cellular depolarisation. Since intracellular calcium concentrations regulate flagellar activity and sensory transduction from the photoreceptors to flagella in *C. reinhardtii* (Harz and Hegemann 1991; Yoshimura et al. 1997), anatoxin-a may inhibit C. *reinhardtii* motility by affecting the calcium ion channels. On the other hand, microcystin-LR is a potent inhibitor of specific phosphatases (Dawson 1998) and microcystin-sensitive phosphatases have been shown to be important for flagellar movement in *Chlamydomonas* (Yang et al. 2000). Using microplate assays, Babica et al. (2007) investigated the effects microcystin-LR and -RR in the concentration range 1–25,000 μg L^{-1} on growth of five green algae (*C. reinhardtii*, *Parachlorella* (*Chlorella*) *kesslerii*, *Pediastrum duplex*, *Raphidocelis* (*Pseudokirchneriella*) *subcapitata*, *Scenedesmus communis* (as *S. quadricauda*)) and the cyanobacterium *Microcystis aeruginosa*. They found different susceptibility of these planktonic organisms to the microcystins; in some species (*C. reinhardtii*, *P. kesslerii*, *P. duplex*, *M. aeruginosa*), microcystin-RR induced more pronounced effects on growth than microcystin-LR. However, environmentally relevant concentrations of microcystins (1–10 μg L^{-1}) did not cause significant changes in the growth of the target species. Growth of *P. subcapitata* was strongly inhibited only at concentrations of microcystin-LR or -RR ≥1000 μg L^{-1} after 4 days of exposure, whereas *S. communis* was affected only at the very high concentration of 25,000 μg L^{-1}. Microcystin-LR isolated from *Nostoc* sp. BHU001 was found to be a general growth inhibitor active at nanomolar range (25–100 μg L^{-1}) of five closely related cyanobacteria (*Nostoc muscorum*, *Desmonostoc* (*Nostoc*) *commune*, *Trichormus fertilissimus* (as *Anabaena fertilissima*), *Trichormus* (*Anabaena*) *doliolum*, and *Cylinderospermum majus*) isolated from different habitats (Bajpai et al. 2013). Microcystin-LR affected a number of metabolic processes such as photosynthesis, respiration, and nitrogen fixation. Nitrogenase activity showed maximum sensitivity, followed by respiration, photosynthesis, and general growth. The photosynthetic electron transport activity was maximally inhibited at PSI, followed by whole chain and PSII activities. It has also been found that the growth of *Microcystis wesenbergii* was inhibited by the addition of cell-free filtrates of *M. aeruginosa* whereas *M. aeruginosa* was promoted by the addition of cell-free filtrates of *M. wesenbergii*, but the active compounds have not been identified (Yang et al. 2014). Semipurified *Microcystis* extract containing microcystins affected age-induced cell differentiation in the filamentous cyanobacterium *Trichormus variabilis* (Bártová et al. 2011); heterocyst and akinete formation was significantly decreased after exposure to an extract containing 2 or 20 nM of microcystins within 10 days of exposure. About 89 isoforms of microcystin have been described (Welker and Von Döhren 2006), however little is known how the structural differences might affect their function as allelochemicals.

The question remains whether these toxins are released by the living cells or whether they only enter the water on cell death or other processes leading to cell lysis. Rapala et al. (1993) have shown that anatoxin-a was released to the medium by living cells of *Aphanizomenon flos-aquae*, but not by cells of *Anabaena*. On the other hand, several studies have reported that *Microcystis aeruginosa* only releases microcystin on cell death, especially in older cultures (Orr and Jones 1998; Rohrlack and Hyenstrand 2007), but recently Cordiero-Araújo and Bittecourt-Oliveira (2013) have demonstrated active release of microcystins under the control of an endogenous rhythm in *M. aeruginosa*. This release is probably via an ABC-type transporter under the control of the mcyH gene (Pearson et al. 2004).

The cell-free filtered growth medium of the freshwater cyanobacterium *Oscillatoria* sp. LEGE05292 shows allelo-

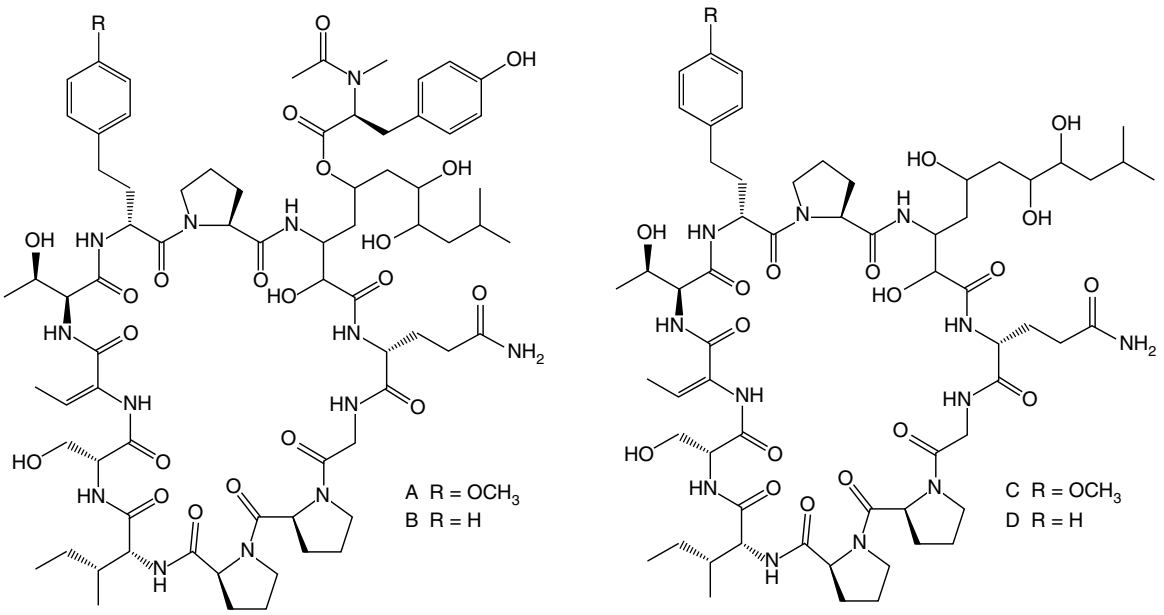


Fig. 6 Portoamides A, B, C and D

pathic activity against eukaryotic microalgae (Leão et al. 2009a; Antunes et al. 2012). The effect is cytostatic as the exposed microalgae recovered and grew normally when placed in regular growth medium (Leão et al. 2009a). Bioassay guided isolation let to the purification and characterisation of large cyclic depsipeptides containing several modified amino acids, including a β-amino acid and an *N*-Ac-*N*-Me-Tyr moiety (Leão et al. 2010). These compounds have been called portoamides. Portoamide A and B (Fig. 6) are most abundant in the biomass, whereas the derivatives without the doubly modified Tyr (Portoamides C and D, Fig. 6) were the major components in the culture medium. A mixture of Portoamide A and B was active against *C. vulgaris* completely inhibiting its growth at 30 μg mL^{-1} with an IC_{50} of 12.8 μg mL^{-1}, unlike a mixture of portoamides C and D, or the individual pure Portoamide A or B (Leão et al. 2010). A mixture of portoamides A and B also inhibited the growth of the green alga *Ankistrodesmus falcatus* and the cyanobacterium *Cylindrospermopsis raciborskii*, but not the diatom *Cyclotella menenghiniana* and the cyanobacteria *Anabaena* sp., *Aphanizomenon* sp. and *Microcystis aeruginosa*. Addition of an extract of the medium from *Oscillatoria* sp. LEGE05292 also caused changes to a mixed phytoplankton community dominated by *M. aeruginosa* over 15 days (Leão et al. 2012b). Interestingly, different genotypes of *M. aeruginosa* were differently affected. Higher temperature, higher irradiance and P-limitation also were all found to enhance the allelopathic activity against the green alga *A. falcatus* (Antunes et al. 2012). More study of the contribution of the portoamides to the allelopathic activity of this alga and their action clearly is required.

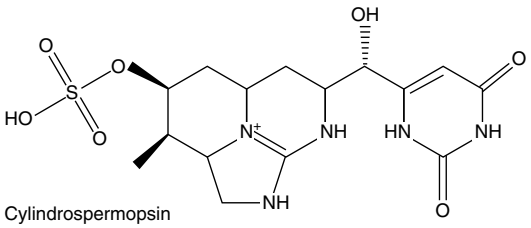


Fig. 7 Structure of cylindrospermopsin

The cyanobacterial alkaloid cylindrospermopsin (Fig. 7) is produced by at least 11 species of cyanobacteria including *Cylindrospermopsis raciborskii* and *Aphanezomenon ovalisporum*. Bar-Yosef et al. (2010) showed that *A. ovalisporum* and cylindrospermopsin induced extracellular alkaline phosphatase activity in other phytoplankton cells including *C. reinhardtii* and *Debrya* sp. The laboratory studies were supported by enzyme-labelled fluorescence of ALP studies in the field where alkaline phosphatase activity was promoted in other phytoplankton but not in *A. ovalisporum*. The authors argued that by 'enslaving' other algae to increase alkaline phosphatase activity more P was made available for growth of the N-fixing *A. ovalisporum*. Recently, Rzymski et al. (2014) found that cylindrospermopsin at 1–5 μg L^{-1} significantly up-regulated alkaline phosphatase activity in *M. aeruginosa* without having any marked effect on growth. Higher concentrations (10 and 50 μg L^{-1}) caused significant growth inhibition and cell necrosis. At all the concentrations of cylindrospermopsin tested, the production of microcystin-LR by *M. aeruginosa* was strongly inhibited. Spent growth medium of *C. raciborskii*

also inhibited *M. aeruginosa* growth and microcystin-LR production.

Mello et al. (2012), using a *C. raciborskii* strain (strain CYRF) that does not produce cylindrospermopsins but saxitoxins, found that this strain also inhibited the growth of *M. aeruginosa* and induced colony formation. The saxitoxin may not be responsible for the effect as Perreault et al. (2011) have reported that saxitoxin did not induce any toxic effect on *C. reinhardtii*. At this time it is not known whether *M. aeruginosa* responds to saxitoxin or not, or whether other, as yet unknown, allelochemicals are produced by *C. raciborskii*.

The allelopathic relationships between species can be complex and there may be reciprocal, density-dependent inhibition of growth. For example, *Microcystis* sp. inhibits photosynthesis in the dinoflagellate *Peridinium gatunense* by inhibiting intracellular carbonic anhydrase activity (Sukenik et al. 2002). The allelochemical responsible for this inhibition has been identified as microcarbonin A (Fig. 4) (Beresovsky et al. 2006). However, the effect of *Microcystis* sp. on *P. gatunense* depends on the concentration of *P. gatunense* (Vardi et al. 2002). When the initial *P. gatunense* inoculum was 200 cells mL^{-1} its growth was completely inhibited by *Microcystis* sp. MG. When the inoculum density was 560 cells mL^{-1}, growth was inhibited by 60 % and, when the initial density was 2300 cells mL^{-1} growth was little affected by *Microcystis*. Conversely, the growth of *Microcystis* sp. was hardly affected by the presence of *P. gatunense* as long as the initial inoculum of *P. gatunense* was less than 1000 cells mL^{-1}. At higher cell densities of *P. gatunense* growth of *Microcystis* sp. was severely depressed, even in the presence of an adequate nutrient supply. Spent *P. gatunense* medium induced sedimentation of *Microcystis* cells and subsequent lysis after 24 h. Interestingly, there was a concurrent large rise in the level of McyB which is involved in toxin biosynthesis in *Microcystis* (Dittmann et al. 2013). Conversely, *Microcystis*, probably through the action of microcystin-LR, elicited a biphasic oxidative burst and the activation of certain protein kinases in *P. gatunense*.

Cross-talk between different algae has also been observed by Bittencourt-Oliveira et al. (2015) in mixed culture experiments. Both the microcystin-producing *M. aeruginosa* and the non-microcystin-producing *Microcystis panniformis* significantly inhibited the growth of the green algae *Monoraphidium convolutum* and *Scenedesmus acuminatus*, with *M. convolutum* being most affected. *S. acuminatus*, in turn, was able to inhibit the growth of both cyanobacteria. In the presence of the green algae *M. aeruginosa* also increased its microcystin production. Extracts of the cyanobacteria had no significant inhibitory effect on the green algae, whereas extracts of the green algae significantly inhibited the growth of *M. aeruginosa*. It was suggested that the absence of an inhibitory effect of the cyanobacterial extracts was because the algae had been grown in monoculture where production of allelochemicals was not induced. Mello et al. (2012) found that growth inhibition of *M. aeruginosa* was only observed when it was exposed to exudates from a mixed culture of *M. aeruginosa* and a high proportion of *C. raciborskii*. Exudates of monocultures had no effect.

4.3 Diatoms

The diatoms are amongst the best studied microalgae with respect to chemically-mediated interactions and provide an excellent example of the nature and complexity of these interactions and of how laboratory studies and field studies can be used to try and understand these. Diatoms have been shown to produce allelopathic compounds in a number of field and laboratory studies (Sharp et al. 1979; Tamoko et al. 2003; Yamasaki et al. 2007, 2010) and polyunsaturated fatty acids (PUFAs) and polyunsaturated aldehydes (PUAs, Fig. 8) have been implicated as the possible allelopathic compounds, although other allelopathic compounds also are produced (Yamasaki et al. 2012). A likely allelopathic role of the PUAs has been supported by the observation of Vidoudez and Pohnert (2008) who described the release of PUAs (octadienal and heptadienal) from intact cells of *Skeletonema marinoi* over a short period at the end of the exponential growth phase just before the culture enters stationary phase, whereas it appears that the PUFAs are only released into the medium as a result of cell lysis (Jüttner 2001, 2005). Ribalet et al. (2007b) have also reported that *S. marinoi* produces PUAs upon cell disruption during the exponential, stationary and declining phase of the culture, with a maximum wound-activated PUA production per cell in the early stationary phase. The pathways of the synthesis of PUAs from chloroplast galactolipid-derived C16:n-4 fatty acid and phospholipid-derived C20:5n-3 fatty acid is shown in Fig. 8.

PUAs have been shown to have negative effects on many organisms including diatoms. For example, 2*E*,4*E/Z*-decadienal is an effective inhibitor of diatoms (Casotti et al. 2005; Hansen and Eilertsen 2007), and 2*E*,4*E/Z*-decadienal, octadienal and heptadienal also inhibit other phytoplankton taxa (Ribalet et al. 2007a). Ribalet et al. (2007a) found that the effective inhibitory concentration for 2*E*,4*E/Z*-decadienal ranged from 0.15 to 0.377 μg mL^{-1}. These concentrations are much higher than those detected for heptadienal and octadienal by Vidoudez and Pohnert (2008) in cultures of *Skeletonema marinoi*. 2*E*,4*E*-decadienal also inhibits benthic diatom adhesion at concentrations of 2.5 μg mL^{-1} (Leflaive and Ten-Hage 2011).

In assays with oyster haemocytes, 2*E*,4*E*-decadienal exhibited a dose-dependent inhibition of cytoskeleton organisation, rate of phagocytosis and oxidative burst, and a dose-dependent promotion of apoptosis (Adolph et al. 2004). The effective photochemical efficiency F_v/F_m of diatoms also is reduced by this metabolite (Leflaive and Ten-Hage 2011).

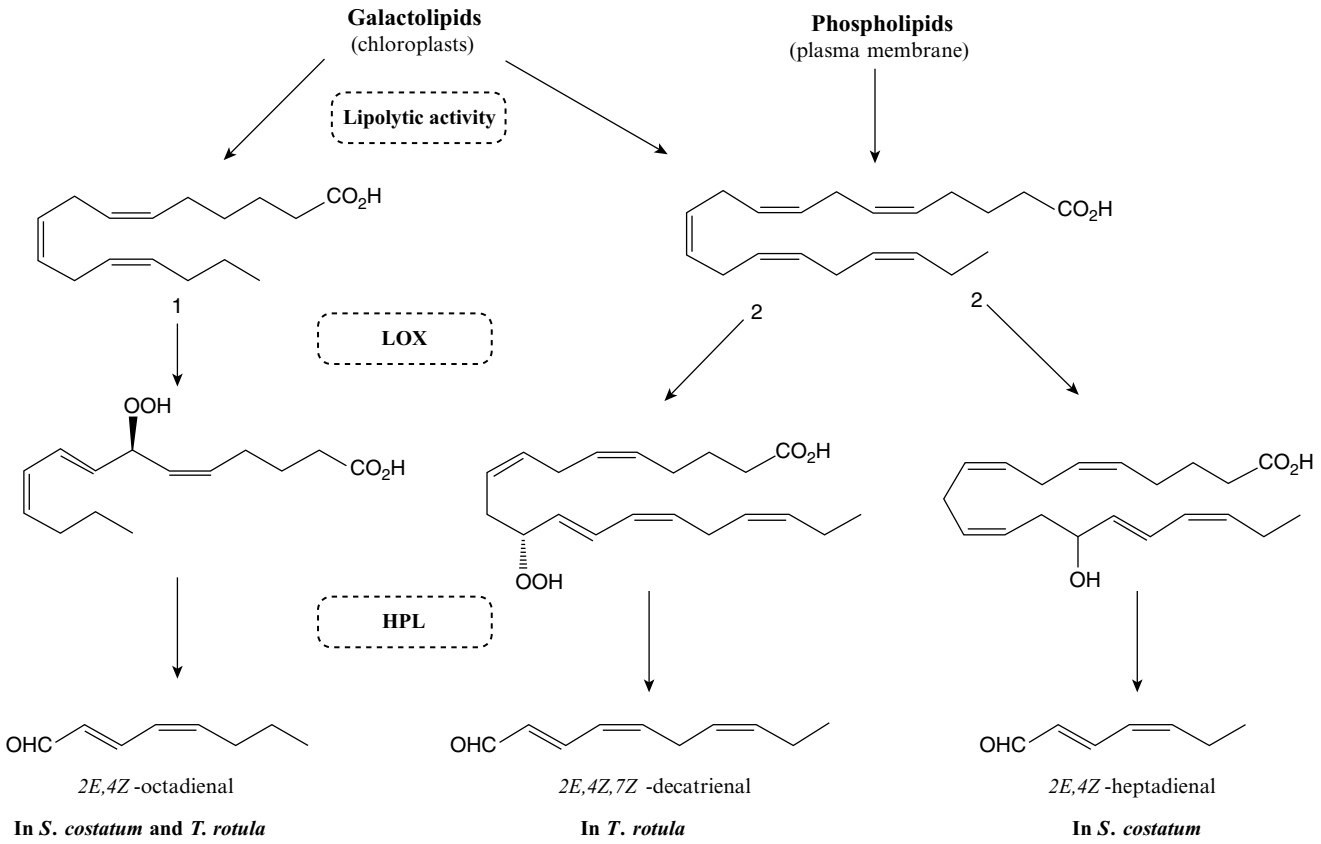


Fig. 8 Pathways of synthesis of PUAs from chloroplast galactolipid-derived 6,9,12-hexadecatrieonic acid (C16:3n-4) (*1*) and phospholipid-derived eicosapentaenoic acid (C20:5n-3) (*2*) in the diatoms *Skeletonema costatum* and *Thalassiosira rotula* (current name = *Thalassiosira gravida*). *LOX* lipoxygenase, *HPL* hydroperoxide lyase (Redrawn from Fontana et al. (2007))

On the other hand, exposure to 2*E*,4*E*/*Z*-octadienal did not affect the F_v/F_m of *S. marinoi* (Gallina et al. 2014).

When the diatom *S. marinoi* (a PUA-producing species) was exposed to either 2*E*,4*E*/*Z* -decadienal, 2*E*,4*E*/*Z*-octadienal, 2*E*,4*E*/*Z* -heptadienal, or a mix of the latter two compounds a reduction in NO was observed in proportion to the concentration of the PUA concentration (Gallina et al. 2014). However, only 2*E*,4*E*/*Z* -octadienal, 2*E*,4*E*/*Z* -heptadienal, or their mixture also induced a parallel increase of reactive oxygen species (ROS). Interestingly, the diatom *P. tricornutum* (a non-PUA-producing species) produced NO in response to 2*E*,4*E*/*Z* -decadienal but not to 2*E*,4*E*/*Z* -octadienal. Gallina et al. (2014) suggest that *S. marinoi* perceives 2*E*,4*E*/*Z* -octadienal and 2*E*,4*E*/*Z* -heptadienal as intra-population infochemicals, whereas *P. tricornutum* perceives them as allelochemicals, indicating multiple ecological functions of these compounds.

4.4 Haptophyta

The most widely studied haptophyte alga with respect to allelopathy is *Prymnesium parvum* because of the common and widespread blooms formed by this alga and the ichthyotoxic effects of these blooms causing severe damage to marine ecosystems and fish farming (Guo et al. 1996; Edvardsen and Paasche 1998; Johnsen et al. 2010). The extracellular toxin of this alga has strong haemolytic activity (Yariv and Hestrin 1961). Compounds released by *P. parvum* have also been shown to have allelopathic effects, usually causing cell lysis, on a wide range of phytoplankton species including cyanobacteria, dinoflagellates, cryptophytes and diatoms, but not on the closely related *Prymnesium parvum* cf. *patelliferum* (Fistarol et al. 2003; Granéli and Johansson 2003). However, Olli and Trunov (2007) showed that cell-free filtrate of *P. parvum* can inhibit the growth and even lyse the cells of the same strain, unless the strain has been previously adapted to gradually increasing toxin levels in the environment. The mechanism of adaptation to the allelochemical(s) is not known.

Prymnesium is not only a mixotroph, but also feeds by phagocytosis. Tillman (1998) proposed that the toxins produced by this alga also serve to immobilise and kill potential food algae allowing the *Prymnesium* to capture and phagocytose this prey. Indeed, several studies have now shown that the medium of *P. parvum* cultures inhibits the swimming of flagellate algae before cell lysis and allows capture and

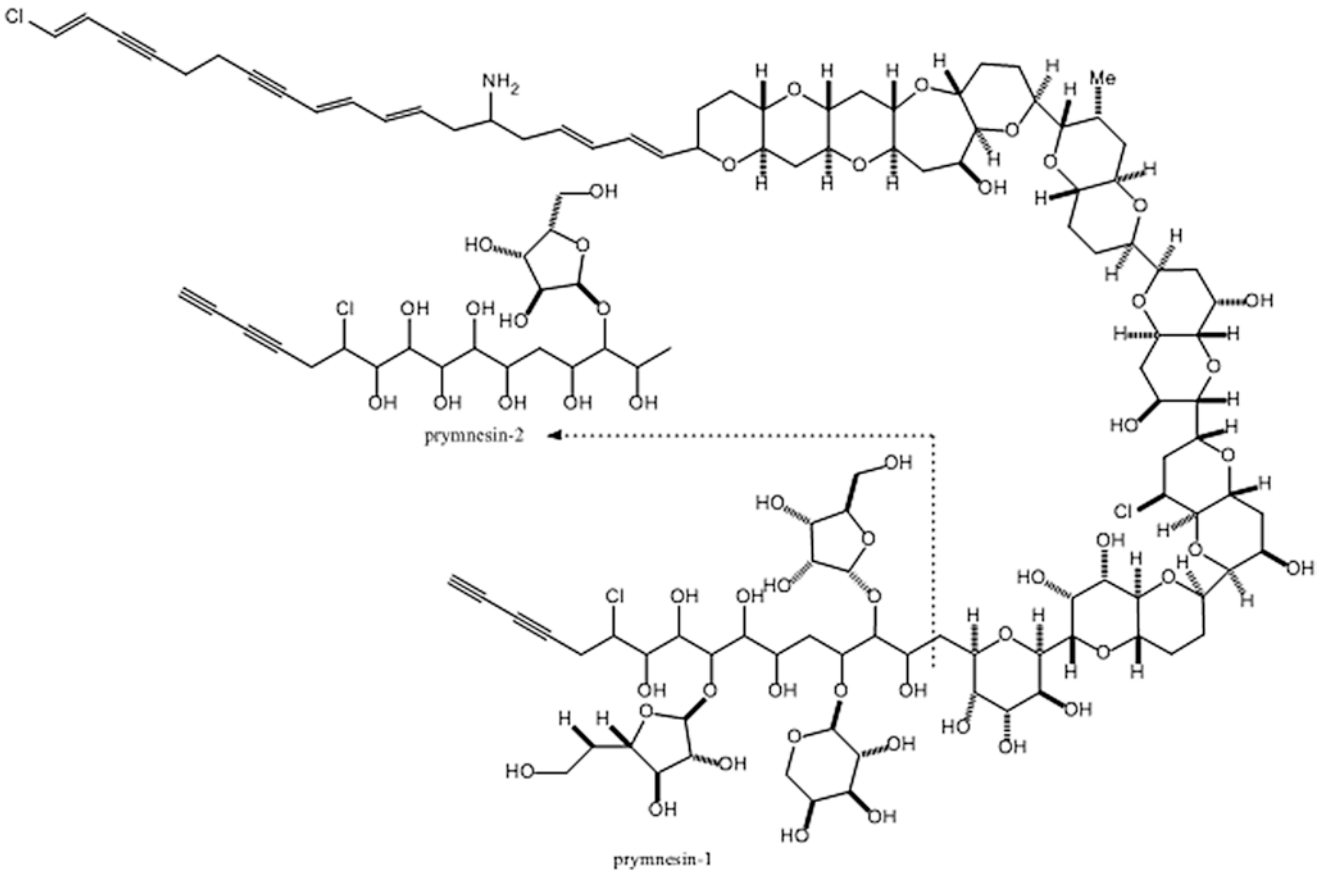


Fig. 9 Prymnesin 1 and 2

ingestion of these algae by *Prymnesium* (Skovgaard and Hansen 2003; Tillmann 2003).

The toxins produced by *P. parvum* have been termed the prymnesins (Manning and La Claire 2010). Although two active compounds, prymnesin-1 and prymnesin 2 (Fig. 9), have been isolated from whole cells and their structure determined (Igarashi et al. 1998, 1999), the actual number of different substances that comprise the 'prymnesins' is not known, but given the wide range of biological activities of extracts of cells and cell-free culture medium, the prymnesins are likely to represent a complex and diverse mixture of toxic metabolites (Manning and La Claire 2010). Prymnesin-1 and prymnesin-2 are ladder-like polycyclic ethers that possess several interesting features (Fig. 9). These complex compounds are remarkable in that they have double and triple carbon-carbon bonds in the unsaturated head and tail regions, an amino group, several chlorines, four 1,6-dioxadecalin units, and an assortment of sugar moieties. They have been shown to have both ichthyotoxic and haemolytic activities and these toxins are assumed to be the same as the allelopathic substances based on an apparent similar mode of action (Granéli and Salomon 2010). The mode of action of the prymnesins on algae is still only partially understood and most attention has been placed on the ichthyotoxic and haemolytic activities. The current knowledge is summarised and discussed in the review by Manning and La Claire (2010). Clearly, as the main effect on other algae is cell lysis, the haemolytic activity is of most interest within the context of this chapter.

The production of the prymnesins by *Prymnesium* is influenced by a number of environmental factors as is the release of these to the external environment. Release may be by secretion or excretion from the cell or by cell lysis (Yariv and Hestrin 1961) and to date no studies have attempted to differentiate between these modes of release. The amount of toxins and/or allelopathic compounds produced by *P. parvum* appears to be strain specific (Larsen and Bryant 1998), but is also influenced by environmental factors (Granéli and Flynn 2006). The haemolytic activity of *P. parvum* toward co-occurring algae is lowest in the exponential phase and highest in the stationary phase (Shilo 1967; Johansson and Granéli 1999; Granéli and Johansson 2003). The impact of the allelopathic compounds on the target species is also affected by cell density, with higher cell numbers of the allelopathic species having greater detrimental effects. The cell-free medium of *P. parvum* grown under N and P limited conditions shows enhanced toxicity and an enhanced allelopathic effect on phytoplankton, whereas nutrient replete cells

show little allelopathic activity (Granéli and Johansson 2003). Uronen et al. (2005) observed a similar effect using dilute co-cultures of *P. parvum* with *Rhodomonas salina* as the target species. Interestingly, nutrient deficient cells of the diatom *Conticribra* (*Thalassiosira*) *weissflogii* were more sensitive to cell-free filtrate of *P. parvum* than were cells grown under nutrient replete conditions (Fistarol et al. 2005). Non-optimal salinity and temperatures also enhance the toxicity of *P. parvum* cultures (Baker et al. 2007). Repeated exposure to the cell free filtrate also made the diatom cells more susceptible to the allelopathic compounds.

4.5 Dinoflagellates

Dinoflagellates show allelopathic activities against a wide range of phytoplankton and protozoa. For example, cell-free filtrates of *Alexandrium tamarense* negatively affected the growth of several members of a natural plankton sample (Fistarol et al. 2004b). Microscopic examination showed that the filtrate caused cell blistering and lysis in all phytoplankton groups and species as well as an increase in empty diatom frustules. Tests on single species (*Conticribra weissflogii* and *Rhodomonas* sp.) confirmed the allelopathic effect and also showed that the effect was independent of whether or not the *Alexandrium* strain produced PSP toxins, indicating that these were not the allelopathic agents. The possibility of the observed effect being due to the increased bacteria in the culture was also examined and excluded. Cell-free filtrates of the dinoflagellates *Alexandrium tamarense* and *Karenia mikimotoi* not only cause cell death but also induce cyst formation in the dinoflagellate *Scrippsiella trochoidea* (Fistarol et al. 2004a).

The outcome of co-cultivation experiments can be influenced by the relative concentration of the species involved and also by the growth stage. When studying the growth interactions between *Scrippsiella faeroensis* (=*Scrippsiella trochoidea*), *Prorocentrum micans* and *Gymnodinium splendens* (=*Akashiwo sanguinea*), Kayser (1979) found that in mixed batch cultures, growth depended strongly on the ratio between cell numbers of the two species inoculated at the start of the experiments. When two species were inoculated at different relative cell densities in pair-wise co-cultures, the more abundant species prevailed and suppressed the competitor, even during exponential growth. Growth inhibition was most apparent in older cultures.

The allelopathic impact of dinoflagellates on phytoplankton communities can be complex. For example, Weissbach et al. (2010) studied the impact of two strains of *A. tamarense*, one with lytic activity and one without, on a *Phaeocystis globosa* dominated phytoplankton community from the North Sea. The cell-free medium of the lytic strain suppressed phytoplankton growth whereas the medium from the non-lytic strain had a positive impact on diatoms. *Phaeocystis globosa* which was present as single cells in the initial community increased in abundance and formed colonies in all treatments. However, the total abundance and number of colonies was reduced with addition of cell-free medium from the lytic *A. tamarense*; addition of non-lytic *Alexandrium* medium affected the shape of the *Phaeocystis* colonies but not their abundance. Allelopathic compounds from *A. tamarense* have been characterized, but as yet their structure has not been elucidated. Ma et al. (2009) confirmed that these compounds were unrelated to the known toxins of this genus – the paralytic shellfish toxins (Tillmann and John 2002) and the spirolides (Tillmann et al. 2007). Bioassay-guided fractionation using the cryptophyte *Rhodomonas salina* as the target species and mass spectroscopic characterisation has shown them to be a suite of amphipathic high molecular weight (7–15 kDa) compounds (Ma et al. 2011b). Treatment with trypsin had no effect on the lytic properties of these compounds suggesting that the active part(s) are not proteinaceous, and other tests suggest they may contain polysaccharides. They seem to target steroids in the cell membrane as liposomes containing cholesterol were lysed to a greater extent than liposomes without cholesterol (Ma et al. 2011a). The compounds also do not appear to interact with Ca^{2+} channels in the membrane.

Not all species of *Alexandrium* cause cell lysis in the target species. Using flow cytometry and PAM fluorometry, Lelong et al. (2011) showed that cells of the diatom *Chaetoceros neogracile* exposed to *Alexandrium minutum* (either in co-cultures or as cell-free medium) showed reduced red chlorophyll fluorescence, cell size and cell complexity, as well as having fewer active photosynthetic reaction centers and sharply decreased photosynthetic efficiency (F_v/F_m), however no cell lysis was observed. Unlike the rather stable allelopathic compound(s) of *A. tamarense* (Ma et al. 2011b), the majority of the allelopathic compounds from *A. minutum* remained active for less than 9 h. The allelopathic compounds have not yet been characterised.

When co-cultured, *Heterosigma akashiwo* reduced the growth of *Akashiwo sanguinea* (Qiu et al. 2012). Culture filtrates of *H. akashiwo* also reduced the total cell yield and growth rates of *A. sanguinea*, whereas filtrates of *A. sanguinea* only reduced the final cell yield of *H. akashiwo* without affecting the growth rate. Yamasaki and coworkers (2009) have isolated allelopathic polysaccharide-protein complexes (APPCs) from *H. akashiwo* which inhibit the growth of *A. sanguinea* (Qiu et al. 2012). Ecologically realistic concentrations of APPCs (~40 μg mL^{-1}) did not produce any morphological changes in *A. sanguinea*, but at the very high concentration of 100 μg mL^{-1} the *A. sanguinea* cells became smooth and flattened. When these two species were

co-cultured, marked morphological changes and eventual cell lysis were observed in *A. sanguinea*, however when co-cultured but separated by a 0.3 µm pore size membrane there was no effect. It is hypothesised that the APPCs are located on the cell surface of *H. akashiwo* and that cell contact is required for them to induce cell lysis in other algae. Similar surface mediated chemistry to lyse cells or inhibit competitor growth has been proposed for the allelopathic lytic effect of *Heterocapsa circularisquama* on *Prorocentrum dentatum* in co-culture (Yamasaki et al. 2011).

The allelopathic activity of dinoflagellates of the same species is variable. For example, of the 16 lipophilic extracts from the medium of separate exponential phase cultures of the same strain of *Karenia brevis*, only 7 were allelopathic to the diatom *Asterionellopsis gracialis* (Prince et al. 2008). The allelopathic effect of cultures may vary because of nutrient limitation, associated bacteria, lack of selection pressure allowing mutations to accumulate, growth stage and pH (e.g., Schmidt and Hansen 2001; Granéli and Salomon 2010). Kubanek et al. (2005) found some variation in the allelopathic effects of *K. brevis* between strains and growth stages. The allelopathic effects are not due to brevetoxin, but other as yet identified compounds (Kubanek et al. 2005; Poulson et al. 2010; Prince et al. 2010). HPLC fractionation of the organic compounds from the medium showed that multiple chemical compounds produced by *K. brevis* inhibited the growth of four phytoplankton competitors, and that these competitors were susceptible to different combinations of compounds (Poulson et al. 2010). The active compounds were polar, neutral or positively charged and had molecular weights between 500 and 1000 Da and possessed aromatic functional groups (Prince et al. 2010). The relative production of the various multiple allelopathic compounds may vary with strain, growth conditions and growth stage and this may account for the variability observed in different studies. Strain variation in allelopathic activity has also been found in *Alexandrium tamarense* (Tillmann et al. 2009), *Alexandrium fundyense* (Hattenrath-Lehmann and Gobler 2011) and *Alexandrium ostenfeldii* (Hakanen et al. 2014). Furthermore, different species of phytoplankton respond variably to *K. brevis* and some show resistance to the allelopathic effects of this alga (Poulson-Ellestad et al. 2014a).

4.6 Chlorophyta

Based on observations that the cell-free medium of axenic cultures of the green alga *Scenedesmus* sp. (strain *huji*) severely inhibited the growth of the cyanobacterium *Microcystis aeruginosa* PCC7005 and other *Microcystis* strains, Harel et al. (2013) isolated weakly ionic allelochemicals with a molecular size of <10 kDa from the *Scenedesmus* medium and concentrated the extract 20,000-fold. Exposing *Microcystis* to 10–20 % of this active material led to a significant decline in the photosynthetic quantum yield within 20 min and to complete lysis of the cells within 24 h. Within 3 h there was also a marked increase in phycobilin leakage and leakage of various ions, especially iron. The authors concluded that the addition of the active material altered the functionality and possibly the integrity of the cell membrane of *Microcystis* leading to the leakage of the ions. It also appears that the thylakoids were affected as indicated by the rapid effect on variable fluorescence. Cell-free medium of the green alga *Scenedesmus obliquus* also inhibits the growth of *Microcystis aeruginosa* (Chen and Guo 2013).

Since the initial observation by Pratt and Fong (1940) that *Chlorella* produces an inhibitory substance, later named chlorellin (Pratt et al. 1944), and which has been shown to consist of a mixture of fatty acids and hydrocarbons (Spoehr et al. 1949), there have been several reports that free fatty acids (FFAs) apparently released by green algae can be both autoinhibitory and inhibit the growth of other algae (Proctor 1957; McGrattan et al. 1976; McCracken et al. 1980; Arzul et al. 1995; Ikawa et al. 1997; Chiang et al. 2004; Wu et al. 2006; Fergola et al. 2007; DellaGreca et al. 2010). Free fatty acids have also been shown to have wide antibacterial and other bioactivities (Desbois and Smith 2010). It is not clear whether the fatty acids are released by living cells or are released mainly by cell rupture. However, several studies on a taxonomically diverse range of microalgae algae suggest release from intact living cells (Parrish et al. 1993; Gladyshev et al. 1996; Sushchik et al. 2001, 2003).

Extracts of the benthic/epiphytic green alga *Uronema confervicola* contain putative allelopathic compounds that induce oxidative stress and growth inhibition in the planktonic *Desmodesmus quadrispina* (= *Scenedesmus quadrispina*?) (Leflaive et al. 2008). The culture medium filtrate of *U. confervicolum* has no effect on growth, but does induce the formation of four- and eight-celled coenobia in *D. quadrispina*. In the agar diffusion test both *U. confervicolium* and *D. quadrispina* inhibited growth of the cyanobacterium *Anabaena* PCC 7120.

5 Mechanisms of Allelopathic Action and the Production of Allelochemicals

For allelochemicals to be able to affect the target species they must be released from the cell. Release can be either by diffusion out of the cell and across the plasma membrane, active transport out of the cell, or by cell lysis. Differentiating between these mechanisms can be very difficult and, unfortunately, in most studies where allelochemical activity and/or allelochemical(s) have been detected it is not known which of these mechanisms is operational. Using filtration to prepare a cell-free medium supernatant can result is some

cell breakage and leakage of cell contents in many relatively fragile cell types such as the dinoflagellates, even with gentle filtration. Culturing the host and target strains separated by a fine screen or permeable membrane which allows exchange of soluble compounds is a better experimental system for the detection of allelopathic activity which reduces the likelihood of cell breakage which might occur during filtration. However, cultures under nutrient and environmental stress (as occurs in laboratory cultures in stationary phase for example) have been shown in several studies to undergo programmed cell death (apoptosis) (e.g., Brussaard et al. 1997; Vardi et al. 1999; Peperzak et al. 2000; Bidle and Falkowski 2004; Timmermans et al. 2007) and this results in the release of intracellular products. The decline in cell numbers in old cultures and the more spectacular culture 'crashes' sometimes observed all indicate that cells have lysed by one mechanisms or another. Factors such as aeration or other means to 'mix' the cultures can also result in cell damage (Thomas and Gibson 1990; Garcıa Camacho et al. 2000; Moheimani et al. 2011) and there is great variability between species in their sensitivity to such damage.

There have been only a few studies showing the actual excretion of allelochemicals by living cells. However, excretion of organic compounds, especially polysaccharides, by microalgae is extremely widespread and possibly universal (Allen 1956; Lèon and Galván 1994; Romera-Castillo et al. 2010). The secretion of polysaccharides (mucilages) is by the fusion of Golgi-derived mucilage vesicles with the plasma membrane followed by discharge to the cell exterior (Oertel et al. 2004; Weiss et al. 2012). Some algae have also been shown to excrete lipophilic compounds such as hydrocarbons, lipids and free fatty acids (Parrish et al. 1993, 1994; Gladyshev et al. 1996). The best known example of this is the trebouxiophycean green alga, *Botryococcus braunii* (Metzger and Largeau 2005) which excretes significant quantities of lipids and hydrocarbons. The secretion of free fatty acids by microalgae has also been shown by Sushchik et al. (2001, 2003) and others (e.g., Billmire and Aaronson 1976; Parrish et al. 1993). They found that saturated and unsaturated fatty acids are excreted, whereas polyunsaturated fatty acids and not excreted, or only excreted in minor amounts. The mechanism by which these compounds traverse the cell membrane is not known for algae. However, it is probably and active transport process (Abumrad et al. 1998; Benning 2009) and the mechanism may be is similar to the transport of cuticular lipids in the higher plant *Arabidopsis* which involves a group of ATP binding cassette (ABC) transporters (McFarlane et al. 2010).

Once the allelopathic metabolites have entered the water in which the target algae live, they must reach the target cells in sufficient concentrations to elicit an effect. The stability of the metabolites in water is also important as this affects the likely concentrations which may build-up over time.

Microalgae range in cell size from about 1 μm in diameter of the eukaryote *Ostreococcus tauri* (Chrétiennot-Dinet et al. 1995), to over 1000 μm in the centric diatom *Ethmodiscus rex* (Moore and Villareal 1996). Microalgae with a diameter of less than ~100 μm are smaller than the Kolmogorov scale and are surrounded by a static thin layer of fluid known as the diffusive boundary layer. Mixing with the surrounding (bulk) fluid does not occur because turbulence is not sustainable at such small scales (Lazier and Mann 1989). Therefore, transport through this layer is entirely diffusive rather than advective.

In the absence of relative water motion the molecular diffusion of water soluble metabolites from the cell surface of a spherical cell of radius r_0 to the bulk medium can be described by

$$C_r = \frac{Q_D}{4\pi Dr} + C_\infty \tag{1}$$

where Cr is the concentration at distance r from the center of the cell ($r \geq r0$), Q_D is the flux out from the cell surface, D is the diffusion coefficient, and C_∞ is the concentration in the bulk solution (Karp-Boss et al. 1996). Sinking of the cells, swimming or turbulence induced shear mean that the bulk solution moves relative to the algal cell distorting the shape of the concentration field and resulting in steeper concentration gradients in parts (see Fig. 4 in Amin et al. 2012).

The effectiveness of advective mass transport compared to molecular diffusion is described by the dimensionless Péclet number (Pe):

$$Pe_{swim} = \frac{Ur_0}{D} (swimming\ or\ sinking\ motion) \tag{2a}$$

$$Pe_{shear} = \frac{r_0^2}{D} \left(\frac{\epsilon}{2v}\right)^{1/2} (motion\ due\ to\ turbulent\ shear) \tag{2b}$$

where U is the swimming or sinking velocity, ε is the turbulent dissipation rate, and ν is the kinematic viscosity of water (Karp-Boss et al. 1996). Turbulent dissipation rates range from about 10^{-10} m^2 s^{-1} in the calm deep ocean to 10^{-4} m^2 s^{-1} in the well mixed surface layer (Brainerd and Gregg 1993). The ratio of release in the presence of flow to release due solely to diffusion is the Sherwood number (Sh):

$$Sh = \frac{Q}{Q_D} \tag{3}$$

where Q is the flux under relative fluid motion and Q_D is the flux under pure molecular diffusion. Thus, under pure diffusion $Sh = 1$. Clift et al. (1978) have suggested the following relationship between the Péclet and the Sherwood numbers for swimming and sinking cells:

$$Sh_{swim} = \frac{1}{2}\left[1 + (1 + 2Pe_{swim})^{\frac{1}{3}}\right] \tag{4}$$

And Karp-Boss et al. (1996) have provided the following relationship for turbulent shear:

$$Sh_{shear} = 1 + 0.34 Pe_{shear}^{1/2} \quad (5)$$

Using these equations, Guasto et al. (2012) calculated that a phytoplankter with a radius of 100 µm, swimming at typical speeds of 50–500 µm s^{-1} ($Pe = 5-50$), obtains an Sh -1 $= 62-183$ % increase in potential uptake of small organic molecules ($D = 10^{-9}$ m^2 s^{-1}). The effect of swimming (or sinking) would be even greater with larger molecules whose diffusivity (D) is $>10^{-9}$ m^2 s^{-1}.

Most studies to date have looked at the effects of fluid motion generated by sinking or swimming mainly for spherical cells; however most microalgae are not spherical, and several form stiff or flexible chains, or have other colonial morphologies. Some of this variation is shape is well illustrated by the range of geometrical models developed by Sun and Liu (2003) for the calculation of algae cell volumes. The non-spherical shapes of cells as well as their mode of swimming affects solute transport in the vicinity of the cells in ways beyond those for non-motile spherical cells. In most cases the non-sphericity and the action of flagella result in a deformed and thinner boundary layer and more rapid exchange of solutes between the cell surface and the bulk medium (Pahlow et al. 1997; Koehl et al. 2003; Musielak et al. 2009; Guasto et al. 2012).

The motion of flagellae can not only partially disrupt boundary layers, but the type of flagellar motion can affect the rate of (advective) transport towards or away from the cell. For example, the biflagellate *Chlamydomonas reinhardtii* swims by switching between two types of stroke patterns – an undulatory stroke and a breaststroke (Tam and Hosoi 2011). In the breaststroke the alga tends to pull the fluid in the front towards its surface thus speeding up the ability to sense any chemical signals in the bulk medium. On the other hand, when swimming using the undulatory stroke tends to push away the fluid in the front thus markedly delaying the transmission of information about the upcoming conditions. It would be interesting to analyse in more detail the effects of the gyratory swimming pattern of dinoflagellates.

Although most studies have focused on nutrient supply to the cells, their findings are equally relevant to the transmission of allelochemicals and similar metabolites from the donor species and the likelihood of sensing of these metabolites by other species.

Jonsson et al. (2009) carried out a metaanalysis of 21 published reports of allelopathic effects of algae on the growth or biomass of target algae species. The experiments included in the analysis were conducted with a total of 16 potentially allelopathic and 29 target species covering several taxonomic groups. The studies were categorized according to the Chl *a* content of the potentially allelopathic species from which the media extracts, cell-free filtrates or supernatants were prepared. Studies were categorized into low (<5 g L^{-1}) or high (≥5 g L^{-1}) Chl *a* content. These classes are typical for coastal waters and many harmful algal blooms. They could only detect statistically significant allelopathic effects in studies using high, but not low, Chl *a* content; i.e. at high cell concentrations typical of well-developed plankton blooms. The also used modeling to consider whether at low cell densities cell-cell interactions could account for allelopathic effects, and concluded that these were unlikely because the spatial dispersion of cells in turbulent flow would make it difficult for an allelopathic cell to receive an exclusive benefit, and a dispersion model showed that dividing cells were rapidly separated constraining clone selection. Their modeling was based on spherical cells, however as shown above, non-spherical cells and swimming can lead to a more rapid increase in the concentration of an allelochemical in the bulk water and can also increase the concentration at the surface of the target cell. Furthermore, phytoplankton including toxic species are known to form what are called 'thin layers' through gyrotactic trapping, convergent swimming and other mechanisms (Durham and Stocker 2012). In these 'thin layers' cell densities are as high as found in bloom conditions where allelopatically active concentrations of allelochemicals may be reached.

5.1 Toxins

The chemical and genetic basis for the synthesis of many of the cyanobacterial toxins, some of which appear to have allelopathic activity, has been extensively studied (Welker and Von Döhren 2006; Neilan et al. 2013). Some of these toxins have been proposed to serve as allelochemicals; however actual proof of this is limited. Their inhibitory effects on microalgae in laboratory studies are often found only at high concentrations which not observed in the environment. In some studies it is also unclear whether confounding factors such as pH and nutrient levels have been controlled for. In several studies algal toxins when tested in pure form had little or no effect on toxic algae (e.g. saxitoxin at 2–128 nM had no effect on *C. reinhardtii* (Perreault et al. 2011)).

The microcystins are potent serine/threonine protein phosphatase inhibitors (MacKintosh et al. 1990). In the field microcystin concentrations are usually below 10 µg L^{-1} (Park et al. 1998; Chorus 2001), however concentrations can be higher following the decline of cyanobacterial blooms. Culture studies have shown that microcystins are release from cells, but only in low concentrations (Rapala et al. 1997; Böttcher et al. 2001). Babica et al. (2006) reviewed the available data on the effects of the cyanobacterial toxins, the microcystins, on terrestrial plants, macrophytes, macroalgae and planktonic algae and concluded that the ability of MCs to act as allelopathic compounds against microalgae seemed

unlikely as they only seem to exert an effect at concentrations very much higher than observed in nature. In a later study Babica et al. (2007) showed that there were large differences between species of microalgae in their sensitivity to microcystin-LR and -RR with growth of *Pseudokirchneriella subcapitata* being affected at concentrations of 100–5000 μg mL^{-1}, whereas concentrations of 25,000 μg L^{-1} and exposure times of 4 days or longer were required to elicit a response in other species. Similarly, (Ma et al. 2015) observed no effect of microcystin LR at 250 and 500 μg L^{-1} on *Aphanizomenon flos-aquae*. Pinheiro et al. (2013) also observed no negative allelopathic effects of microcystin-LR at environmentally relevant concentrations on a number of marine and freshwater phytoplankton species. However, semipurified extracts of *Microcystis* containing microcystins did inhibit heterocyst and akinete formation in *Trichormus variabilis* at concentrations of 2–20 μg L^{-1} (Bártová et al. 2011), but it is unclear whether the effect observed is due to the microcystins or other compound(s) in the extracts. In a few studies lower concentrations of microcystins did result in the increased formation of ROS in the target cells. Exposure of *Peridinium gatunense* to 50 μg L^{-1} microcystin-LR led to increased ROS within 24 h and also affected the activity of protein kinases (Sukenik et al. 2002; Vardi et al. 2002). Exposure to 100 μg L^{-1} of microcystin-RR for 6 days led to growth inhibition and elevated levels of ROS in *Synechococcus elogatus* (Hu et al. 2005). Interestingly microcystin-LR at 0.01–10 μg L^{-1} had no effect on growth but enhanced O_2 uptake in *Euglena gracilis* (Duval et al. 2005). However, whether the microcystins have an allelopathic function remains unclear, but seems unlikely.

The cyanotoxin, cylindrospermopsin enhances alkaline phosphatase activity in *Chlamydomonas reinhardtii*. The response to spent medium from the cylindrospermopsin producer, *Aphanizomenon ovalisporum*, was greater than that observed following application of purified cylindrospermopsin (50 mg L^{-1}), although its concentration in the used media (7–8 mg L^{-1}) was considerably smaller (Bar-Yosef et al. 2010). This suggests a synergistic involvement of other secondary metabolites in the used media. Cylindrospermopsin at the significantly lower concentrations of 1 and 5 μg L^{-1} also caused very rapid up-regulation of alkaline phosphatase activity in *Microcystis aeruginosa*. At higher, but still environmentally relevant concentrations of 10 and 50 μg L^{-1} the cylindrospermopsin caused growth inhibition and cell lysis (Rzymski et al. 2014). Cylindrospermopsin also decreased the production of microcystin-LR in *M. aeruginosa*. Although these studies show the effect of cylindrospermopsin, similar effects on *M. aeruginosa* were observed using the spent medium on non-cylindrospermopsin-producing strains of *Cylindrospermopsis raciborskii* (Mello et al. 2012; Rzymski et al. 2014) indicating that these strains are able to produce different extracellular compounds with a similar allelochemical mode of action. Similar to microcystin-LR, cylindrospermopsin at 0.13–12.5 μg mL^{-1} increased the productivity and the O_2 uptake rate of *Euglena gracilis*. At the higher concentration photosynthesis and greening of the cells was drastically inhibited and reduced glutathione increased by 80 % indication the production of ROS (Duval et al. 2005). The possible environmental role of cylindrospermopsin has recently been reviewed by Rzymski and Poniedziałek (2014).

The dominance of harmful dinoflagellate blooms has been proposed to be mediated by the production of the toxins, okadaic acid (OA) and diphysiotoxin-1 (DTX-1) (Plumley 1997; Windust et al. 1996) suggested that OA and DTX-1 from *Prorocentrum lima* may have allelopathic effects on non-toxin forming microalgae. OA is a potent inhibitor of serine/threonine protein phosphatases. 1 μM OA inhibited protein phosphatase activity in the dinoflagellates *Amphidinium klebsii* (Gymnodiniales), *Coolia monotis*, *Gambierdiscis toxicus* and *Ostreopsis lenticularis* (Gonyocaulales), but stimulated activity in the OA-producing *Prorocentrum lima* (Prorocentrales) (Sugg and VanDolah 1999). Preconditioned medium of *P. lima* strongly inhibited growth in three of the four dinoflagellates (but not *A. klebsii*), and inhibited phosphatase activity in all four species. Fractionation of the *P. lima* preconditioned medium showed that growth inhibition and phosphatase inhibition occurred in different fractions. The growth inhibiting fraction contained about 0.7 nM OA (determined by ELISA). This is much less than the ≫1 μM OA required to inhibit growth in the species tested by Windust et al. (1996, 1997) (*Conticribra* (*Thalassiosira*) *weissflogii*, *Diacronema* (*Pavlova*) *lutheri*, *Isochrysis galbana*). Furthermore, the conditioned medium of *P. lima* only contained ~5 nM OA. These results suggest that OA does not have an allelopathic role in inhibiting growth of other algae and that growth inhibition is due to another unknown substance.

5.2 Fatty Acids and Oxylipins

Fatty acids have been widely reported to have algicidal (allelopathic) activity (McGrattan et al. 1976; Ikawa 2004; Alamsjah et al. 2007, 2008; Bosma et al. 2008; Wang et al. 2012). In fact free fatty acids have been shown to exhibit a wide spectrum of antibiotic activities (Desbois and Smith 2010). The exact mode of action of FFAs on microalgae is not known. However, the prime target seems to be the outer cell membrane and the various processes that occur within the membrane or at the membrane surface. The detergent property of FFAs, resulting from their amphipatic structure, allows them to interact with the cell membrane to create transient or

permanent pores of variable size. In the bacterium *Escherichia coli* short chain free fatty acids have been shown to alter membrane fluidity and inhibit growth, presumably by affecting membrane associated enzyme function (Royce et al. 2013). At higher concentrations these FFA detergents can solubilize the membrane to such an extent that various membrane proteins or larger sections of the lipid bilayer are released, and this may ultimately lead to cell lysis (Parsons et al. 2012).

Wu et al. (2006) proposed that the fatty acids interact more easily with target organisms if their molecular structures are similar to those in the plasma membranes of the target organisms. This hypothesis is supported by the structure-activity studies of Huang et al. (2014). The algicidal activity of free fatty acids (FFAs) is influenced by their structure and shape. Based on studies on both the antibacterial and antialgal activity of FFAs the following general observations can be made (Desbois and Smith 2010 and references therein; Huang et al. 2014):

- The – OH group of the carboxyl group seems to be important as methylated FFAs generally have reduced or no activity.
- Unsaturated FFAs show greater activity that saturated FFAs of the same chain length.
- Within the monounsaturated FFAs the most potent usually have 14 or 16 carbon atoms.
- A direct correlation between the number of double bonds in an unsaturated FFA's carbon chain is often directly correlated with its activity.
- The double bonds in naturally occurring FFAs typically have *cis* orientation and these tend to have greater activity than FFAs with double bonds in *trans* orientation, probably because the structures of *trans*-bonded unsaturated FFAs resemble saturated FFAs.

Clear evidence for membrane damage by FFAs was shown by Wu et al. (2006) who showed that C18 fatty acids induced K^+ leakage from cells of *C. vulgaris* and *Monoraphidium contortum*, and the level of K^+ leakage was strongly correlated with the EC_{50}. Similarly, treatment of the cyanobacterium *Anabaena* P-9 with α-linolenic acid (C18:3 n-3) resulted in leakage of the phycobilin pigments after about 40 min indicating major membrane damage. Low concentrations of palmitoleic acid (C16:0; 10 μg mL^{-1}) caused growth inhibition of *Alexandrium tamarense*, but the alga recovered after several days indicating that there was no permanent damage (Li et al. 2014). However, at higher concentrations plasmolysis was observed after about 2 days, and the cells eventually lysed.

The sensitivity of cells to free fatty acids appears to be dependent on the composition of the membranes, especially their degree of saturation. For example, the free fatty acid yields of *Synechococcus* strains which have been metabolically engineered to overproduce free fatty acids are limited by the negative effects of free fatty acids on the host cells (i.e. autoinhibition) (Ruffing and Jones 2012; Ruffing 2013). However, the cyanobacterial strain *Synechococcus* sp. PCC 7002 is less susceptible to this inhibition than *Synechococcus elongatus* PCC 7942 (Ruffing 2014). The enhanced tolerance to FFA production of *Synechococcus* sp. PCC 7002 was found to be temperature-dependent, with physiological effects such as reduced photosynthetic yield and decreased photosynthetic pigments observed at higher temperatures. Temperature-induced changes in the chemical composition of the cell membrane may be responsible for the low free fatty acid tolerance at higher temperatures. One of the hypotheses proposed by Ruffing (2014) is that the increase in saturated membrane fatty acids at higher temperatures may influence the ability of free fatty acids to pass through the cell membrane, ultimately leading to intercalation of the free fatty acids and membrane damage.

Oxylipins, which are metabolites of polyunsaturated fatty acids, are the bioactive allelochemicals in diatoms. The production of diatom oxylipins, such as the polyunsaturated aldehydes (PUAs), occurs in at least three steps (Fig. 8). First, chloroplast membrane glycolipids and plasmalemma phospholipids are hydrolised to generate free polyunsaturated fatty acids (Pohnert 2002; d'Ippolito et al. 2004, 2005). Then, lipoxygenases act on the free polyunsaturated fatty acids to generate hydroperoxy fatty acids. Finally, the hydroperoxy fatty acids are transformed into PUAs through the action of hydroxyperoxide lyases (Wichard and Pohnert 2006; Barofsky and Pohnert 2007). Unlike in higher plants where oxylipins play a role in abiotic stress resistance (Savchenko et al. 2014), the diatom-derived PUAs are synthesized mainly after cell damage or lysis (Pohnert 2000) and PUA production takes place as long as the enzymes remain in contact with the precursor free fatty acids (Fontana et al. 2007). The stability of PUAs in seawater is quite long, but is affected by temperature. For example, the half-lives of octadienal and heptadienal were reduced from 200 h at 10 °C to 60 h at 20 °C (Bartual and Ortega 2013); decatrienal was stable for longer periods at all temperatures. This stability allows the local concentrations to build-up to concentrations where they can exert biological activity.

Oxylipins have also been isolated from the growth medium of the freshwater diatom *Asterionella formosa* (Jüttner and Müller 1979) and cell extracts of the green alga *Chlamydomonas debaryana* and the eustigmatophyte *Nannochloropsis gaditana* (de los Reyes et al. 2014). However, it is not known whether in these algae they have any role as allelopathic or anti-grazer metabolites as do the oxylipins of the diatoms.

5.3 Inhibition of Photosynthesis

Inhibition of photosynthesis is often reported in studies of allelopathic interactions of algae and several putative allelochemicals have been shown to inhibit photosynthesis, and these are particularly common in the cyanobacteria (Smith and Doan 1999).

The major allelopathic compound, an unidentified but slightly hydrophobic compound, isolated from the medium of the cyanobacterium *Trichormus doliolum* has been shown to inhibit both $^{14}CO_2$ fixation and photosynthetic electron transport in *Trichormus* (*Anabaena*) *variabilis* (Von Elert and Jüttner 1996, 1997). Fischerellin A isolated from *Fischerella muscicola* is a potent inhibitor of photosynthetic organisms with a minimum inhibitory concentration (MIC) of 14 nM against *Synechococcoccus* PCC 6911 in a 54 h growth assay (Hagmann and Jüttner 1996). In a detailed study using *Chlamydomonas reinhardtii*, *Anabaena* sp. P9 and pea (*Pisum sativum*) leaves, Srivastava et al. (1998) showed that fischerellin A acts at several sites which appear with increasing half-time of interaction in the following sequence: (1) effect on the rate constant of Q_y reoxidation; (2) affects the trapping of the primary photochemistry; (3) inactivation of PSII A reaction center; and (4) segregation of individual units from grouped units. Fischerellin B has also been shown to be a photosystem II inhibitor (Papke et al. 1997). The chlorine-containing compound cyanobacterin from *Scytonema hofmanni* has a MIC of 2 mg mL^{-1} (4.6 μM) against *Synechococcus* cells, but has no effect on purple photosynthetic bacteria that lack photosystem II (Gleason and Baxa 1986). Its site of action was shown to be near photosystem II (Gleason and Paulson 1984), specifically on the oxidizing side of the Q_B electron acceptor (Gleason and Case 1986) inhibiting electron flow from Q_A (Mallipudi and Gleason 1989). Its site of action is different from that of DCMU as it was effective against a mutant of *Anacystis nidulans* which was resistant to DCMU (Gleason et al. 1986). Compounds with a similar name ('cyanobacterin') but with different chemical structures have been isolated from *Nostoc linckia* and partially characterized. Cyanobacterin LU-1 from *N. linckia* CALU 892 and cyanobacterin LU-2 from *N. linckia* CALU 893 are reported to inhibit light-dependent O_2 evolution in cyanobacteria and the site of action was reported to be electron transport to Q_B (Gromov et al. 1991; Vepritskiĭ et al. 1991). At low concentrations the metabolite nostocyclamide produced by *Nostoc* sp. 31 increased photosynthetic O_2 evolution and respiratory O_2 uptake in *Anabaena* and *Synechococcus* as well as in spinach chloroplasts suggesting it acts as an uncoupler (Jüttner 1997).

Lipophilic organic compounds extracted from the medium of *Karenia brevis* cultures Inhibited the photosynthetic efficiency, F_v/F_m, within 1 h of exposure in several species of phytoplankton (*Akashiwo* cf. *sanguinea*, *Prorocentrum minimum* (=*P. cordatum*), *Amphora* sp., *Asterionellopsis glacialis*, *Skeletonema costatum*) during at least one stage of their growth (Prince et al. 2008). When exposed to the extracellular *K. brevis* extract the photosynthetic efficiency of *S. costatum* was suppressed by 65–77 % in all growth stages, whereas *A. glacialis* and *P. cordatum* were most inhibited during lag phase (49–67 %), rather than in exponential growth (39–48 %) or stationary phase (20 %). The photosynthetic efficiency of *Akashiwo* cf. *sanguinea* was lowered by 33–53 % in lag and exponential phases, but not in stationary phase. Interestingly, *Amphora* sp., whose growth over 48 h was actually stimulated by the *K. brevis* extracellular extract, had a 65 % reduction in F_v/F_m when exposed to the extract for 1 h in lag phase, but F_v/F_m was unaffected if *K. brevis* extracts were added at later growth stages. Exposure to the *K. brevis* extracellular extracts increased the proportion of cells with permeable (damaged) cell membranes in *Akashiwo* cf. *sanguinea*, *A. glacialis* and *P. cordatum*, but not in the other species. These results suggest that the effect on photosynthesis is a secondary one, and that the primary allelopathic effect is on membrane function and integrity. This hypothesis is supported by a recent metabolomic and proteonomic analysis of the allelopathic effects of *K. brevis* on the diatom *T. pseudonana* showed effects on multiple physiological pathways including disruption of energy metabolism and impeded cellular protection mechanisms including altered cell membrane components, inhibited osmoregulation, and increased oxidative stress (Poulson-Ellestad et al. 2014b).

6 Conclusions

Clearly, chemically-mediated interactions play an important role in the ecology and physiology of microalgae, and also in the relationships with other organisms such as bacteria and protozoa (Ianora et al. 2011). This chapter has not considered the chemically-mediated interactions between microalgae and organisms other than microalgae, although these are very important. For example, the chemically-mediated interactions between microalgae and their grazers have been discussed and reviewed by Wolfe (2000), Tillmann (2004), and Pohnert et al. (2007) and others. Similarly Paul et al. (2013) have shown that bacteria can have important, apparently allelopathic effects, on microalgae. The diversity of the microalgae, their metabolism and the environments they grow in is reflected in the diversity of the types of chemical interactions described and the variety of metabolites that are involved. However, despite extensive research, the diversity and complexity of the potential interactions means that much remains unknown as yet. In particular, only a small number of allelo-

chemicals, or potential allelochemicals, have actually been identified and without knowing the identity and structure of these chemicals it is difficult to study their actual mode of action. Furthermore, relating the results of laboratory studies to the actual conditions in the field often is difficult and requires extensive further study and close collaboration between chemists, physiologists and ecologists (Pohnert 2010). New advances in metabolomics and genomics are beginning to have a major impact on how we study these interactions and are providing important new discoveries in the chemical language of microalgae.

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Coping with High and Variable Salinity: Molecular Aspects of Compatible Solute Accumulation

Martin Hagemann

1 Introduction to the Basal Salt Acclimation

Algae comprise a large and taxonomically highly diverse group of photoautotrophic organisms living in aquatic habitats. The size of algae is very diverse. While macroalgae can reach length of more than 45 m, microalgae are of much smaller size between half of a micrometre up to several millimetres. The group of microalgae includes cyanobacteria, small members of the Archaeplastida (glaucophytic, red, and green algae) with plastids of primary endosymbiotic origin from an ancient cyanobacterium, as well as many eukaryotic algal groups, which received the plastids via secondary or tertiary endosymbiosis such as brown algae and diatoms (Keeling 2010). Despite their high taxonomic and morphological diversity, algae especially microalgae are regarded as a functional group of important primary producers in the oceans and freshwater ecosystems, which is responsible for at least half of the annual primary production on the Earth. Thus, these organisms are of high importance for the biogeochemical carbon as well as nitrogen (N_2-fixing cyanobacteria serve as major fertilizers of aquatic systems), which shaped our environment not only during the last billion years but also today (Chavez et al. 2011). Marine microalgae such as diatoms and coccolithophorids represent the main sink for anthropogenically released CO_2, because these algae sink relatively quickly into deeper water due to their heavy siliceous cell walls or coccolith-containing cell coverings (Sigman and Boyle 2000; Bixler and Porse 2011). This algal burial removes high amounts of carbon out of the cycle and may help to decrease the extent of the present climate change. Last but not least, algae are also of high economic importance. Several million tonnes of macroalgae are each year used for human nutrition or for the isolation of valuable natural products such as agar, carrageenan or laminarin (e.g. Pomin 2010). The production of microalgae is much lower, only about 15,000 tons are produced each year especially as food additive and for some fine chemicals. However, due to the increase in oil price and ecological concerns, microalgae are discussed to provide a promising source for green bioenergy and many attempts are under way to produce them in much higher amounts (Jones and Mayfield 2012). Due to limitations in freshwater supply, the future mass production of microalgae has to be done in sea or brackish waters (Borowitzka and Moheimani 2013; Chisti 2013).

The majority of liquid water on the Earth surface is characterized by rather high amounts of inorganic ions. At average, 1 l of sea water contains about 35 g of dissolved salt (35‰ total salinity, 35 practical salt units (PSU), or 35 absolute salinity (*SA*) according to TEOS-10; Wright et al. 2010), mainly NaCl. While the salinity is rather constant in the open oceans, in estuaries, coastal waters, and especially in tidal zones, it can vary to high extents when rain causes dilution and evaporation increases salinity. These changes affect mostly macroalgae fixed to the shore of the coast (Karsten 2012); however, benthic microalgae also cannot escape these alterations. Another interesting marine habitat with highly variable salinity is sea ice, in which the freezing of water results to an increase of salinity in internal brines up to 150‰. It is known that specialized diatom species not only survive these conditions but proliferate in sea ice to high biomass densities (Mock and Junge 2007). High amounts of inorganic ions and especially fluctuations in the salinity are challenging for organisms living in this habitat. Marine organisms including microalgae are obviously well adapted to this environment.

Growth in high salt environments has to solve basically two problems. First, water has to be obtained and kept by the cells in an environment characterized by high external osmolarity, which can be only done when the cell interior is hyperosmotic (i.e. it contains a higher concentration of osmotic active compounds such as ions and small water

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soluble organic molecules) compared to the surrounding. Water is not only the universal solvent for cellular biochemistry; its uptake via osmosis generates also the turgor pressure, the driving force for growth. Second, high concentrations of inorganic ions (particularly Na^+) exhibit direct toxic effects on metabolic processes (discussed in more detail by Serrano et al. 1999; Hagemann 2011); therefore, the internal ionic concentrations need to be carefully regulated. To acclimate to high and changing salinities, all algae apply the salt-out strategy (Trüper and Galinski 1990). This strategy was defined in contrast to the salt-in strategy used by halophilic archaea and some halophilic bacteria. These prokaryotes can accumulate high amounts of inorganic ions (mostly KCl) inside the cell, which maintains water flux via osmosis and turgor. The direct use of inorganic ions is regarded to be metabolically cheap; however, it needed the adaptation of the whole metabolic machinery to remain functional in the high salt environment (Oren 2007). The latter requirement was quite difficult to achieve during evolution, which explains why only few highly specialized prokaryotes can tolerate high internal salt concentrations. Organisms applying the salt-out strategy rely on active transport systems to maintain a rather low and constant ionic composition in metabolic active compartments such as cytoplasm, mitochondria, plastids and the nucleus, whereas ions are exported out of the cells or are accumulated in the metabolically less active vacuole (if one exist) (Fig. 1). To become hyperosmotic, instead of ions they accumulate low molecular mass organic compounds, which are highly soluble and compatible with the metabolism. These organic molecules form the functional group of compatible solutes (Brown 1976). Additional to its osmotic function, it also has been shown that these compounds exert direct protective effects on macromolecules, i.e. they prevent denaturation of enzymes or membrane complexes in high ionic environments as well as against freezing or high temperatures (e.g. Empadinhas and da Costa 2008). The continuous pumping of ions and the synthesis of large amounts of compatible solute is metabolically expensive, but the proteins and membranes of these organisms did not need a special adaptation to a highly saline environment. Thus, this strategy also allows a flexible readjustment of the cellular osmotic potential allowing euryhaline organisms to thrive in waters of different salinities.

The physiology and biochemistry of algal salt acclimation received much attention in the past. These initial experiments were summarized by Kirst (1990) and Reed (1990). These reviews do not only reflect many of the primary findings how eukaryotic algae can thrive in marine and hypersaline waters. These authors also provided many accurate definitions of terms in the field of salt acclimation. More recently, the knowledge on salt acclimation of cyanobacteria alone (Hagemann 2011) or in comparison to eukaryotic algae (Erdmann and Hagemann 2001; Oren 2007) as well as of macroalgae (Karsten 2012) has been summarized. Here, I will present some of the available data on the ionic regulation and compatible solute accumulation among algae. During the last years, many new "omics" technologies have been developed, which tremendously improved our data base for an improved understanding of many environmental acclimation processes. Especially the knowledge of genomes allows a much better survey whether or not the organism is able to perform specific stress acclimation strategies. Among cyanobacteria, we have now more than 130 complete genome sequences (Shih et al. 2013), while the number of genomes for eukaryotic algae is only slowly increasing. End of 2013, about 20 genomes of mostly marine microalgae were available. In the main part, I will here discuss how genomics and transcriptomics increased our knowledge on algal salt acclimation.

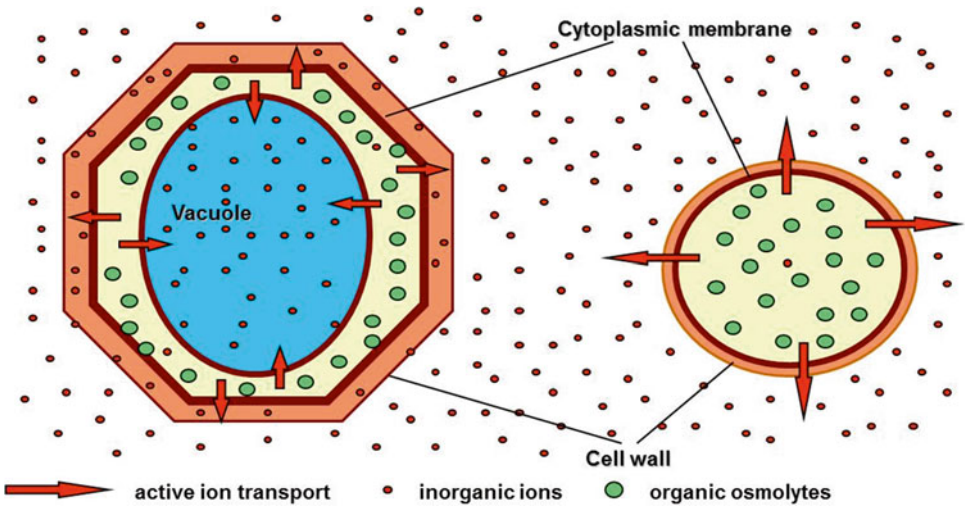

Fig. 1 Schematic drawing of cells (with and without large internal vacuoles) using the salt-out strategy to acclimate toward high external salinity. Cells accumulate compatible solutes in metabolically active compartments and export ions out of the cell or into the vacuole using active transport systems

2 Ionic Relations

Inorganic ions are important players in the osmotic equilibrium of microalgae. Since seawater is dominated by Na^+ and Cl^-, the regulation of cellular contents of these ions is particularly important. It is long known that Na^+ is found in much lower concentrations inside marine algae compared to the external milieu (Scott and Hayward 1953). Among eukaryotic algae there exists a general trend that cells without, or only very small internal vacuoles contain rather low contents of Na^+ and possibly also Cl^-, whereas cells with large internal vacuoles are characterized by much higher ion contents (data summarized by Kirst 1990; Reed 1990). Like halophytes, algal cells with large vacuoles are able to concentrate inorganic ions in this metabolically less active compartment (Fig. 1), which allows a metabolically cheap decrease of the overall osmotic potential of the cell. Due to methodological limitations (discussed in Blackwell and Gilmour 1989; Reed 1990; Erdmann and Hagemann 2001), true values for inorganic ions in metabolically active compartments are difficult to obtain.

Na^+ measured with NMR or radiotracer equilibrium experiments revealed rather low cytoplasmic levels, which were clearly below the levels of the surrounding sea water. The cyanobacterium *Synechococcus elongatus* PCC 6301 contained 120 mM Na^+ inside the cell after long-term cultivation in the presence of 0.5 M NaCl (Nitschmann and Packer 1992). In an elegant study using sodium isotopes, Reed et al. (1985a) showed a characteristic kinetic of Na^+ content changes in *Synechocystis* sp. PCC 6714 upon addition of 0.5 M NaCl. In this experiment Na^+ increased immediately after salt addition almost to equilibrium with the external Na^+ amount. Then the Na^+ became quickly reduced during the next hours to rather low steady state values around 40 mM. Such transient increases of internal Na^+ shortly after the elevation of salinity were also observed with cells of the green algae *Dunaliella salina*[1] (Weiss and Pick 1990) and *Chlorococcum submarinum* (Blackwell and Gilmour 1991a). However, long-term salt-acclimated *D. salina* cells contained only around 50 mM Na^+ after cultivation in media with 0.1 up to 4 M NaCl (Bental et al. 1988; Blackwell and Gilmour 1989), whereas Pick et al. (1986) reported an increase from about 30 to 100 mM Na^+ within this salinity range. Similarly, rather low and unchanged Na^+ levels were found in *Tetraselmis viridis* cultivated at salinities between 0.01 and 1.2 M NaCl (Strizh et al. 2004).

The reliable cellular Na^+ concentrations are much lower than those of the external salinity and in disequilibrium with the membrane potential (see discussion in Reed 1990). These findings clearly point to active transport processes guaranteeing the low, nontoxic internal Na^+ contents Microalgae without vacuoles certainly have plasmalemma localized Na^+ exporters, which extrude Na^+ out of the cell, while cells with internal vacuoles need additional transporters at the tonoplast keeping the Na^+ accumulated inside the vacuole. Na^+-transporters are not well defined at the molecular level among algae. Biochemical studies indicated that Na^+ export seems to be coupled to H^+ transport by Na^+/H^+ antiporters in salt-loaded cells of *Dunaliella* (Katz et al. 1991, 1992). The necessary proton motive force is generated by H^+-ATPases. Interestingly, these and further studies on the P-type H^+-ATPase (e.g. Smahel et al. 1990) showed that the transport activities of these proteins are directly stimulated by inorganic ions, while the expression of the plasma membrane H^+-ATPase was not increased with increasing external salinity (Wolf et al. 1995). Among cyanobacteria, mutant studies revealed that different Na^+/H^+ antiporters are indeed crucial for the Na^+ extrusion from salt loaded cells (Inaba et al. 2001; Elanskaya et al. 2002; Wang et al. 2002). Alternatively, a direct coupling of Na^+ export to an electron transport chain at the plasmalemma was supposed for *Dunaliella* (Katz and Pick 2001).

The contributions of Na^+/H^+-antiporters as main mechanism for Na^+ export from marine microalgae has been questioned, because in the rather alkaline sea water it seems to be difficult to generate the necessary proton motive force at the plasmalemma (e.g. Ritchie 1991; Gimmler 2000). Therefore, primary Na^+ transporting mechanisms have been sought among microalgae. Only recently the unequivocal proof for the existence and function of a Na^+-ATPase has been published for cyanobacteria. The genomes of a few strains contain an operon coding proteins with features of a Na^+-specific F_1F_0 ATPase (Dibrova et al. 2010). Recently, its function as Na^+ exporter in the plasmalemma has been verified in the halophilic cyanobacterium *Aphanothece halophytica* (Soontharapirakkul et al. 2011). Clear biochemical evidence for the functioning of a Na^+-ATPase also has been provided for the green alga *Tetraselmis viridis* (Popova et al. 1998; Balnokin et al. 2004) and the raphidophyte *Heterosigma akashiwo* (Shono et al. 1995). From the latter organism, the complete cDNA for a Na^+/K^+-ATPase was cloned, which was the first example for such an enzyme among eukaryotic phototrophs (Hara et al. 2003). Recently, genes for Na^+ pumps, which also show similarities to animal Na^+/K^+-ATPase, have been identified in the red alga *Pyropia* (*Porphyra*) *yezoensis* (Uji et al. 2012). Moreover, cDNA fragments of different ATPase types among them some resembling animal Na^+/K^+-ATPases have been cloned and sequenced from different algal lineages (Barrero-Gil et al.

[1]Wherever possible the currently accepted names for species are used. The name used in the paper cited is also indicated. For details of names see chapter "Systematics, Taxonomy and Species Names: Do They Matter?" of this book (Borowitzka 2016).

2005). However, none of these transporters coded by these gene sequences have been directly linked to the active Na^+ export in these algal cells. With the increasing number of genomes, systematic searches for specific Na^+ exporters among algae will become possible. Recently, the occurrences of monovalent cation/H^+ antiporters have been searched for in algal genomes. At least three different classes of such proteins were found which seem to function as Na^+/H^+ antiporters, as judged from the functionally characterized homologous proteins in cyanobacteria, or possibly as K^+/H^+ antiporters (Chanroj et al. 2012).

The Cl^- contents inside microalgae are largely differing in the literature. For *Dunaliella acidophila* (Hirsch et al. 1992) the internal Cl^-remained around 30 mM when grown between 0.1 and 300 mM external chlorides, while it increased from 30 to 90 mM in cells of *Platymonas subcordiformis* (= *Tetraselmis subcordiformis*) in the presence of 0.15–1 M NaCl (Dickson and Kirst 1986). However, rather high Cl^- contents were reported for marine red algae (reviewed in Reed 1990). Nevertheless, even the rather high Cl^- levels are usually lower than the chloride in the external medium and are not in equilibrium with the membrane potential. The work with *D. acidophila* (Hirsch et al. 1992) provided clear evidence for an active Cl^- uptake in the presence of very low external Cl^- concentrations, whereas at high external Cl^- an active Cl^- export must be functioning. Unfortunately, the molecular basis for chloride transport is largely unknown for eukaryotic algae as well as for cyanobacteria (discussed in Hagemann 2011). Moreover, in many cases it has been observed that the amount of Cl^- is lower than the amount of cations, i.e. the sum of Na^+ and K^+. Thus, other anions or negatively charged organic compounds are necessary to balance the charge differences.

In contrast to Na^+, K^+ is much more compatible with cellular metabolism and represents usually the major cation inside living cells as was shown for *Ulva lactuca* already 60 years ago (Scott and Hayward 1953). For example K^+ levels between 250 and 700 mM were found in *Tetraselmis subcordiformis* grown in the presence of 0.15–1 M NaCl (Dickson and Kirst 1986), 110 and 190 mM K^+ in cells of *Chlorococcum submarinum* (Blackwell and Gilmour 1991a) at 0.1 and 0.5 M NaCl, respectively, and of ca. 115 mM K^+ in *Dunaliella parva* irrespective of the external salinity (Blackwell and Gilmour 1989), while macroalgae often accumulate higher internal K^+ levels due to the high proportion of vacuolar volume in the cells (Reed 1990). Escassi et al. (2002) revealed the crucial role of K^+ uptake for growth in the red alga *Pyropia* (*Porphyra*) *leucosticta*, which showed daily cycles in K^+ enrichment and subsequent turgor-driven enlargement that were disturbed by K^+ limitation or inhibitors of K^+ transport. As discussed for Na^+ and Cl^-, the mechanisms for regulating the internal K^+ levels are rather poorly characterized among eukaryotic algae. For red algae it has been discussed that the K^+ levels are nearly in equilibrium with an electrogenic uptake through K^+ channels driven by the membrane potential (Reed 1990). However, assuming different concentrations in different cell compartments, active transporters have to be involved in the regulation of defined internal K^+ levels. In the cyanobacterium *Synechocystis* sp. PCC 6803, the action of Ktr and Kef like K^+ transporters using the proton motive force have been proven to be responsible for the K^+ accumulation in salt-loaded cells (Berry et al. 2003; Matsuda and Uozumi 2006). Genes coding proteins for K^+/H^+ transporters with similarities the mentioned bacterial systems have been identified in the genomes of eukaryotic algae (Chanroj et al. 2012). Last but not least, the putative Na^+/K^+-ATPases in algae (Hara et al. 2003; Barrero-Gil et al. 2005; Uji et al. 2012) also could contribute not only to Na^+ export but also K^+ uptake.

Future experiments (e.g., expression of genes for putative algal ion transporters in yeast mutant or attempts to knock down these genes in the algal cell) are needed to proof which of these transporters is really involved in ion transport in salt-acclimated cells. This knowledge also will allow the estimation of the energetic costs for the ion regulation in marine algae. Even in cells or thalli completely acclimated to a certain salinity, inorganic ions are constantly moving into the cells following the electrochemical gradient or as driving force for nutrient uptake co-transporters. Depending on the flux and the transport mechanisms, the energy burden for ion homeostasis may vary to a large extent and could limit for example the productivity of microalgae for biofuel production.

3 Overview on Compatible Solute Accumulation in Different Algal Groups

During the last 50 years numerous algae have been screened regarding the accumulation of compatible solutes, which are responsible for the osmotic equilibrium in metabolically active compartments. These screenings revealed that in many cases a cocktail of compatible solutes is accumulated in one algal cell among them one compound is often dominating. Moreover, different algal groups seem to prefer special chemical structures as compatible solutes.

Among cyanobacteria, more than 150 strains were screened resulting in a well-supported correlation between the main compatible solute and the final salt tolerance level (reviewed in Hagemann 2011). Strains of low salt tolerance, which usually occur in freshwater and/or terrestrial habitats, accumulate sucrose and/or trehalose (Fig. 2). These carbohydrates, especially trehalose, act not only as osmolytes in salt-stressed cells, but exhibit also a high protective potential for enzymes under desiccation and/or high temperature.

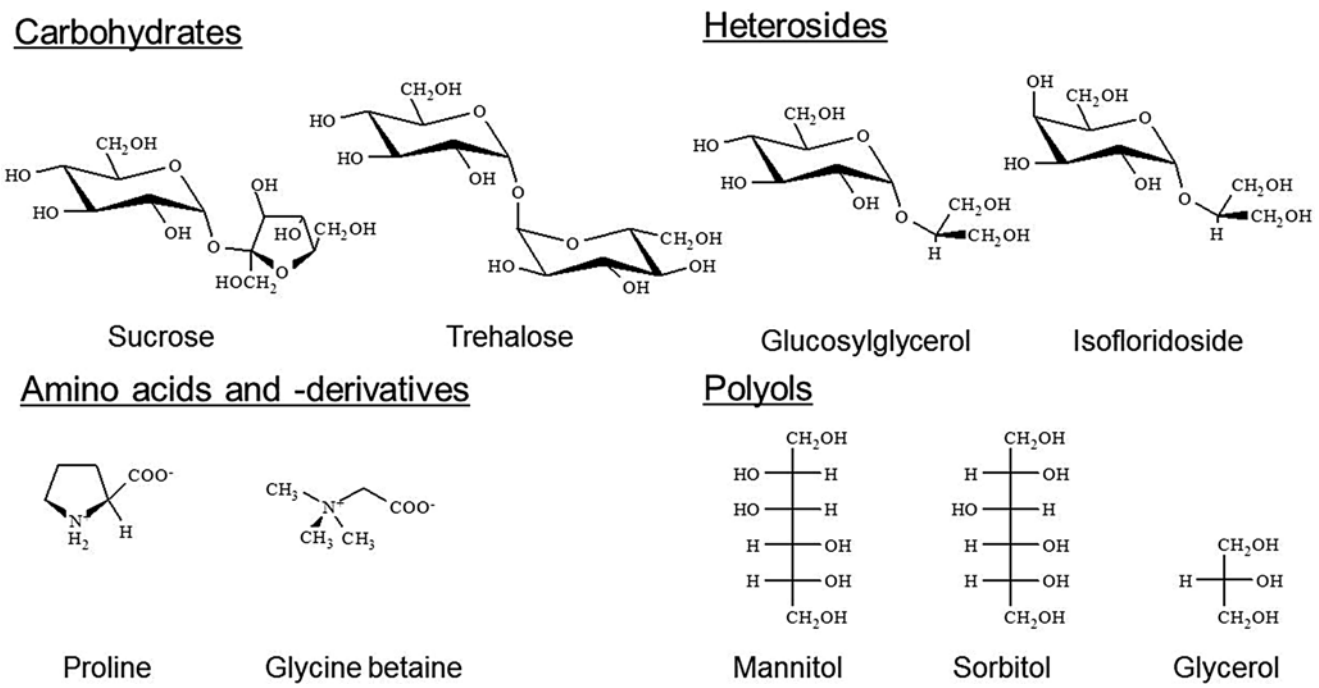

Fig. 2 Major groups and structures of selected compatible solutes found to be accumulated in salt-treated cells of microalgae

Moderate halotolerant strains, which occur mostly in the marine system, accumulate glucosylglycerol as characteristic compatible solute (Hagemann 2013). Strains of the highest salt tolerance, which are often true halophiles that need a certain amount of NaCl in the growth medium, accumulate glycine betaine or rarely glutamate betaine as major compatible solute (Reed et al. 1984).

Only recently, glucosylglycerate was found as a new, minor compatible solute in marine cyanobacteria (Klähn et al. 2010a). Glucosylglycerate is an unusual compatible solute (Empadinhas and da Costa 2008), because it is charged at physiological pH. Among marine cyanobacteria it was discussed that the charged glucosylglycerate might not only acting as compatible solute but rather as counterion for cytosolic K^+ especially under N-limiting conditions, which are characteristic for the open ocean. This hypothesis is based on two findings (Klähn et al. 2010a). Firstly, glucosylglycerate accumulation is much higher when cyanobacterial cells are salt-shocked under N-limiting compared to N-replete conditions. Interestingly, glutamate, which is usually the major organic molecule acting as counterion for cellular K^+, showed a reverse behaviour, it increased in salt-shocked cells under N-replete but decreased under N-deplete conditions. Secondly, genes for enzymes catalysing the glucosylglycerate biosynthesis are usually found in genomes of marine cyanobacteria not able to perform N_2-fixtion, whereas the genomes of diazotrophic oceanic strains are not harbouring these genes (Hagemann 2013). Obviously, N_2-fixation makes these cyanobacteria independent from external N-sources; therefore, their glutamate level is sufficient to balance the charge of intracellular K^+.

The correlation between salt tolerance level and preferred compatible solute implies that certain chemical structures of compatible solute are better suited for the osmotic purposes, e.g. glycine betaine, while others are superior in protecting membranes under combined stress conditions, e.g. trehalose. Indeed, differences in the protective effects of different compatible solutes towards enzymes or membranes have been found in in vitro assays (Borges et al. 2002; Hincha and Hagemann 2004). Another example represents the selective accumulation of sucrose or trehalose in many low salt tolerant as well as terrestrial strains. Despite the existence of genes for the biosynthesis of sucrose as well as trehalose, salt-stressed cells of e.g. *Anabaena* sp. PCC 7119 (Porchia and Salerno 1996) or *Nodularia spumigena* CCY9414 (Möke et al. 2013) accumulate exclusively sucrose, whereas the trehalose synthesis genes became activated by desiccation stress in *Anabaena* (Higo et al. 2006).

The molecular mechanisms underlying the synthesis of the different compatible solutes are well-characterized among cyanobacteria. In all cases, we know the genes, enzymes etc. (summarized in Hagemann 2011). This knowledge allows the annotation of the salt tolerance strategy to cyanobacterial strains, which are difficult to cultivate but for which the genome sequence has been released to public data bases (see Hagemann 2013). In many cases it has been shown that alterations in external salinity influence the expression of the genes for enzymes involved in compatible solute

biosynthesis (e.g. genes for the compatible solute glucosylglycerol; Klähn et al. 2010b). Additionally, it has been often shown that the activity of enzymes catalysing the compatible solute biosynthesis is directly stimulated by high salt contents in crude protein extracts. Recently, the mechanism for the salt dependence of the enzyme for glucosylglycerol synthesis has been revealed. Novak et al. (2011) showed that the glucosylglycerol synthesis enzyme is inhibited by phosphate-rich, negatively charged compounds, i.e. DNA, RNA or nucleotides, under low salt conditions. These organic molecules bind sequence-independent to the positively charged protein. This electrostatic interaction is released in the presence of increasing amounts of ions such as Na^+ or K^+ leading to the activation of the enzymatic activity. Since salt-shocked cells or cells acclimated to higher salt concentrations contain transiently or permanently increased ion levels, the amount of cellular ions titrates the enzyme activity to an activity level, which calibrates the compatible solute synthesis capacity to the demands of the external salinity (Novak et al. 2011). It would be highly interesting to know if this mechanism regulating the activity of salt-acclimation proteins by inorganic ions is more widespread among other organisms including eukaryotic microalgae.

The data basis for eukaryotic algae especially regarding molecular mechanisms of compatible solute synthesis is less comprehensive. Nevertheless, there are some interesting trends showing that specific compatible solutes are preferentially used by certain groups of eukaryotic algae. Green algae of lower salt tolerance often accumulate sucrose and proline as major compatible solutes (Ahmad and Hellebust 1984; Edwards et al. 1987; Setter and Greenway 1983). It has been shown that the enzymes for sucrose biosynthesis among green algae and land plants were acquired from cyanobacteria during the endosymbiotic events leading to plastids (Lunn and MacRae 2003; Kolman et al. 2012; Blank 2013). Proline biosynthesis also seems to use the plant-like proline biosynthesis pathway with pyrroline 5-carboxylate reductase as key enzyme (Laliberté and Hellebust 1989). However, other compatible solutes such as sorbitol (Jacob et al. 1991) or α-laminaribiose (Vazquez-Duhalt and Arredondo-Vega 1991) were also found among green algae. Well characterized is the salt acclimation of *Dunaliella* spp. (e.g. Borowitzka and Brown 1974; Ben-Amotz and Avron 1981). These walless green algal species have served as model organisms to understand high salt acclimation for many years, because some of them can propagate in saturated salt brines. As discussed above, even in the presence of very high external salt concentrations the cytoplasm of *Dunaliella* contains only very low amounts of inorganic ions; instead glycerol is accumulated to very high levels balancing the osmotic potential (glycerol biosynthesis is discussed in more detail below). Thus, glycerol is the compatible solute allowing the highest salt tolerance level among eukaryotic algae (Fig. 2). Compared to other compatible solutes, its biosynthesis is metabolically cheap because glycerol biosynthesis branches early from Calvin Benson cycle (Erdmann and Hagemann 2001). Glycerol accumulation was also found in the green alga *Chlorococcum submarinum* (Blackwell and Gilmour 1991b).

Red algae also accumulate several compatible solutes. However, with the exception of the group of Ceramiales, the heteroside floridoside (α-D-galactopyranosyl-(1-2)-glycerol) was found in many species as the dominating compatible solute (Bisson and Kirst 1979; Kirst 1980, 1990; Reed 1990; Wiencke and Läuchli 1981). It also has been regarded as the main soluble photosynthesis product among red algae (Bondu et al. 2009). Later on, the isomer isofloridoside (α-D-galactopyranosyl-(1-1)-glycerol) also has been identified as compatible solute. The proportion of isofloridoside and floridoside seems to vary in different species but also growth conditions (Karsten et al. 1993; Karsten 1999). The accumulation of (iso)floridoside has been used as a reliable marker to identify symbiotic Rhodophyta (Kremer et al. 1980) or unicellular species such as *Cyanidium caldarium* (Reed 1983). Isofloridoside also acts as the major compatible solute in the chrysophyte *Poterioochromonas malhamensis* (Kauss 1977). Structurally, isofloridoside and floridoside as galactosylglycerols are very similar to the cyanobacterial compatible solute glucosylglycerol (Fig. 2). As shown for glucosylglycerol, (iso)floridoside also protects enzymes against denaturation in in vitro assays (Karsten et al. 1996). Not only the structure and function but also the two step biosynthetic pathway of these compounds is very similar. (Iso) floridoside synthesis was analyzed with crude and purified enzyme preparations from several red algal species such as *Pyropia* (*Porphyra*) *perforata* (Meng and Srivastava 1991, 1993) or *P. malhamensis* (Kauss 1977, 1979) and will be discussed in more detail below. In many red algae, including species of the Ceramiales, digeneaside [α-D-mannopyranosyl-(1-2)-glycerate] has been found as minor osmolyte (Kremer and Vogel 1975; Bondu et al. 2009). As discussed above for glucosylglycerate, digenaside is a charged compatible solute and also might be serving for more specialized tasks than simply osmotic equilibrium. Additionally, the accumulation of several polyols (Reed 1990) such as sorbitol, mannitol, and dulcitol has been reported from red algal species (Fig. 2). Especially species of the red algal genus *Caloglossa* use mannitol as typical compatible solute (Karsten et al. 1997), which has been previously found mostly in brown algae.

Brown algae evolved from a secondary endosymbiosis, in which an ancient rhodophyte served as ancestor of their plastids (Keeling 2010). Mannitol has been regarded for years as the characteristic compatible solute in species of this algal lineage (Kirst 1977; Reed et al. 1985b); however, it also is accumulated by other algae, e.g. the red alga. It should be mentioned that mannitol is not only functioning as an

osmolyte. It also has been shown that mannitol can serve as antioxidant, carbon storage and for some other purposes (reviewed by Iwamoto and Shiraiwa 2005).

Proline (Fig. 2) represents another widespread compatible solute. It is found in bacteria, cyanobacteria, land plants, and animals as compatible solute. Proline is also a bit exceptionally among compatible solutes. Usually, these compounds are not participating in the active metabolism of the cell; they often represent end products of some metabolic branches. This separation from the highly active primary metabolism is thought to be advantageous in terms of regulation and ensures also its high accumulation levels. Proline is clearly 1 of the 20 proteinogenic amino acids, which participates in the protein turnover and primary N-metabolism. Nevertheless, this amino acid is often used as osmolyte, because of its high solubility and compatibility with cellular metabolism. Among algae, diatoms are known to use proline as main compatible solute (Krell et al. 2007; Bromke et al. 2013). However, it is also accumulated in cells of salt-treated green algae. Additional to proline, cyclohexanetetrol (Fujii et al. 1995) and mannose (Paul 1979) have been identified as organic osmolytes in salt-treated cells of diatom species.

4 Molecular Mechanisms of Selected Compatible Solute Syntheses

4.1 Glycerol Accumulation in *Dunaliella*

Generally, the glycerol accumulation is best studied in yeast (reviewed by Hohmann 2002, 2009). In *Saccharomyces cerevisiae*, glycerol is accumulated under osmotic as well as salt stress. The genes and proteins for the glycerol synthesis and transport are known as well as the signal transduction chain guaranteeing its stress-proportional expression. Among algae, glycerol accumulation leads to the highest level of salt tolerance and its biosynthesis is best studied in *Dunaliella* species (Borowitzka and Brown 1974; Borowitzka et al. 1977; Ben-Amotz and Avron 1981; Belmans and Van Laere 1987). In *Dunaliella* as in yeast, glycerol is synthesized via the so-called glycerol cycle. The biosynthesis starts from Calvin Benson cycle or glycolysis intermediate dihydroxyacetone phosphate (DHAP), which is converted into glycerol 3-phosphate by a DHAP/glycerol 3-phosphate dehydrogenase. Glycerol 3-phosphate is then dephosphorylated by a specific phosphatase. In hypo-osmotically shocked cells, glycerol can be converted back into DHAP via dihydroxyacetone using dihydroxyacetone/glycerol dehydrogenase and then a specific glycerol kinase. All enzymes have been characterized on the biochemical level. These studies showed that the DHAP/glycerol 3-phosphate dehydrogenase seems to be the crucial enzyme for glycerol accumulation, since its activity was found to be rate limiting for glycerol biosynthesis. Interestingly, the activity of this enzyme is directly stimulated in vitro by raising the amount of NaCl in enzyme assay mixtures. In vivo, salt influx represents a situation characteristic for salt-shocked *Dunaliella* cells, thus the salt stimulation of the biosynthetic enzyme guarantees a gene-expression-independent fast increase in the glycerol pool. The glycerol cycle also involves a close cooperation of the chloroplast, where the DHAP/glycerol 3-phosphate dehydrogenase is localized, and the cytosol, in which the remaining steps occur.

Two isozymes for the DHAP/glycerol 3-phosphate dehydrogenase have been characterized in *D. tertiolecta*, one in the cytosol and one from the chloroplast. The latter one showed much higher and salt-stimulated activities (Gee et al. 1989) necessary for the osmotic-stress-stimulated glycerol biosynthesis. Cells of *D. salina* seem to harbour even five different isozymes of DHAP/glycerol 3-phosphate dehydrogenases, which also responded differently towards cultivation at different salinities (Chen et al. 2009). However, this high number of isozymes was found using activity stains of native gels, which also could reflect rather modified forms (e.g. by phosphorylation) of identical proteins than truly different isozymes. In contrast to yeast, the molecular basis for glycerol biosynthesis remained largely unknown in *Dunaliella* due to missing sequence information, which also would directly show the numbers of genes for such isozymes.

Only recently, attempts started to identify genes coding for enzymes of the glycerol cycle among algae. An expressed sequence tag (EST) profiling approach with salt-shocked cells of *D. salina* resulted in a large collection of sequences regulated after NaCl addition (Alkayal et al. 2010). Among them, many cDNAs for enzymes of the glycerol cycle from *Dunaliella* have detected, including several isoforms of putative DHAP/glycerol 3-phosphate dehydrogenases as well as ESTs for putative dihydroxyacetone or glycerol kinases. Additionally, several full length cDNAs for different isoforms of chloroplastidial DHAP/glycerol 3-phosphate dehydrogenases have been cloned and sequenced from different *Dunaliella* species (He et al. 2009; Cai et al. 2013). Interestingly, in some of these proteins an N-terminal phosphatase-like domain was found, which is fused to the C-terminal dehydrogenase domain. This protein structure offers the possibility of bifunctional glycerol synthesis enzymes performing the two step conversion of DHAP towards glycerol in *Dunaliella*. The increasing amount of genetic information on glycerol cycle enzymes offers the avenue to characterize the molecular basis and regulation of the glycerol cycle in green algae.

For many years, it was an open question how *Dunaliella* and other glycerol accumulators can keep the high internal glycerol concentrations, because biological membranes are usually highly permeable for this compound that can even

penetrate through a special form of aquaporins. The rather high content of sterols in membranes of *Dunaliella* was thought to diminish the glycerol permeability of its membranes (Gimmler and Hartung 1988). In yeast it was shown that in the course of hypo- and hyper-osmotic treatments of cells specific glycerol exporters are opened to release glycerol and glycerol importers are used to take up or recover external glycerol, respectively (Hohmann 2002). Recently, glycerol transport also was shown for salt-treated *Dunaliella* cells (Lin et al. 2013). Moreover, the authors identified and cloned the responsible glycerol uptake protein. Interestingly, silencing of the expression of the transporter gene not only affected the glycerol uptake, it also resulted in a loss of resistance toward salt shocks. These results clearly show that *D. tertiolecta* cells need a tight coordination of glycerol biosynthesis and retention by reuptake of leaked glycerol inside the cell to achieve its remarkable high salt tolerance.

4.2 (Iso)Floridoside Accumulation in Red Algae

As for glycerol, the biochemistry of (iso)floridoside biosynthesis was analysed in great detail. Using some model red algae, and especially the chrysophyte *Poterioochromonas*, it was shown that the synthesis depends mostly on the activity of an (iso)floridoside phosphate synthase. This enzyme uses UDP-galactose (synthesized from UDP-glucose by the UDP-glucose-4-epimerase) and glycerol-3-phosphate to synthesize the intermediate (iso)floridoside-phosphate, which then is dephosphorylated by a specific phosphatase to the non-charged (iso)floridoside. The precursors can be synthesized via photosynthesis in the Calvin Benson cycle or via degradation of floridean starch. The enzymes from *P. malhamensis* were investigated in great detail by the group of Kauss (1977, 1979). The isofloridoside phosphate synthase was found to be directly activated after salt addition in crude protein extracts of this alga (Kauss et al. 1979). The activation seemed to involve a protein modification via a protease, thus not the enzyme itself but the protease seems to be directly salt-activated (Brunner and Kauss 1988; Rausch et al. 1991).

Despite the comprehensive knowledge on biochemistry and physiology of (iso)floridoside accumulation among rhodophytes and chrysophytes, the genetic basis for the synthesis of this osmolyte was only recently identified analysing (iso)floridoside synthesis in the extremophilic, unicellular red alga *Galdieria sulphuraria* (Pade et al. 2015). This study took advantage from generated genome sequence of *G. sulphuraria*, which recently became publically available (Schönknecht et al. 2013). Using protein sequences from plants, yeast, and cyanobacteria for functionally characterized enzymes synthesizing trehalose or glucosylglycerol, which are compatible solutes using a biochemical pathway similar to that of (iso)floridoside, related protein sequences were identified in the genome of *G. sulphuraria*. The phylogenetic clustering showed separated clusters for enzymes involved in special compatible solute synthesis. One cluster comprised enzymes for glucosylglycerol synthesis from cyanobacteria, whereas other clusters harbored enzymes for trehalose synthesis from prokaryotes or from eukaryotes. Finally, one cluster was detected comprising one protein from *G. sulphuraria* (encoded by the gene *gasu_10960*) and many related proteins from diverse eukaryotic algae, mostly from rhodophytes (Fig. 3). The *Galdieria* protein of this cluster was the most promising candidate for an enzyme catalyzing the (iso)floridoside synthesis. The proteins in this cluster have been automatically annotated as putative trehalose synthesis enzymes, which all harbor a glucosyltransferase and a phosphatase domain. The protein structure prediction implies that these enzymes may catalyze the two-step biosynthesis of (iso)floridoside, i.e. the synthase and phosphates reaction, by one composite enzyme.

The predicted function of the *G. sulphuraria* candidate protein was verified by two strategies: the biochemical characterization of the recombinant protein and its expression in a salt-sensitive mutant of the cyanobacterium *Synechocystis* sp. PCC 6803 defective in glucosylglycerol accumulation (Pade et al. 2015). The recombinant protein was able to synthesize isofloridoside from the substrates UDP-glucose and glycerol 3-phosphate. Correspondingly, the cyanobacterial mutant expressing the candidate gene from *G. sulphuraria* accumulated isofloridoside. These results clearly showed that the *gasu-10960* gene from *G. sulphuraria* encodes for an enzyme catalyzing the complete isofloridoside synthesis, i.e. it is an isofloridoside phosphate synthase/phosphatase (Pade et al. 2015). Additionally, another gene, gasu_26940 was shown to encode for the floridoside phosphate synthase/phosphatase in *G. sulphuraria*. These findings verified that separate enzymes are responsible for synthesis of either floridoside or isofloridoside (Pade et al. 2015). Moreover, the first functional verification of the enzymes for (iso)floridoside synthesis allowed to conclude that the related proteins in the cluster also are most probably enzymes for (iso)floridoside synthesis. Interestingly, those enzymes were not only found in genomes of different red algae, but closely related proteins also occur in genomes of brown algae and a green alga. The more wide spread occurrence of such genes/proteins in algae not reported to synthesize (iso)floridoside could indicate that these closely related proteins synthesize different products or these proteins are not expressed in the brown and green algal hosts. Another possibility could be that these genes might be involved in digenaside (mannosylglycerate) biosynthesis. Digenaside is probably synthesized by a pathway quite similar to that of (iso)floridoside, i.e. UDP-mannose and phosphoglycerate serve as precursors for the intermediate mannosylglycerate phosphate, which is

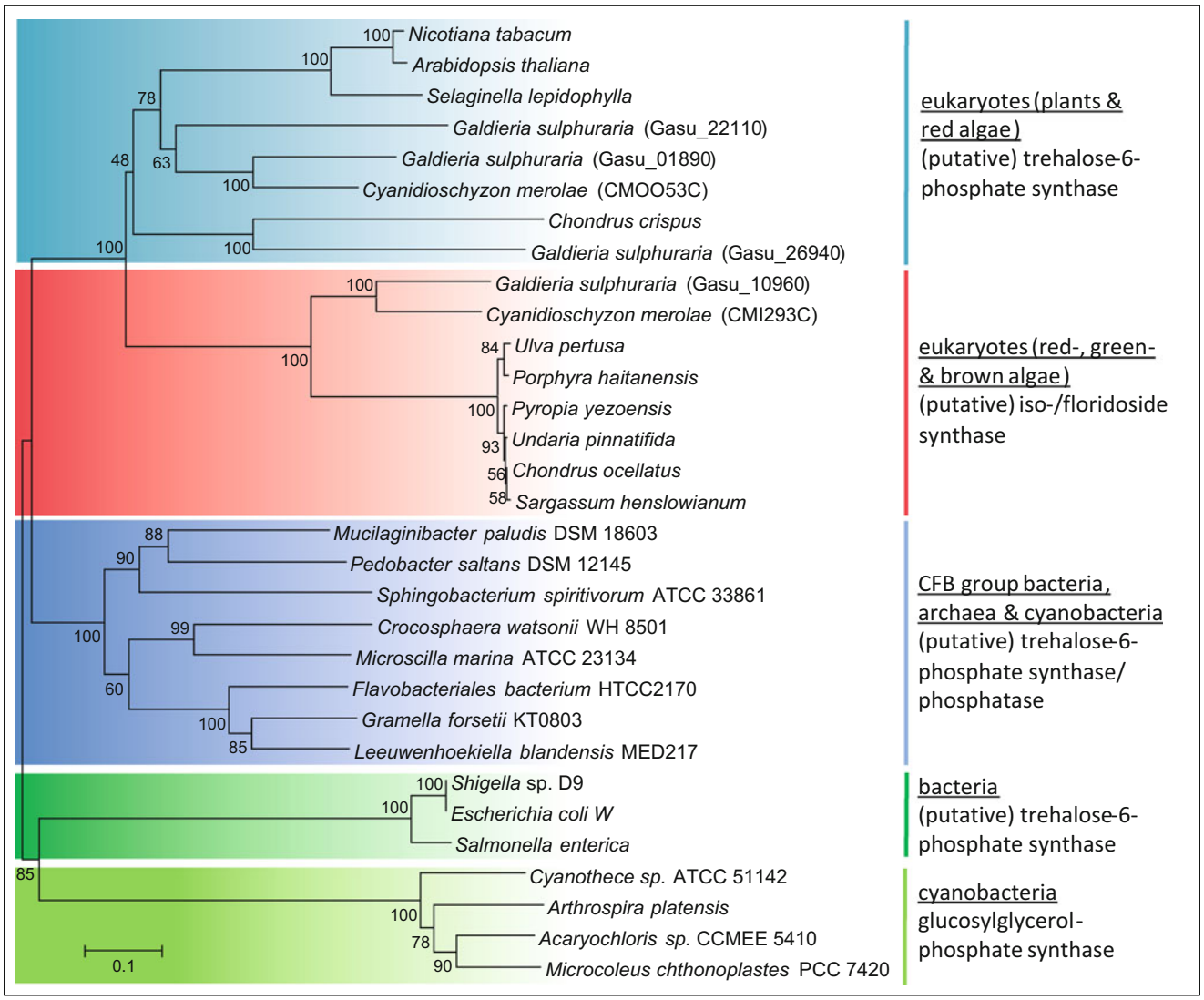

Fig. 3 Unrooted phylogenetic tree of amino acid sequences from putative (iso)floridoside-phosphate synthase and related enzymes using the neighbor joining algorithm. The distance scale is shown in the bottom left-hand corner and bootstrap values are added to nodes

then hydrolyzed to mannosylglycerate among bacteria (Empadinhas and da Costa 2008). However, the mannosylglycerate/glucosylglycerate biosynthesis pathway was recently identified in the first eukaryotic organism, the primitive land plant *Selaginella moellendorffii* (Nobre et al. 2013). The author's showed that in *Selaginella* a direct mannosyl/glucosylglycerate biosynthesis without phosphorylated intermediate occurred. The responsible enzyme was obtained via horizontal gene transfer from mannosylglycerate-accumulating bacteria. Interestingly, closely related proteins, which are clearly different from the Gasu_10960 protein of *G. sulphuraria* for isofloridoside synthesis, are encoded by algal genomes qualifying them as the best candidates for digenaside synthesis proteins.

4.3 Mannitol Accumulation in Brown Algae

Mannitol is usually the major compatible solute among brown algae, but it is also found in some red algal lineages (discussed above). Mannitol synthesis is done by the so-called mannitol cycle, which was characterized among brown algae (Ikawa et al. 1972; Richter and Kirst 1987) and red alga (Karsten et al. 1997) but also occurs in other organisms (Iwamoto and Shiraiwa 2005). Fructose 6-phosphate (Fru6P) is used as precursor, which is synthesized via photosynthesis or breakdown of reserve polysaccharides. Fru6P is converted into mannitol 1-phosphate by mannitol 1-phosphate dehydrogenase. Then, the intermediate is dephosphorylated by specific mannitol 1-phosphate phosphatase. Under hypo-

osmotic conditions, mannitol can be reverted back into Fru6P by the subsequent action of mannitol dehydrogenase and hexokinase. Studies with crude extracts or partially purified enzyme preparations showed that the entrance enzyme, mannitol 1-phosphate dehydrogenase, seems to be rate limiting. As found in many other cases, the activity of this enzyme also is directly stimulated by the addition of NaCl or other salts to the assays.

The genetic basis for mannitol biosynthesis in algae was unknown for a long time. In contrast, mannitol biosynthesis genes and their regulation were intensively characterized among bacteria and yeast. These organisms are not only natural mannitol accumulators; they also are presently used for biotechnological mannitol production (Saha and Racine 2011). The publication of the first eukaryotic algal genome sequence from the phaeophyte *Ectocarpus siliculosus* (Cock et al. 2010) stimulated subsequent molecular work. For example, the genetic basis for the complex sugar biosynthesis pathways of *E. siliculosus* was analysed (Michel et al. 2010). Among them, the mannitol cycle was annotated. These analyses revealed that for most enzymes, particularly for the mannitol 1-phosphate dehydrogenase small gene families exist. Moreover, the mannitol synthesis enzymes seem to originate from Actinobacteria and were obtained via an earlier horizontal gene transfer event (Michel et al. 2010; Dittami et al. 2011a). In a subsequent study by Rousvoal et al. (2011), the three mannitol 1-phosphate dehydrogenase proteins were characterized whether or not they are involved in salt-regulated mannitol biosynthesis. The corresponding genes showed differential expression under different environmental stresses including salt. The gene called *EsM1PDH1* was identified as the most highly expressed one of this small gene family. Expression of the EsM1PDH1 cDNA in *E. coli* allowed the biochemical characterization of the protein, which showed the expected mannitol 1-phosphate dehydrogenase activity, a very narrow substrate spectrum, and, last but not least, the activity of the recombinant enzyme was stimulated by salt. Collectively, these results make it very likely that EsM1PDH1 is the first functionally verified gene/protein for the biosynthesis of the compatible solute mannitol among brown algae (Rousvoal et al. 2011). This annotated sequence will now help to search for related enzymes in the increasing amount of genome information from different algae and to annotate the occurrence of the mannitol biosynthetic cycle among them.

4.4 Proline Accumulation in Diatoms

The biochemistry and molecular biology of proline biosynthesis among eukaryotic algae is not well understood. As discussed above, this compatible solute is accumulated in cells of different algal lineages, particularly in diatoms and green algae. In contrast to algae, the molecular biology of salt-stress-induced proline accumulation is well-understood for *E. coli* (Kempf and Bremer 1998) and land plants (Verbruggen and Hermans 2008). Proline is usually synthesized using glutamate as precursor. In bacteria three enzymes, γ-glutamyl kinase, glutamic-γ-semialdehyde dehydrogenase, pyrroline 5-carboxylate reductase (P5CR), are converting glutamate via γ-glutamyl phosphate, glutamic-γ-semialdehyde, and pyrroline 5-carboxylate into proline. In plants and other eukaryotes, the enzyme pyrroline 5-carboxylate synthetase (P5CS) comprises the first two enzyme activities and converts glutamate directly into the direct proline precursor pyrroline 5-carboxylate. P5CS is feedback-regulated by proline in plants, while in bacteria the glutamate 5-kinase activity is diminished by accumulated proline. Specific mutations in plant P5CS decreased the feedback inhibition of the enzyme leading to proline overaccumulation and increased salt as well as osmotic stress tolerance (reviewed in Kishor et al. 2005). The Gram-positive bacterium *Bacillus subtilis* also uses proline as major compatible solute. Genetic analysis revealed that different enzymes were used for the anabolic proline biosynthesis or the salt-induced proline accumulation (Brill et al. 2011) ensuring the stress proportional regulation of the proline biosynthesis on genetic and biochemical level independently from the proline demand of cells for the normal growth.

There is a second proline biosynthesis route starting from ornithine, which is used by organisms having the urea cycle, i.e. animals. The enzyme ornithine cyclodeaminase converts ornithine directly into proline. The appearance of the first genome sequences from diatoms allowed to annotation of metabolic pathways to these ecological important organisms (Kroth et al. 2008). These searches revealed that diatoms have not only the plant-like proline biosynthetic pathway, but that diatoms also perform an active urea cycle from which ornithine can be used for the animal-like proline biosynthesis (Allen et al. 2011). It was also shown that this ornithine-based proline biosynthesis pathway clearly originates from the animal-like host of heterokonta, which received the plastid via a secondary endosymbiotic event. Initial investigations of gene expression and of proline biosynthesis showed conflicting results. One of this early studies indicated that the urea-cycle may be the main source for salt-stimulated proline biosynthesis due to an increased expression of the ornithine cyclodeaminase gene (Krell et al. 2007), whereas another study reported an increased expression of the P5CS gene from the plant-like pathway (Mock and Junge 2007). Recently, a study including gene knockdown approaches revealed that rather the ornithine pathway plays the main role for salt-induced proline accumulation in the diatom *Phaeodactylum tricornutum* (Allen et al. 2011).

5 Concluding Remarks

The increasing amount of genome information will allow receiving a much better picture on salt acclimation among the diverse groups of algae in the near future. The boost of knowledge on salt acclimation after publishing the *Ectocarpus* genome represents an impressive example. For this model organism, a system biology view on metabolism and its variability under environmental stresses is emerging including acclimation to salt (Dittami et al. 2011b; Tonon et al. 2011). The comprehensive analysis of genetic basis for salt resistance also will stimulate studies on the evolution of this trait, i.e. whether or not high salt tolerance was a primary feature or only secondary acquired (discussed by Dittami et al. 2012; Blank 2013). To accomplish such studies, the development of an efficient genetic system would be high value for marine model microalgae, which would allow the directed mutation or silencing of genes putatively involved in compatible solute biosynthesis as well as the overexpression of foreign genes for high salt tolerance. First examples of successful genetic manipulation were published for *Cyanidioschyzon merolae* (unicellular red alga; e.g. Imamura et al. 2009) or *Phaeodactylum tricornutum* (diatom; e.g. Allen et al. 2011; Haimovich-Dayan et al. 2013), two model microalgae with known genome sequence.

Moreover, the detailed knowledge on salt acclimation strategies also will become of value for the biotechnological applications of microalgae. As discussed above, mass cultivation of microalgae for biotechnological purposes will certainly be done in seawater, because freshwater is becoming a limiting resource on our planet. Therefore, the optimization of salt acclimation, for example by introducing novel compatible solute biosynthesis that are metabolically cheaper or less interfering with the pathways for wanted products, will receive high attention in the future generation of real production strains. These requirements will certainly revitalize the research on mechanisms for salt acclimation among microalgae.

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Effects of Global Change, Including UV and UV Screening Compounds

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1 Introduction

More than 70 % of the Earth surface is covered by water and more than 99 % of this is constituted by the oceans (Charette and Smith 2010). The marine ecosystems provide food for the growing human population and moderate extreme temperature increases. Even though macroalgae are more obvious to the casual observer, the vast majority of the biomass producers in marine habitats is represented by prokaryotic and eukaryotic microorganisms which include bacterioplankton, cyanobacteria and phytoplankton (Häder et al. 2007a). The standing crop of these organisms constitutes only 1 % of the biomass of all terrestrial ecosystems combined, but their productivity equals that of all land plants taken together (Tardent 2005). This is due to the fact that, depending on the environmental conditions, these cells can divide as often as once every few hours, and also microalgae are known to be among the fastest growing plants.

The CO_2 concentration in the air has increased from about 280 ppm at 1880 to a value above 400 ppm today due to fossil fuel burning and tropical deforestation, to name just the two major sources (Etheridge et al. 1996; NOAA 2013). CO_2, being a greenhouse gas, causes global increases in terrestrial and aquatic temperatures. Additionally, large quantities of CO_2 are released from the Arctic permafrost soils, which are thawing because of the fast rising temperatures in the area (Cory et al. 2013). The simultaneous release of methane from the same areas due to the melting of the Arctic permafrost soil (Cory et al. 2013) augments this increase because this gas is an about 20 times more potent greenhouse gas (Bruggemann et al. 2009; Stohl et al. 2009). The marine ecosystems absorb about the same amount of CO_2 from the atmosphere as terrestrial ecosystems (Falkowski 1997), and being a major sink of atmospheric CO_2 play a major role in regulating the CO_2 concentration in the air (Chester and Jickells 2012).

Currently an estimated amount of 9.5 billion tonnes of carbon are annually released. However, this amount is not found in the atmosphere. So it must be assumed that part of this is sequestered in global sinks. Part of this is stored in terrestrial sinks in the form of wood and plant litter. In addition, marine primary producers absorb about one million tonnes of CO_2 per hour (Gao et al. 2012b) which sums up to about 50 Gt carbon per year (Falkowski 2002; Sabine et al. 2004). Part of this absorbed CO_2 is removed from the system due to the effect of what is called the biological pump (Behrenfeld et al. 2006; Girard 2013): part of the absorbed CO_2 sinks to the deep sea bottom when the organisms decay or are consumed by primary and secondary consumers which release part of the bound carbon dioxide in the form of fecal pellets. The amount of organic matter taken out of the system, called oceanic snow, can be estimated by measuring the sedimenting material in traps deployed at a given depth (Witherow and Lyons 2008). The CO_2 in deep sea sediments is stored there for hundreds of thousands of years (Archer and Maier-Reimer 1994). By this mechanism the oceans moderate an otherwise even faster global warming.

In addition to playing a significant role in modifying global climate change, phytoplankton are major producers at the basis of freshwater and marine food webs feeding fish, crustaceans, mollusks and other consumers (Häder et al. 1998). During the past five decades fish consumption has increased faster than the human population, and fish production from capture and aquaculture will exceed that of meat during the next decade (FAO 2012). Marine products are a vital subsistence for about one billion people in Asia alone (UNEP 2006). Tourism and recreation are further significant aspects of aquatic ecosystems. For example, coral reefs are

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estimated to produce an annual profit of close to 10 billion US$ (Cesar et al. 2003).

The productivity of phytoplankton depends on various external chemical and physical factors. Light is the single most important factor for marine and freshwater aquatic habitats driving photosynthesis in photosynthetic bacteria including cyanobacteria, phytoplankton and macroalgae. Consequently these organisms live in the photic zone, the water column between the surface and a depth where solar radiation has decreased to 1 %; this lower limit defines positive net photosynthesis.

Terrestrial plants are adapted to a habitat with a defined light level only modulated by the circadian rhythm and changing cloud cover. In contrast, phytoplankton need to adapt to rapidly changing irradiances as they are passively moved within the mixing layer by wind and waves (Häder 1997b). However, some phytoplankton are capable of active vertical migration superimposing the passive movement using cilia or flagella or by changing their buoyancy (Häder 1995). Active vertical motility is controlled by positive and negative phototaxis (movement toward or away from the light at the surface) (Neale et al. 1998b; Häder 2004) as well as positive and negative gravitaxis (movement toward or away from the center of gravity of the Earth), respectively (Häder et al. 2005). The active and passive movement in the water column results in rapid changes in the level of photosynthetically active radiation (PAR, 400–700 nm). Simultaneously the cells are exposed to variable irradiances of solar UV radiation, which can have negative effects on morphological and physiological processes and can cause DNA damage (Häder et al. 1998). By their oriented movements within the water column the cells try to optimize the light conditions for photosynthesis and to diminish exposure to detrimental UV radiation (Gallegos et al. 1983). On a population level these detrimental effects inhibit primary productivity as shown by model calculations in an Arctic bay (Wängberg et al. 2006) but not food quality as found in Arctic diatoms (Leu et al. 2007).

Temperature is another vital environmental factor controlling phytoplankton productivity (Raven 1991). Most biochemical processes are temperature-dependent and only a few organisms thrive below freezing temperature. Enzymatic repair mechanisms are also temperature-dependent. E.g. the repair of cyclobutane dimers, induced by UV-B radiation in the DNA, is facilitated by an enzymatic process and therefore is more effective at higher temperatures (Häder et al. 2003; Lamare et al. 2006). Other important environmental factors controlling productivity of phytoplankton are pH, salinity (Roncarati et al. 2008), nutrient availability (Villafañe et al. 1991), competition and predator pressure (Hessen et al. 2004).

Most of these important chemical and physical environmental factors change under climate change scenarios, including increasing temperature, ocean acidification and increasing CO_2 concentrations as well as nutrient availability (Schindler and Smol 2006; Gao et al. 2012b) and increasing exposure to damaging solar UV-B radiation caused by stratospheric ozone depletion (Ferreyra et al. 2006).

Visible and UV radiation, CO_2 supply, temperature, pH and salinity are well known physical and chemical parameters affecting photosynthesis in primary producers. But their interactions are not well understood (Gao et al. 2012a). These interactions can only be revealed by multifactorial analysis (Boyd 2011; Häder 2011). As mentioned above, most studies have been performed in short-term experiments under controlled conditions in the laboratory. The impact of fast changing solar radiation, temperature and nutrient supply can only be revealed in the open ocean (Gao et al. 2012a). This is not an easy task due to the large areas to be studied and the low phytoplankton concentrations in the ocean. Long-term studies and multi-generation experiments need to be carried out to reveal physiological and genetic adaptations of the organisms to changing environmental conditions (Hays et al. 2005).

It is known that different taxa possess different capabilities to adapt to changing conditions such as temperature (Huertas et al. 2011), and therefore changes in the species composition will occur with far reaching consequences for the extensive food webs.

Another ambitious goal of future research will be to disentangle the various feedback mechanisms; for example, higher surface temperatures could create a heavier cloud cover, resulting in lower exposure of phytoplankton to excessive UV and PAR. Both macroalgae and phytoplankton species synthesize the organosulfur dimethylsulfoniopropionate (DMSP, $(CH_3)_2S^+CH_2CH_2COO^-$), which serves as an osmolyte. Upon excretion this zwitterionic metabolite can be metabolized to dimethylsulfide (DMS, CH_3SCH_3) (Karsten et al. 1990; Merzouk et al. 2004). Once this substance enters the atmosphere it functions as cloud nuclei (Buckley and Mudge 2004). Higher temperatures favor the proliferation of DMSP-producing phytoplankton which may result in denser cloud formation.

In both Arctic and Antarctic waters light exposure is diminished by dissolved and particulate organic and inorganic material by runoff during ice melting (Schindler and Smol 2006). The massive increase in temperature observed in the Polar Regions results in considerable changes in transparency of the water. In order to understand future changes in the Arctic and Subarctic due to global warming, models have been produced for small lakes using dissolved organic carbon (DOC) concentrations, weather conditions and water acidification which all affect the light penetration and thus productivity of the phytoplankton (Keller et al. 2006; Paterson et al. 2008).

2 Increasing Atmospheric CO_2 Concentrations and Ocean Acidification

It is well known that the CO_2 concentration in the atmosphere constitutes a bottleneck for the efficiency of photosynthesis in terrestrial plants (Ziska and Bunce 2006). Many experiments have demonstrated that an increase of CO_2 in the atmosphere augments growth and productivity. Therefore CO_2 is applied to many vegetable crops in commercial greenhouse cultures (Jones 2008). Increasing the atmospheric CO_2 concentration should also augment the CO_2 availability in the surface layers of the oceans and thus enhance photosynthetic activity. As a consequence the productivity of the whole oceanic food webs should increase (Schippers et al. 2004; Hutchins et al. 2007; Riebesell and Tortell 2011). This hypothesis was not confirmed by the experimental results. The reason for this is that most cyanobacteria and phytoplankton have developed CO_2 concentration mechanisms using a carbonic anhydrase. This enzyme increases the internal CO_2 concentration to far higher levels than the external available concentration, thus mitigating the bottle neck (Aizawa and Miyachi 1986; Sültemeyer 1998; Bozzo and Colman 2000). For instance, it has been found that the coccolithophorid *Emiliania huxleyi*[1] increases it's intracellular CO_2 concentration tenfold above that in the surrounding water (Hayakawa et al. 2003). This is however not a general feature since this concentration mechanism was not found in another coccolithophorid (Nimer and Merrett 1992; Israel and Gonzalez 1996). The CO_2 concentration mechanism results in only a small enhancement of productivity when the CO_2 concentration increases in the water column. This was confirmed in a diatom-dominated phytoplankton association in coastal Pacific waters where no net increase in photosynthesis was found (Tortell et al. 2000). Increasing the water CO_2 concentrations down-regulates the carbonic anhydrase (Wu et al. 2010; Hopkinson et al. 2011). This finding also does not represent a universal rule, since in another diatom increasing CO_2 concentrations augmented photosynthesis, but this was partially compensated by higher respiratory and photorespiratory losses (Wu et al. 2010; Gao et al. 2012b). Thus, it is still controversial whether increasing atmospheric CO_2 concentrations augment phytoplankton net productivity in aquatic ecosystems (Riebesell and Tortell 2011).

Seawater is usually well buffered in the range between pH 7.5 and 8.4. Increasing the atmospheric CO_2 and uptake into surface waters has acidified the ocean since it forms carbonic acid with water, which dissociated into bicarbonate and releases protons (Doney et al. 2009).

$$CO_2 + H_2O \rightarrow H_2CO_3$$

$$H_2CO_3 \rightarrow H^+ + HCO_3^-$$

$$HCO_3^- \leftarrow H^+ + CO_3^{2-}$$

By now the pH has been lowered by about 0.1 units. This does not seem much, but means an increase in the proton concentration by 30 % (Caldeira and Wickett 2003a, b). If the atmospheric CO_2 concentration will increase to the values assumed by the IPCC A1F1 model we expect atmospheric CO_2 concentrations between 800 and 1000 ppm by the end of the century, boosting the pH reduction to 0.3–0.4 units, corresponding to an increase in the proton concentration by about 100–150 % (Hein and Sand-Jensen 1997; Gao et al. 2012b). Acidification of the water column affects biological processes and furthermore hinders the uptake and availability of minerals such as iron and ammonium (Hutchins et al. 2009; Shi et al. 2010; Beman et al. 2011).

Many phytoplankton and macroalgae, as well as animals such as mollusks, worms, corals and echinoderms use the process called calcification to produce exo- or endoskeletons which consist of calcium carbonate (Zondervan et al. 2001)

$$2HCO_3^- + Ca^{2+} = CaCO_3 + CO_2 + H_2O$$

These incrustations protect the organisms from predators, mechanical damage as well as excessive solar UV radiation. Decreasing pH interferes with this calcification.

Coccolithophorids are major phytoplankton organisms in the oceans. They produce coccoliths, which are small plates made of $CaCO_3$ which cover the cells. Biosynthesis of these coccoliths is hindered by acidification (Delille et al. 2005; De Bodt et al. 2010; Beaufort et al. 2011). Other photosynthetic organisms are likewise affected (Israel and Gonzalez 1996; Riebesell et al. 2000; Riebesell and Tortell 2011). *Corallina* as well as other marine macroalgae use calcification to support their thallus structure; this is also disturbed by acidification (Gao et al. 1993; Langer et al. 2006; Gao and Zheng 2010). In addition to excessive heat stress, corals suffer from acidification, which is also a problem for many mollusks (Fine and Tchernov 2007; Anthony et al. 2011; Melzner et al. 2011; Sinutok et al. 2011).

Calcified exoskeletons absorb and scatter UV radiation and thus protect the organisms form excessive solar UV, and acidification augments the damage by short-wavelength radiation. In the prymnesiophyte *E. huxleyi*, the coccoliths have been found to reduce solar UV by 26 % (Gao et al. 2009). At ambient CO_2 concentrations solar UV stimulates the formation of $CaCO_3$ coccoliths in *E. huxleyi*, increasing the protection against UV radiation. Since this is a metabolic burden for the cell, this results in a reduced growth rate

[1]Wherever possible the currently accepted names for species are used. The name used in the paper cited is also indicated. For details of names see Chap. Systematics, Taxonomy and Species Names: Do They Matter? of this book (Borowitzka 2016).

(Guan and Gao 2010). In *Phaeocystis globosa* the photochemical yield and the growth rate are affected by acidification; simultaneously applied UV-B radiation synergistically further reduced these functions (Chen and Gao 2011).

The effects of ocean acidification have been studied mostly in the laboratory using short-term experiments (Gao et al. 1991, 1993, 1999). Consequently, little knowledge is available for natural ecosystems; but it can be assumed that drastic consequences will occur (Turley et al. 2010). Other climate change effects such as increased temperature and a shoaled mixing layer will contribute to this development (Hays et al. 2005; Wu et al. 2008; Capotondi et al. 2012). Acidification and increased UV radiation will affect the marine ecosystems (Boyd et al. 2010; Boyd 2011). Given the relatively short lifetime of phytoplankton, genetic alterations may occur which might allow the organisms to adapt to the new conditions with lower pH values in the ocean (Collins et al. 2006). Since this is speculative, predictions regarding phytoplankton productivity and species composition due to global climate change are still limited (Steinacher et al. 2010; Tagliabue et al. 2011).

3 Effects of Temperature on Phytoplankton Productivity

Temperature is one of the most influential physical variables for phytoplankton productivity. Only few organisms are capable of converting inorganic CO_2 into organic material at temperatures below 0 °C (Neori and Holm-Hansen 1982; Davey 1989; Krause and Somersalo 1989). A useful description of the enhancement of biochemical processes by a temperature increase of 10 °C is the Q10 value. Organisms reach an optimum at a species-specific temperature beyond which photosynthetic biomass production decreases again (Zou et al. 2011). Respiration and photorespiration usually further increase with rising temperatures. Enhanced CO_2 concentrations as well as acidification likewise boost respiration as was shown in the diatom *Phaeodactylum tricornutum* (Wu et al. 2010). Temperatures well above the optimum can kill aquatic organisms; the large scale coral dying has been attributed to temperatures exceeding 36 °C (Fitt et al. 2001).

Ocean surface temperatures have risen by 0.13 °C per decade since 1900 and a further rise of 2–3 °C is predicted for the end of this century (Boyd and Doney 2002). Because of the large buffer capacity of the oceans this increase is only half of that on land. Short-term effects such as El Niño cause a warming of the Pacific Ocean and generally higher global temperatures, while La Niña events have the opposite effect. The increased temperatures affect the water column down to 700 m; this influences the ocean currents and the global weather (Fischetti 2013). This warming also affects most organisms, since they dwell in the upper 400 m. It also substantially governs phytoplankton growth and thus productivity of the aquatic food webs (Halpern et al. 2008).

On subtidal rocky habitats algal assemblages are predicted to show higher productivity at higher temperatures and CO_2 concentrations (Russell et al. 2009). Since different species have different temperature optima and thermal windows, increased temperatures will result in altered species composition with prominent effects on the trophic food web (Raven and Geider 1988; Davison 1991; Pörtner and Farrell 2008); e.g., the cyanobacterium *Synechococcus* has a higher productivity at enhanced temperatures and CO_2 concentrations, whereas in *Prochlorococcus* this is not found, so that the former species has an advantage with increasing temperatures and CO_2 concentrations (Fu et al. 2007). Growth and nitrogen fixation were markedly augmented in the cyanobacterium *Trichodesmium* when the temperature was increased by 4 °C (Hutchins et al. 2007; Levitan et al. 2010). Likewise in the prymnesiophyte *E. huxleyi*, which had been isolated from the Sargasso Sea, productivity was enhanced by both increased temperatures and higher CO_2 concentrations (Feng et al. 2008). However, enhanced temperatures affected calcification in both *E. Huxleyi* (De Bodt et al. 2010) and in the coccolithophorid *Syracosphaera pulchra* (Fiorini et al. 2011). Growth and productivity was also boosted in a natural North Atlantic spring bloom assemblage when temperature and CO_2 were increased simultaneously (Feng et al. 2009); also in this case calcification of the cells was notably lower. The same is true for the calcified chlorophyte *Halimeda* in response to acidification and enhanced temperatures; in addition, chlorophyll content and photochemical yield were reduced (Sinutok et al. 2011). Maximal photosynthetic carbon incorporation was boosted in the bloom-forming raphidophyte *Heterosigma akashiwo* only when CO_2 concentration and temperature were increased simultaneously. In contrast, in the dinoflagellate *Prorocentrum minimum* (= *Prorocentrum chordatum*) only increased CO_2 had an effect (Fu et al. 2008).

These and other differences between species will result in changes in the species composition of natural phytoplankton communities due to global climate change. During a cruise in the Bering Sea incubation studies of natural phytoplankton assemblages showed a shift from diatoms to nanoflagellates when the temperature was enhanced (Hare et al. 2007). The same phenomenon was found in a diatom-dominated North Atlantic bloom (Feng et al. 2009). In Daya Bay (China) a shift from diatoms to dinoflagellates was observed caused by heating of the water by a nuclear power plant (Li et al. 2011). It is not clear if these effects are directly due to the higher temperature or temperature-induced changes of the nutrient cycling within the mixing layer (Wohlers-Zöllner et al. 2011).

UV-B radiation hits a wide range of targets in phytoplankton. In the photosynthetic electron transport chain the D1 protein in photosystem II is a main target of short-wavelength

radiation (Wängberg et al. 1996; Bouchard et al. 2005b, 2006). In addition, UV-B damages the DNA. This wavelength range induces the formation of cyclobutane dimers (CPD) (Sinha et al. 2001a; Klisch et al. 2005). In addition, UV-B can cause the generation of reactive oxygen species (ROS) which in turn damage cellular components (Frost and Xenopoulos 2002; Ferreyra et al. 2006; Pelletier et al. 2006; Roncarati et al. 2008). CPDs can be eliminated by the enzyme photolyase, which uses the energy of blue light or UV-A photons (Buma et al. 2001; Helbling et al. 2001; van de Poll et al. 2001). When phytoplankton are passively circulated within the upper mixed layer they are exposed to excessive solar UV-B radiation near the surface which causes the formation of CPDs. The repair occurs when the cells are transported downward out of the excessive UV radiation (Helbling et al. 2008). The activity of the photolyase is increased by higher temperatures (Häder et al. 2007a; Gao et al. 2008).

UV-B-induced damage of the photosynthetic apparatus is repaired by replacing the affected D1 molecules. The resynthesis of these proteins is also enhanced by higher temperatures (Sobrino and Neale 2007; Gao et al. 2008; Halac et al. 2010; Helbling et al. 2011). Due to temperature increases in the water column changes in abundance and habitat selection have already been observed in the major phytoplankton groups such as dinoflagellates (Peperzak 2003; Cloern et al. 2005; Hallegraeff 2010), diatoms and coccolithophorids (Mericoa et al. 2004; Hare et al. 2007). Higher temperatures also enhanced growth and changes in species composition in an assemblage of phytoplankton and zooplankton in a mesocosm experiment (Lewandowska and Sommer 2010).

In coastal waters the situation is more complicated because in these habitats CO_2 concentration and temperature show marked daily changes which affect phytoplankton productivity. Hinder and coworkers (2012) found a substantial decrease in dinoflagellates between 1960 and 2009 in the northeast Atlantic and North Sea which they believe is due to ocean warming and changed wind patterns.

Over the last few decades the temperature increase has been most extreme in the Arctic significantly boosted by a feedback mechanism. Snow and ice reflect most of the solar radiation back into space. When they melt water and soil absorb a larger fraction of the incoming radiation which results in a significant additional warming (Deser et al. 2000). As a result, ice melting occurs earlier and freezing later in the year. During the summer the sea ice-covered area in the Arctic has decreased by almost 50 % from the long-time average between 1979 and 2000 (http://nsidc.org). This marked temperature increase results in higher phytoplankton productivity, and even tropic *Radiolaria* have been found recently to invade the Arctic ocean (http://earth.columbia.edu).

In addition to earlier melting and later freezing, the thinning of the remaining sea ice exposes the phytoplankton to excessive visible and detrimental solar UV radiation. This is partly offset by lower transparency of the water due to higher terrestrial runoff with high dissolved organic matter (DOM) concentrations (Carmack 2000). One novel phenomenon struck the scientists when they first observed it: When the ice melts it creates pools on the surface which act like "skylights" or greenhouse windows, causing an explosion of phytoplankton blooms below (http://earth.columbia.edu). A similar phenomenon was recorded in Antarctica: In spring large phytoplankton blooms develop under the ice the formation of which is seeded by cells trapped in the ice during winter (Lancelot et al. 1993; Kang et al. 2001). Sea water is diluted by freshwater from thawing ice and glaciers. This effect also shoals the thickness of the upper mixing layer, so that phytoplankton are exposed to higher PAR and UV solar radiation. In addition, it decreases nutrient availability (Steinacher et al. 2010).

4 Underwater Light Climate

Light is the single most important factor for photosynthesis in primary producers. Both terrestrial and aquatic plants are adapted to the daily light and dark cycle and the changing cloud cover. Terrestrial plants are adapted to shade or sun conditions and the different irradiances as well as day length depending on the latitude. Most macroalgae are fixed in their position and are adapted to the ambient light conditions. Furthermore, the light climate is subject to the tidal rhythm which does not coincide with the daily light-dark cycle (Jiménez et al. 1998). Another factor modulating the irradiance of the transmitted light is the transparency of the water column. Most macroalgae are shade plants adapted to low light conditions. When exposed to excessive irradiation, e.g. during low tide, they shut down the photosynthetic quantum efficiency, a phenomenon known as reversible photoinhibition. This mechanism regulates the amount of energy funneled into the photosynthetic electron transport (Häder et al. 2001; Helbling et al. 2010).

Most phytoplankton are, in contrast, not sessile. Therefore they can adapt to the current light conditions by altering their vertical position in the water column. Some organisms rely on active motility such as flagellates (Häder and Griebenow 1988) while others change their buoyancy such as cyanobacteria and diatoms by producing gas vacuoles or synthesizing oil droplets, respectively (Walsby 1987). Actively swimming cells often use phototaxis to guide their vertical movements in the water column. At low light intensities they move toward the surface (positive phototaxis) and at higher intensities they swim downward (negative phototaxis) (Matsunaga et al. 2003). Often the vertical orientation movements are

supported by gravitaxis which brings the cells closer to the surface (negative gravitaxis) (Lebert et al. 1996; Eggersdorfer and Häder 1991; Roberts 2006). When exposed to excessive visible and/or UV intensities many phytoplankton use photo- and graviorientation to move into deeper waters (Lenci et al. 1983; Matsuoka 1983; Josef et al. 2005).

These orientation mechanisms are mostly effective in fresh water ecosystems, but in marine habitats they are often superimposed by passive movement by wind and waves (Helbling et al. 2005; Barbieri et al. 2006). Here phytoplankton are moved within the mixing layer (Yoshiyama and Nakajima 2002). But still then active movement superimposes the passive mixing resulting in typical vertical distribution patterns in the water column (Piazena and Häder 1995).

The light exposure of the phytoplankton is largely affected by the transparency of the water body (Boss et al. 2007). The transparency of the water is determined by dissolved organic matter (DOM) (Vione et al. 2009) or dissolved inorganic matter (DIM) (Xie et al. 2009), or by particulate organic matter (POM) (Mayer et al. 2006) or particulate inorganic matter (PIM) (Vahatalo and Jarvinen 2007). POM includes also the phytoplankton. The transparency is correlated with their concentration (Bracchini et al. 2006; Sommaruga and Augustin 2006). Depending on the properties of the dissolved and particulate substances the attenuation of the light can be neutral or wavelength-dependent (Häder et al. 2007b; Smith and Mobley 2007); e.g., some fraction of DOM specifically absorbs at shorter wavelengths, therefore this fraction is called colored or chromophoric DOM (CDOM) (Osburn et al. 2009).

Eutrophic freshwater ecosystems have higher DOM concentrations than oligotrophic ones. The material enters the water with the runoff from the adjacent land. It consists of organic material and stems from decaying plant litter (Boyle et al. 2009). Also the DOM in coastal marine waters is supplied by terrestrial runoff (Day and Faloona 2009); but also decaying macroalgae contribute to the DOM concentration (Hulatt et al. 2007). In the open ocean DOM concentrations are much lower and stem mostly from decaying phytoplankton (Vahatalo and Jarvinen 2007; Behrenfeld et al. 2009). Only in rare cases swimming macroalgae contribute to the DOM pool as in the Sargasso Sea (Bailey et al. 2008).

In the South Pacific Gyre the hyper-oligotrophic water hardly contains any DOM. This results in exceptionally high UV-B and UV-A penetration into the water column. One percent of the radiation at 325 nm hitting the surface reaches 85 m depth (Tedetti et al. 2007). In the clearest lakes studied, 1 % of the 320 nm radiation reaches 27 m in Lake Tahoe (California-Nevada, USA) (Rose et al. 2009) and 62 m in Crater Lake (Oregon, USA) (Hargreaves et al. 2007).

In order to determine the penetration of UV and PAR radiation, broadband radiometers or spectroradiometers can be used. Furthermore, dosimeters can be applied which use a chemical reaction or the damage of biological molecules such as DNA (Schouten et al. 2007, 2008, 2009). During a transect from Bremerhaven (Germany) to Cape Town (South Africa) erythemal UV irradiances and ozone concentrations in various climate zones have been measured using ship-borne instruments (Wuttke et al. 2007).

The massive temperature increase in both Arctic and Antarctic regions causes a significant input of dissolved and particulate organic and inorganic material from runoff during melting, which modifies the light quality and irradiance (Schindler and Smol 2006). In addition to the changes in the transparency, the timing of the melting changes. Models have been developed for Arctic and Subarctic small lakes using DOC concentrations, acidification and weather patterns to predict future changes as a result of global warming causing changes in the transparency and by that the development of organisms (Keller et al. 2006; Paterson et al. 2008).

The optical properties (irradiance and wavelength distribution) of both freshwater and marine waters are governed by CDOM concentrations. These substances also modify biogeochemical cycles (Zepp et al. 2007; Fernandes et al. 2008). The concentration of CDOM varies seasonally and furthermore depends on rainfall activity, since CDOM is produced by the degradation of organic material from phytoplankton and macroalgae and from terrestrial plants for freshwater and coastal habitats (Suhett et al. 2007). High mountain lakes generally have lower CDOM concentrations and thus higher transparency, since they are located above the tree line and thus have lower input of organic material (Rose et al. 2009). In addition, CDOM concentrations are affected by intense UV radiation, such as in Alpine lakes (Hayakawa and Sugiyama 2008), since it breaks down CDOM into smaller particles which can be easier consumed by microorganisms such as bacteria. As a consequence the bacterioplankton populations increase, further decreasing the DOM concentration and increasing the transparency of the water (Piccini et al. 2009). Especially UV-B breaks the double bonds in humic molecules caused by the strong absorption in this wavelength band. By this mechanism the transparency of the water increases (Hudson et al. 2007). Due to the high UV-B absorption of DOM these molecules have a large impact on the biomass productivity and phytoplankton composition. This has been analyzed in predator-free rivers in the Northern U.S.A. (Frost et al. 2007).

Singlet oxygen ($^1O_2$) is generated when dissolved free amino acids in the water are decomposed in the presence of DOM. This mechanism has been monitored in both Pony Lake, Antarctica and several rivers and lakes in the Northern U.S.A. (Boreen et al. 2008). When exposed to UV CDOM is photodegraded (Feng et al. 2006; Tzortziou et al. 2007;

Zhang et al. 2008). This is the main mechanism for mineralization and carbon recycling of DOM (Feng et al. 2006; Anusha and Asaeda 2008; Wang et al. 2009). This removal of DOM from the water increases the penetration of solar UV-B into the water column which exposes the aquatic organisms to higher short-wavelength irradiances (Feng et al. 2006). On the other hand, photodegradation of plankton and macroalgae makes nutrients such as phosphorus and iron available (Bastidas Navarro and Modenutti 2010; Shiller et al. 2006; Navarro et al. 2009).

The concentration of CDOM can be used to determine the transparency of the water column for UV as well as the water quality as has been used for Lake Taihu, China, an important drinking water reservoir (Zhang et al. 2007b). Seasonal changes in the UV penetration are thought to be realistic indicators for environmental changes confirmed by measurements in the subalpine Lake Tahoe, California-Nevada (Rose et al. 2009).

5 Effects of UV and UV-Screening Compounds

The accumulation of atmospheric pollutants, such as chlorofluorocarbons (CFCs), fluorocarbons (FCs) and organobromides (OBs) etc. has eroded the ozone layer with the consequent increase in the availability of ultraviolet-B (UV-B; 280–315 nm) radiation on the Earth's surface (Crutzen 1992; Stolarski et al. 1992) to a level that affects the biota. In addition to the Antarctic ozone hole, ozone depletion has been reported to increase and spread in a broader range of altitudes and latitudes throughout the world since the late 1970s (Hoffman and Deshler 1991). Weatherhead and Andersen (2006) have reported the depletion of the stratospheric ozone layer at rates up to 4 DU (Dobson units) per decade between 1979 and 1995. The Montreal Protocol reduced the use and production of CFCs to prevent ozone depletion, with an expected decrease in UV-B irradiance during the twenty-first century (UNEP 2010). However, the ozone layer is not likely to recover to 1980 levels within the coming decades (Weatherhead and Andersen 2006). Indeed, the area of the ozone hole over Antarctica reached a maximum in 2006 (NASA 2009) and a record destruction of ozone over the Arctic was observed in 2011 (Manney et al. 2011). The high stability of CFCs is mainly responsible for the slow recovery of stratospheric ozone, and it can take 40–50 years to reach the stratosphere, leading to the persistence of their effect for decades (Llabrés et al. 2013).

Natural production of considerable amounts of reactive nitrogen species (RNS) such as nitric oxide (NO^-), peroxynitrite ($ONOO^-$) and nitrous oxide (N_2O), from unpolluted terrestrial and aquatic ecosystems or from anthropogenic sources also contribute to the depletion of the ozone layer (Kramlich and Linak 1994). UV-B radiation (<1 % of total irradiance), being the most detrimental component of the solar spectrum, has the potential to exert adverse effects on photosynthetic terrestrial and aquatic organisms, thereby affecting the productivity of ecosystems (Karentz et al. 1991a; Vincent and Roy 1993; Williamson 1995; Sinha and Häder 1996). They can penetrate into the water column to ecologically significant depths (Jerlov 1950; Fleischmann 1989; Smith et al. 1992), determined by the optical properties of the column. The variables in the atmosphere and the water that affect the amount of UV radiation and wavelength distribution determine the transmission of solar UV radiation into the water column (Häder et al. 2007b; Smith and Mobley 2007). UV-B penetration is relatively low in coastal waters, where 10 % of the incident UV-B radiation reaches about 0.5 m, in turbid estuarine waters, and 12 m in clear coastal waters (Tedetti and Sempéré 2006). UV-B penetrates much more deeply into clear oceanic waters, where UV-B levels that can cause mortality of photosynthetic plankton have been reported down to 60 m in the subtropical Atlantic Ocean (Llabrs and Agustí 2006) and to 26 m in the Mediterranean Sea (Llabrés et al. 2010).

Investigations have shown that UV-B irradiation adversely affects key physiological and biochemical life processes, such as morphology, cell differentiation, survival, growth, pigmentation, motility and orientation, N_2 metabolism, phycobiliprotein composition, protein profile, DNA, CO_2 uptake and membrane permeability (Gao et al. 2007b; Lesser 2008; Sinha et al. 2008a) of various photosynthetic organisms as they are simultaneously exposed to visible and UV radiation in their natural habitats. However, to counteract the damaging effects of harmful radiation, organisms have developed certain mitigation strategies such as avoidance, scavenging by enzymatic and non-enzymatic antioxidants (Middleton and Teramura 1994; Singh et al. 2013), and screening by certain UV-absorbing compounds like mycosporine-like amino acids, scytonemin and sporopollenin (Karentz et al. 1991b; Dunlap and Shick 1998; Sinha et al. 1998). Besides this, repair of UV-induced damage of DNA by photoreactivation and excision repair and resynthesis of proteins (Britt 1995; Kim and Sancar 1995) are also important mechanisms to prevent UV-induced photodamage. The following sections provide an overview on the effects of UVR on cyanobacteria, phytoplankton and macroalgae and the role of photoprotective compounds in mitigating UV-B toxicity.

5.1 Effect of UV-B Radiation on Cyanobacteria

Cyanobacteria, the largest group of Gram-negative, oxygenic photoautotrophic prokaryotes, have cosmopolitan distribution ranging from Arctic to Antarctic regions and are

important biomass producers in both aquatic and terrestrial ecosystems (Stanier and Cohen-Bazire 1977; Häder et al. 2007a). Members of cyanobacteria are valuable sources of various natural products of medicinal and industrial importance (Rastogi and Sinha 2009). In addition, these ecologically important organisms are a dominant flora of wetland soils, especially in rice-paddy fields where they contribute to the fertility as natural biofertilizers (Vaishampayan et al. 2001) by virtue of their ability to fix atmospheric nitrogen using the enzyme nitrogenase. Harvesting of solar energy to perform photosynthesis and nitrogen fixation exposes these cyanobacteria to lethal doses of UV-B and UV-A (315–400 nm) radiations in their natural lit habitats. The highly energetic ultraviolet radiation (UVR) directly or indirectly affects the aquatic cyanobacteria as they can penetrate deep into the water column – up to 20 m in the ocean and to a few centimeters in lakes and rivers. The fluence rate of UV-B radiation impinging on the natural habitats seems to be of major concern since UV-B radiation has been reported not only to impair motility and photoorientation (Donkor and Häder 1991) but also to affect a number of physiological and biochemical processes in cyanobacteria (Sinha et al. 2008a; Singh et al. 2010a). He and Häder (2002b, c) reported UV-B and UV-A-induced reactive oxygen species (ROS) production for the first time in cyanobacteria using a ROS-sensitive probe, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA).

Growth and survival of cyanobacteria have been reported to be severely affected following UV-B irradiation for different durations. Complete killing and loss of survival was reported in several cyanobacteria within 120–180 min of UV-B exposure (Tyagi et al. 1992; Sinha et al. 1995a). The growth of the cyanobacteria *Oscillatoria priestleyi* (=*Phormidium pseudopriestleyi*) and *Phormidium murrayi* (=*Wilmottia murrayi*) was suppressed by 100 % and 62 %, respectively, following exposure to the same UV-B dose (Quesada and Vincent 1997). Inhibition of growth in the toxic bloom-forming cyanobacterium *Microcystis aeruginosa* FACHB 912 and the non-toxic *Microcystis aeruginosa* FACHB 469 following UV-B exposure has been reported by Zhang et al. (2012). Similarly, Han et al. (2003) reported inhibition of growth in a rice-field cyanobacterium *Anabaena* sp. during exposure to PAR + UVR while it was inhibited by up to 40 % by solar UVR in *Anabaena*sp. PCC 7120 (Gao et al. 2007b). Cyanobacteria such as *Scytonema* sp. and *Nostoc commune*, the filaments of which are embedded in a mucilaginous sheath, have been reported to be more tolerant than *Anabaena* sp. and *Nostoc* sp., the filaments of which do not possess such covering (Sinha et al. 1995a). The cellular constituents having absorption maxima in the range of 280–315 nm are destroyed by UV-B radiation which ultimately affects the cellular membrane permeability and induces protein damage eventually resulting in the death of the cell (Vincent and Roy 1993; Sinha et al. 1997).

Cellular differentiation, i.e., differentiation of vegetative cells into heterocysts and akinetes in filamentous cyanobacteria has been reported to be adversely affected by UVR. Exposure of cells to UV-B radiation has been shown to delay the differentiation of vegetative cells into heterocysts and akinetes in *Anabaena aequalis* (Blakefield and Harris 1994). In *Anabaena* sp. PCC 7120 differentiation of heterocysts has also been reportedly suppressed by solar UVR (Gao et al. 2007b) and alteration in the C:N ratio following UV-B exposure was suggested to be responsible for the altered spacing pattern of heterocysts in the filament (Sinha et al. 1996). The spiral filaments of *Arthrospira platensis* have recently been reported to be broken and compressed under solar UVR (Wu et al. 2005; Gao et al. 2008). The filaments of the cyanobacterium *Anabaena flos-aquae* were also broken by UVR while PAR had no effect (Singh et al. 2010a). In addition, major heterocyst polypeptides of around 26, 54 and 55 kDa have been shown to be decreased in concentration following UV-B irradiation, suggesting that the multilayered thick wall of heterocysts may be disrupted resulting in the inactivation of the nitrogen fixing enzyme nitrogenase by the penetrating oxygen.

Investigations on photobleaching of photosynthetic pigments of cyanobacteria have shown that the accessory light-harvesting pigment phycocyanin (λ_{max} 620 nm) was bleached more rapidly and drastically than any other pigment such as Chl *a* (λ_{max} 437 and 672 nm) or the carotenoids (λ_{max} 485 nm) (Sinha et al. 1995a, b). High PAR treatment was found to decrease the phycocyanin level by 93 %, the allophycocyanin level by 75 % and the phycoerythrin level by 69 % in *Nostoc spongiaeforme*(=*Nostoc carneum*) (Bhandari and Sharma 2006). UV-B irradiation has been found to be responsible for a decrease in the phycobiliprotein content and a disassembly of the phycobilisome complex in a number of cyanobacteria (Sinha et al. 1995b, 1997). The pattern of fluorescence emission spectra of phycobiliproteins observed after UV-B irradiation from these studies also suggested an impairment of the energy transfer from the accessory pigments to the photosynthetic reaction center. Sinha and Häder (2003) showed a loss of αβ monomers of phycocyanin, rod-core and core-membrane linker polypeptides after 1 h of UV-B irradiation using SDS-PAGE. Sinha et al. (2005) reported that the exposure of cyanobacterial cultures to intense UV-B radiation causes bleaching of phycocyanin and phycoerythrin. Reports have shown that UV-B radiation also influences the chlorophyll and carotenoids contents in cyanobacteria (He and Häder 2002b; Han et al. 2003; Gao et al. 2007b; Lesser 2008). Kumari (2010) also showed a drastic decline in the absorbance at 620 nm after 30 and 60 min of UV-B irradiation in *Nostoc* sp. HKAR-2 and *Scytonema* sp. HKAR-3, respectively. She also reported a distinct decrease

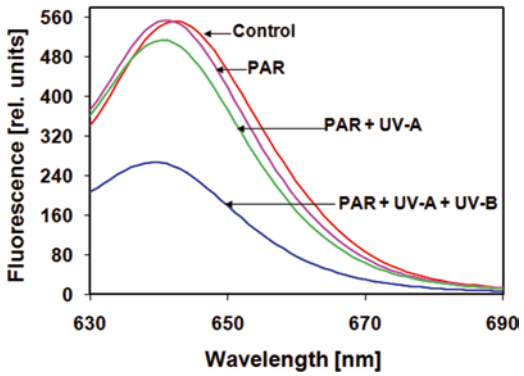

Fig. 1 Fluorescence emission spectra of phycocyanin of *Nostoc* sp. HKAR-2 under various radiation conditions when excited at 620 nm (Modified from Kumari 2010)

in fluorescence of phycobilisomes after 3 h of UV-B irradiation using fluorescence spectroscopy (Fig. 1).

Certain photosynthetic parameters such as $^{14}CO_2$ uptake, O_2 evolution and ribulose-1,5 bisphosphate carboxylase (RUBISCO) activity in cyanobacteria has been shown to be down-regulated by UVR (Sinha et al. 2008b). The D1 and D2 proteins, major constituents of PS-II reaction centers, are also degraded even after exposure to intermediate levels of UV-Bradiation (Sass et al. 1997; Campbell et al. 1998). Zhang et al. (2012) observed a decrease in the content of allophycocyanin or phycocyanin following UV-B exposure. Several groups of mRNAs specifying proteins involved in light harvesting and photosynthesis were also reportedly down-regulated by UV-B treatment, suggesting a reduced photosynthetic activity following UV-B exposure (Huang et al. 2002). The process of nitrogen fixation is also severely inhibited by UV-B radiation due to the extreme sensitivity of the nitrogenase enzyme (Tyagi et al. 1992; Kumar et al. 2003; Lesser 2008). Complete loss of nitrogenase activity within 25–40 min of UV-B exposure was reported in several rice-field cyanobacteria (Kumar et al. 2003). UV-B radiation also induces the lipid peroxidation of polyunsaturated fatty acids (PUFA) via oxidative damage which subsequently affects the integrity of cellular and thylakoid membranes (He and Häder 2002a).

Proteins are one of the main targets of UV-B as the total protein profiles of several cyanobacteria showed a linear decrease in protein content with increasing UV-B exposure time. Kumar et al. (1996a) reported complete loss of protein bands between 14.2 and 45 kDa after 90 and 120 min of UV-B exposure in *Nostoc calcicola*. Similarly, Ehling-Schulz et al. (2002) monitored the proteome change following UV-B exposure using two-dimensional gel electrophoresis in the cyanobacterium *Nostoc commune* and reported that out of 1350 protein spots, 493 were changed by UV-B radiation. Translation activity in *Nostoc* sp. was reportedly inhibited by UVR as indicated by the decreased incorporation of L-[35S]-methionine following exposure to UVR (Araoz et al. 1998). Translation activity was also suppressed by oxidative stress in *Synechocystis* sp. PCC 6803 and oxidation of the elongation factor EF-G was found to be responsible for the inhibited translation activity (Kojima et al. 2007). Aromatic amino acids such as tryptophan, tyrosine, phenylalanine and histidine, having absorption maxima in the UV range (270–290 nm) are also damaged by UV-B radiation (Sinha et al. 1996, 1998).

UV-B radiation has been reported to induce a variety of DNA damage. The main DNA lesions which develop by direct UV-B absorption are dimeric photoproducts such as *cis-syn* cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4 PPs) and their Dewar isomers (Sinha and Häder 2002; Häder and Sinha 2005; Singh et al. 2010a). UVR-induced formation of thymine dimers in three rice-field cyanobacteria, *Anabaena*, *Nostoc* and *Scytonema* sp., was detected using blotting and the chemiluminescence method (Sinha et al. 2001a). It was also found that the frequency of thymine dimers increased with the increase in UVR exposure time, and after 120 min of exposure it reached 35–40 T^T/Mbp in all three studied cyanobacteria. Kumari (2010) reported that UV-B had the most pronounced effects on the formation of thymine dimers in comparison to UV-A and PAR irradiations in *Nostoc* sp. HKAR-2, where the dimer formation started after 30 min of UV-B irradiation, and that continued to increase with increasing duration of irradiations (Fig. 2). UV radiation has been reported to cause single- and/or double-stranded breaks in the native DNA molecules in various cyanobacterial species (He and Häder 2002a). A rapid decrease in the photosynthetic activity of *Scytonema javanicum* occurs after irradiation with UV-B (Chen et al. 2013). Li et al. (2012) showed that UVR irradiation, mainly UV-B, has a detrimental effect on the phosphatase activity of the terrestrial cyanobacterium *Nostoc flagelliforme*.

5.2 Mitigation Strategies in Cyanobacteria

To counteract the damaging effects of UV-B radiation, cyanobacteria have evolved several lines of defense mechanisms (Vincent and Roy 1993; Sinha et al. 1998; Cockell and Knowland 1999). These include avoidance by migration down into the water column with reduced light exposure (Häder 1987; Quesada and Vincent 1997), quenching by enzymatic and non-enzymatic antioxidants (He and Häder 2002a; Singh et al. 2013), synthesis of UV-screening compounds, such as mycosporine-like amino acids (MAAs) and scytonemin (Garcia-Pichel and Castenholz 1991, 1993), repair of damaged DNA and resynthesis of proteins (Britt 1995; Kim and Sancar 1995; Chen et al. 2013). The role of UV-screening compounds in mitigating the harmful UV-B

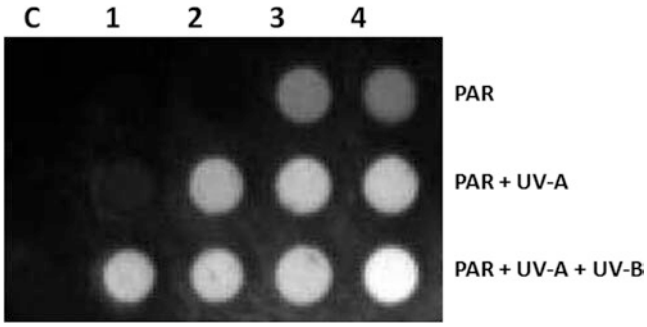

Fig. 2 Dot blot analysis of thymine dimer formation in *Nostoc* sp. HKAR-2 under various radiation conditions. *C* control; *1* 30 min; *2* 60 min; *3* 90 min; *4* 120 min (Adapted from Kumari 2010)

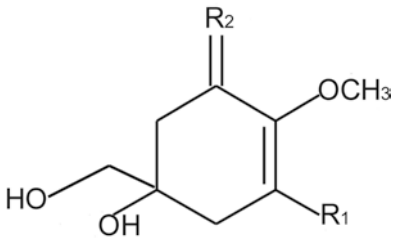

Fig. 3 Basic structure of mycosporine-like amino acids (MAAs). In almost all MAAs R1 is the amino acid glycine, R_2 is an imino-or keto group or an organic moiety

radiation effects in cyanobacteria is discussed below in more detail.

5.3 Mycosporine-Like Amino Acids (MAAs) in Cyanobacteria

Mycosporine-like amino acids (MAAs) are an important group of UV-absorbing, secondary metabolites containing a cyclohexenone or cyclohexenimine chromophore (Fig. 3) conjugated with the nitrogen substituent of an amino acid or its imino alcohol (Singh et al. 2008a). They are small (<400 Da), colorless, water-soluble compounds with a glycine moiety at the third carbon atom of the ring system. In addition, some MAAs also contain sulfate esters or glycosidic linkages through the imine substituent. They absorb maximally in the UV-range (310–362), and variations in the attached side groups and nitrogen substituents are mainly responsible for the different spectral properties of the compounds (Fig. 4). High molar extinction coefficients ($\varepsilon = 28{,}100–50{,}000\ M^{-1}\ cm^{-1}$), photostability in both distilled and sea water in the presence of photosensitizers and high resistance against physico-chemical stressors like temperature, strong UVR, various solvents as well as pH make them successful photoprotectants in various habitats and organisms (Richa and Sinha 2013). MAAs was originally identified in fungi as having a role in UV-induced sporulation (Leach 1965). MAAs protects the cells by absorbing highly energetic UVR and then dissipating excess energy in the form of heat to their environment (Conde et al. 2004). Studies from cyanobacteria have shown that these compounds prevent three out of ten photons from hitting cytoplasmic targets (Garcia-Pichel and Castenholz 1993). To date, 22 MAAs have been reported from terrestrial, marine and freshwater cyanobacteria (Table 1) with known absorbance maxima and retention times during high performance liquid chromatography (HPLC) (Garcia-Pichel and Castenholz 1993; Sinha et al. 1998). MAAs are assumed to be synthesized via the first part of shikimate pathway, but there is still controversy regarding its precise mechanism. 3-dehydroquinate (DHQ), a shikimate pathway intermediate, serves as a precursor for the synthesis of fungal mycosporines (Favre-Bonvin et al. 1987) and MAAs via gadusols (Bandaranayake 1998; Shick and Dunlap 2002; Singh et al. 2008a). MAAs are capable of scavenging the radicals, and their scavenging activities depend on the skeletal structures (Wada et al. 2013).

The MAAs in *Nostoc commune* have been reported to be extracellular and linked to oligosaccharides in the sheath (Böhm et al. 1995). Certain *Anabaena* sp. has been reported to synthesize the MAA shinorine (Sinha et al. 1998, 2007; Sinha and Häder 2008). Among four cyanobacterial species (e.g., *Trichormus* (*Anabaena*) *variabilis* PCC 7937, *Anabaena* sp. PCC 7120, *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 6301), screened for MAAs by Singh et al. (2010b), only *T. variabilis* PCC 7937 was able to synthesize MAAs. However, a novel MAA tentatively identified as dehydroxyl-usujirene was isolated from the cyanobacterium *Synechocystis* sp. (Zhang et al. 2007a). Shinorine and porphyra-334 are the most common MAAs that are present in both freshwater and marine cyanobacteria (Karsten and Garcia-Pichel 1996; Sinha et al. 2001b, c, 2003a, b, 2007; Volkmann et al. 2006; Sinha and Häder 2008; Singh et al. 2010a). Singh et al. (2010a) reported the occurrence of mycosporine-glycine, porphyra-334 and shinorine in *Trichormus* (*Anabaena*) *doliolum*, a common rice-field cyanobacterium. Three species of *Nodularia* (*Nodularia spumigena*, *Nodularia baltica* and *Nodularia harveyana*) from the Baltic Sea have been reported to synthesize the MAAs porphyra-334 and shinorine upon UV-B irradiation (Sinha et al. 2003b). Garcia-Pichel et al. (1998) found a common complement of MAAs in 13 strains of unicellular halophilic cyanobacteria. A symbiotic cyanobacterium, *Prochloron* sp., forming an association with ascidians, contains shinorine and traces of mycosporine-glycine and palythine (Dionisio-Sese et al. 1997). Screening of *Microcoleus* strains revealed the presence of shinorine and three unidentified compounds having absorption maxima at 332, 344 and 346 nm (Karsten and Garcia-Pichel 1996). Recently, Volkmann et al. (2006) have reported the occurrence of a novel MAA containing the

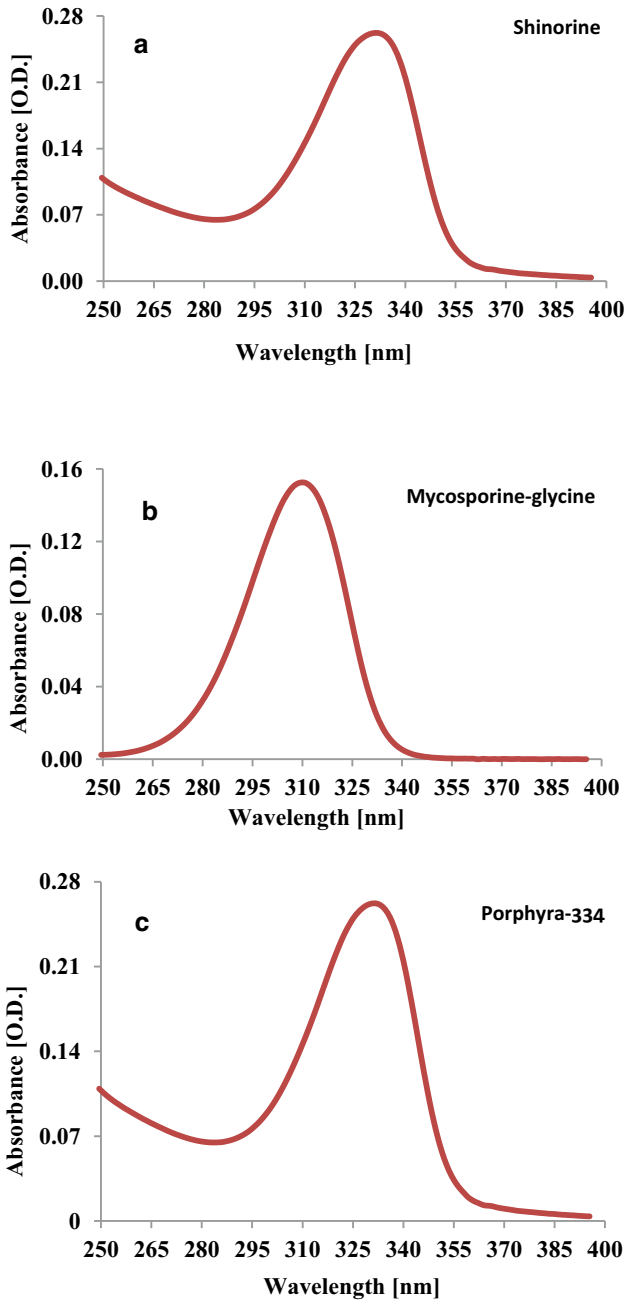

Fig. 4 Absorption spectra of shinorine (**a**), mycosporine-glycine (**b**) and porphyra-334 (**c**) showing peaks at 334 nm, 310 nm and 334 nm respectively

amino acid alanine (2-(e)-2,3-dihydroxipro-1-enylimino-mycosporine-alanine) in the unicellular cyanobacterium '*Euhalothece* sp.' inhabiting a hypersaline pond. Volkmann et al. (2003, 2006) reported that mycosporine-alanine, mycosporine-glutaminol, mycosporine-glutaminol-glucoside provides protection to some terrestrial cyanobacteria against harmful UVR. Sommaruga and Garcia-Pichel (1999) have reported certain MAAs such as asterina-330, shinorine, palythinol and mycosporine-glycine in epilithic cyanobacteria from a freshwater lake. *Microcystis aeruginosa*, a freshwater bloom forming cyanobacterium, also contains high concentrations of MAAs including shinorine and several other unknown MAAs (Liu et al. 2004). The diazotrophic ocean bloom forming cyanobacterium *Trichodesmium* spp. was also reported to contain high amounts of asterina-330 and shinorine. In addition to mycosporine-glycine, porphyra-334 and palythene were also found to be present. However, two of the most abundant compounds were unidentified (Subramanian et al. 1999). Nazifi et al. (2013) have reported the presence of a glycosylated porphyra-334 and palythine-threonine in the terrestrial cyanobacterium *Nostoc commune*, suggesting a unique adaptation for terrestrial environments with conditions that are drastically fluctuating in comparison to stable aquatic environments. The filamentous bloom-forming cyanobacterium *Aphanizomenon flos-aquae* is marketed today as a food supplement and has been used as a source for the preparation of porphyra-334 (Carreto et al. 1990; Torres et al. 2006). Carreto et al. (1990) also reported the role of varying levels of UVR in the interconversions of different MAAs. The conversion of primary MAAs, mycosporine-glycine, into two MAAs, porphyra-334 and shinorine, under the influence by PAR and UVR in the rice-field cyanobacterium *Anabaena doliolum* (=*Trichormus dolium*) has been worked out by Singh et al. (2008b). Rastogi et al. (2012) characterized the highly polar MAAs shinorine, porphyra-334 and mycosporine-glycine and several other unknown MAAs in four cyanobacterial strains isolated from hot-springs in Rajgir, India. MAAs in the cell are regulated by osmotic mechanisms which is reflected by the fact that field populations of halotolerant cyanobacteria contain unusually high concentrations of MAAs (Oren 1997). Yu and Liu (2013) employing HPLC showed that nine MAAs exist in *Nostoc flagelliforme*, a terrestrial cyanobacterium. They also reported that there was an increase in the oligosaccharide-linked mycosporine-like amino acids by 145.5 % after 12 h at 5 Wm^{-2} and 114.5 % after 48 h at 1 Wm^{-2} of UV-B irradiation in the test organism. Singh et al. (2013) identified and characterized three types of MAAs from *Anabaena doliolum* and *Anabaena* strain L31 using absorption spectroscopy, HPLC, electrospray ionization-mass spectrometry (ESI-MS), Fourier Transform Infrared (FTIR) spectroscopy and Nuclear Magnetic Resonance (NMR) spectroscopy (Fig. 5). Singh et al. (2014) have reported the biosynthesis of a single MAAs, i.e., shinorine in *Anabaena* sp., isolated from hot-spring, Rajgir, India. They also showed that PAR+ UVR light regime was most effective for its biosynthesis. Dose-dependent induction of MAAs, mycosporine-glycine (λ_{max}-310 nm) was observed under both PAR as well as UVR in the hot-spring isolates such as *Scytonema* sp. strain HKAR-3 (Rastogi et al. 2010c), *Rivularia* sp. strain HKAR-4 (Rastogi et al. 2014) as well as

Table 1 Mycosporine-like amino acids (MAAs) in various cyanobacteria

Organisms	AS	MG	M2G	MGV	MMS	M-333	M335/360	M328/360	M-320	MT	PNA	PE	PT	PL	PR	SH	US	SME	References
Anabaena sp.	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	Sinha et al. (2001c)
Aphanizomenon .flos-aquae	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	Torres et al. (2006)
Aphanothece halophytica	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	+	–	–	Oren (1997)
Calothrix parietina	–	+	–	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	Garcia-Pichel and Castenholz (1993)
Calothrix sp.	–	+	–	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	Garcia-Pichel and Castenholz (1993)
Chlorogloeopsis sp.	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	Garcia-Pichel and Castenholz (1993)
Chlorogloeopsis sp. PCC6912	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	Portwich and Garcia-Pichel (2000)
Diplocolon sp.	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Portwich and Garcia-Pichel (2000)
Gloeocapsa sp.	+	+	–	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	Sommaruga and Garcia-Pichel (1999)
Lyngbya aestuarii	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	Garcia-Pichel and Castenholz (1993)
Microcystis aeruginosa	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	Liu et al. (2004)
Microcoleus chthonoplastes (= Coleofasciculus chthonoplastes)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	Karsten and Garcia-Pichel (1996)
Microcoleus paludosus	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	Karsten and Garcia-Pichel (1996)
Nodularia baltica	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	Sinha et al. (2003b)
Nostoc commune	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	Sinha et al. (2001c, 2003a), Ehling-Schulz et al. (1997), Böhm et al. (1995)

Nodularia harveyana	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	Sinha et al. (2003b)
Nostoc spumigena	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	Sinha et al. (2003b)
Scytonema sp.	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	Sinha et al. (2001c)
Scytonema javanicum	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	Garcia-Pichel and Castenholz (1993)
Synechococcus sp.	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	Garcia-Pichel and Castenholz (1993)

Presence (+), Absence (−)

AS asterina-330, *M-1* mycosporine-1, *M-2* mycosporine-2, *MG* mycosporine-glycine, *M2G* mycosporine-2-glycine, *MGV* mycosporine-glycinevaline, *MMS* mycosporine-methylamine-serine, *MT* mycosporine-taurine, *PE* palythene, *PL* palythinol, *PNA* Palythenic acid, *PR* porphyra-334, *PS* palythine-serine, *PT* palythine, *SH* shinorine, *SME* shinorine methyl ester, *US* usurijene, *M-333* mycosporine-333, *M335/360* mycosporine-335/360, *M328/360* mycosporine-328/360, *M-320* mycosporine-320

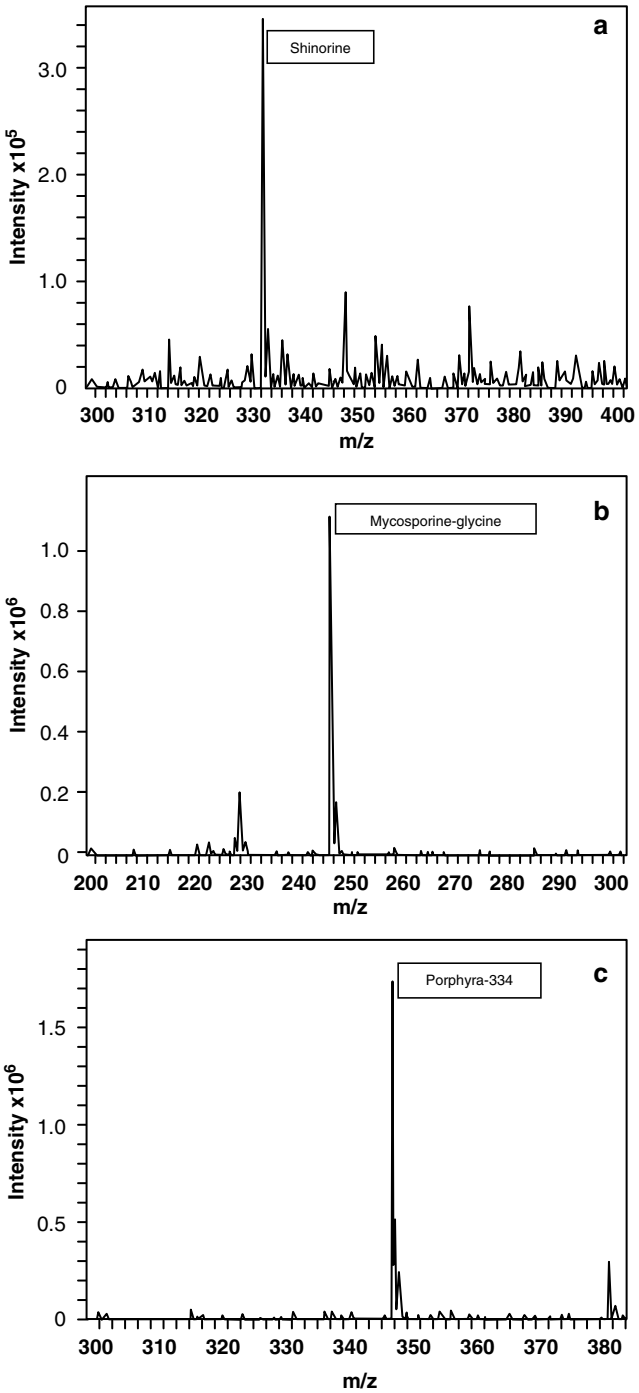

Fig. 5 Electrospray ionization-mass spectrometry (ESI-MS) showing the peaks of MAAs shinorine (**a**), mycosporine-glycine (**b**) and porphyra-334 (**c**) (Adapted from Singh et al. 2013)

in a red alga, *Chondrus crispus* (Karsten et al. 1998a). Recently, the presence of MAA porphyra-334 in two strains of *Nostoc* sp. harbouring different habitats has been characterized using various biochemical tools (Richa 2014; Richa and Sinha 2015).

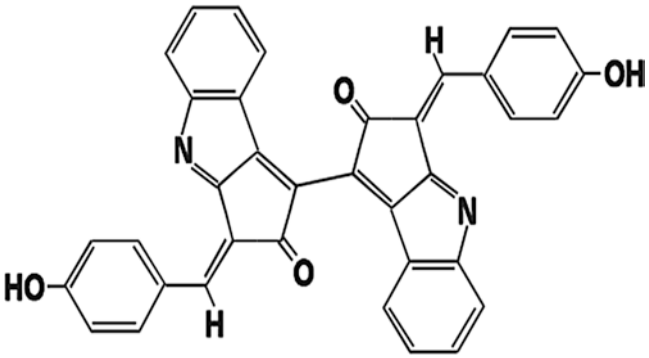

Fig. 6 Molecular structure of scytonemin

5.4 Scytonemin

Scytonemin constitutes the second important class of UV-absorbing compounds in cyanobacteria. It is a yellow-brown lipid-soluble pigment having a molecular mass of 544 Da, present in the extracellular polysaccharide sheath of some cyanobacteria (Fig. 6) (Geitler 1932; Desikachary 1959; Garcia-Pichel and Castenholz 1991; Garcia-Pichel and Belnap 1996). This photoprotective compound was first reported by Nägeli (1849) in some terrestrial cyanobacteria. The presence of scytonemin has been reported in the terrestrial cyanobacterium *Tolypothrix byssoidea* (=*Hassalia byssoidea*), showing maximum absorption at 260 and 384 nm (Adhikary and Sahu 1998). Scytonemin is a dimeric compound composed of indolic and phenolic subunits linked by an olefinic carbon atom, a unique natural product. Scytonemin generally occurs in its green oxidized form, but depending on the redox and acid-base conditions during the extraction process two other forms such as the oxidized (yellow; e.g., fuscochlorin) and the reduced (red; e.g., fuscorhodin) have been reported by Garcia-Pichel and Castenholz (1991). Recently, scytonemin has been extracted from *Lyngbya* sp. inhabiting rocks at the shore near Sao Fransisco do Sul, Brasil (Richter et al. 2006). Similar, results were reported by Sinha et al. (1995c, 1999) from the bark of mango trees in India. Recently, three new pigments, dimethoxyscytonemin, tetramethoxyscytonemin and scytonin from the organic extracts of *Scytonema* sp. have been reported to be derived from the scytoneman skeleton of scytonemin (Bultel-Ponc et al. 2004). Their structures were confirmed on the basis of ^{1}H and ^{13}C NMR and MS experiments. Recently, Varnali and Edwards (2013) have modeled di- and tetramethoxyscytonemin and their iron(III) complexes and studied computationally by using density functional theory calculations at the level of B3LYP/6-31G methodology. Based on this study they proposed new structures that could feature in survival strategy and facilitate the movement of iron through the rock especially for iron-rich stressed terrestrial environments. Scytonemin has an in vivo absorption maximum at 370 nm

in the UV-A region, but also shows considerable absorption in the UV-B region (Richa and Sinha 2011). Purified scytonemin has the highest absorption at 386 nm, although it also absorbs significantly at 252, 278 and 300 nm, and so it probably helps cyanobacteria to survive lethal UV (UV-A and UV-B) radiation. Recently, Fleming and Castenholz (2008) have found that a diazotrophically growing culture of *Nostoc punctiforme* PCC 73102 synthesizes three to seven times higher amounts of scytonemin as compared to a non-diazotrophically growing culture. It has been reported by Castenholz (1997) that scytonemin constitute more than 5 % of the total cellular weight in cyanobacteria. Garcia-Pichel et al. (1992) reported the role of scytonemin as a sunscreen agent in the terrestrial cyanobacterium *Chlorogloeopsis* sp. It has been shown that high scytonemin content is required for uninhibited photosynthesis under high UV flux in *Calothrix* sp. (Brenowitz and Castenholz 1997). Reports indicate that the presence of scytonemin in cyanobacterial sheaths is able to reduce the entry of UV-A radiation into the cells to around 90 % (Garcia-Pichel et al. 1992; Brenowitz and Castenholz 1997). Scytonemins able to persist very long in terrestrial cyanobacterial crusts or dried mats (Brenowitz and Castenholz 1997; Quesada et al. 1999) and performs its screening activity without any further metabolic investment even under prolonged physiological inactivity (e.g. desiccation) (Singh et al. 2010a). Matsui et al. (2012) studied the radical scavenging activity of scytonemin using electron spin resonance spectroscopy and discovered that the IC_{50} value of scytonemin against the ABTS radical was 36 µM. An acidic water stress protein (WspA) of 33.6 kDa, encoded by the wspA gene in the *N. commune* strain DRH1, is secreted to the three-dimensional extracellular matrix under desiccation or UV-A/B stress and was found to bind with scytonemin through noncovalent interactions (Wright et al. 2005). Thus, WspA has an important role in UVR stress by stabilizing the UV-absorbing compounds in the extracellular matrix of cyanobacteria. Chen et al.(2013) utilizing high performance liquid chromatography coupled with electrospray ionization tandem triple quadrupole mass spectrometry (HPLC-ESI-QqQ-MS), reported the presence of oxidized scytonemin in *Nostoc commune* exposed to high temperature and strong solar irradiance. The presence of scytonemin was also reported in *Scytonema* sp. HKAR-3 and *Rivularia* sp. HKAR-4, isolated from hot-springs of Rajgir, India (Rastogi et al. 2012). The photoprotective ability of scytonemin has recently been shown in a cyanobacterium *Rivularia* sp. HKAR-4 (Rastogi et al. 2013).

In addition to scytonemin, *Nostoc commune* has been reported to produce another UV protective agent with absorption maxima at 312 and 330 nm (Scherer et al. 1988). A brown *Nostoc* sp., that produces three UV-absorbing compounds with absorption maxima at 256, 314 and 400 nm, has been reported to be resistant to high visible and UV radiation (de Chazal and Smith 1994). Kumar et al. (1996b) have reported the shielding role of certain cyanobacterial pigments (a brown-colored pigment from *Scytonema hofmannii* and a pink extract from *Nostoc spongiaeforme*) against UV-B radiation. Yu and Liu (2013) have reported the synthesis of scytonemin in the terrestrial cyanobacterium *Nostoc flagelliforme* in response to UV-B radiation.

5.5 Other UV-Absorbing Compounds

A UV-A absorbing pigment, biopterin glucoside (BG, a compound chemically related to the pteridine pigments found in butterfly wings), with absorption maxima at 256 and 362 nm, has been purified from the marine planktonic cyanobacterium *Oscillatoria* sp. collected from the coastal areas of Japan (Matsunaga et al. 1993). The same fluorescent pigment (biopterin-α-glucoside) has also been isolated from the cyanobacterium *Arthrospira* (*Spirulina*) *platensis*, where it prevented the decolorization of the photosynthetic pigments and carotenoids by UVR (Noguchi et al. 1999). Certain cyanobacteria such as *Anacystis nidulans* (= *Aphanothece nidulans*), *Anabaena variabilis* (= *Trichormus variabilis*) and *Nostoc muscorum* (= *Desmonostoc muscorum*) were reported to produce pteridines in relatively large concentrations to counteract the damaging effects of UVR.

6 Effects of UV-B Radiation on Phytoplankton

Phytoplankton are unquestionably the major biomass producers in marine ecosystems thus providing an essential ecological function for all aquatic life. They account for nearly half of the total production of organic matter on Earth via photosynthesis. In addition to playing a major role in the food web, many genera of phytoplankton produce certain volatile substances such as dimethylsulfide (DMS) that serve as an antecedent of cloud condensation nuclei (CCN) and moderate the greenhouse effect (Charlson et al. 1987). The cumulative effect of marine biota in the reduction of CO_2 concentration and emission of DMS has been estimated to lower the atmospheric temperature by up to 6 °C (Watson and Liss 1998). They are also a rich source of dimethylsulfoxide (DMSO) (Simó et al. 1998) having a potential role as cryoprotectant and osmoregulator (Lee and de Mora 1999). The distribution of phytoplankton in the oceans depends upon various factors such as the incidence of solar radiation and nutrient availability. Therefore, dense phytoplankton populations have been reported to be present in the euphotic zone and near the coast because of higher nutrient availability due to upwelling of nutrient-rich water from the deep sea and terrestrial influxes.

The obligatory requirement of phytoplankton communities for solar radiant energy for photosynthesis, growth and survival exposes them to deleterious UV-B radiation. It has been reported that UV-B radiation could alter the morphology, impair motility and photo-orientation, damage proteins and DNA, inhibit growth, nitrogen metabolism, pigmentation, enzymatic functioning, fatty acid composition and photosynthesis of various microalgae species (He and Häder 2002b; Rastogi et al. 2010a; Kottuparambil et al. 2012). Thus, the effects of these ecological consequences on production rates and community structure of primary producers in aquatic ecosystems are of growing concern in recent days (Häder et al. 2011). Gao et al. (2007a) examined the effects of solar ultraviolet radiation on photosynthesis of differently cell-sized phytoplankton, natural phytoplankton assemblages from the coastal waters of the South China Sea. Biologically effective doses of UV-B have been detected down to a depth of 70 m in the water column (Smith et al. 1992) and may thus affect the aquatic ecosystems (Häder et al. 1998). The variables in the atmosphere and the water that affect the amount of UV radiation and wavelength distribution determine the transmission of solar UV radiation into the water column (Häder et al. 2007b; Smith and Mobley 2007). The penetration of UV-B into the water depends on the optical properties of the water column. Colored dissolved organic matter (CDOM), generated through microbial degradation of organic material from macroalgae and plankton as well as terrestrial plants, is a major factor controlling optical characteristics of freshwater and coastal habitats (Hulatt et al. 2007).

UV-B radiation can cause either dynamic or chronic photoinhibition in phytoplankton organisms (Bracher and Wiencke 2000). Dynamic photoinhibition involves the down-regulation of the photosynthetic apparatus and is associated with the dissipation of excess energy as heat; whereas chronic photoinhibition involves photodamage of the PS II reaction center D1 leading to a reduction in the number of functional PS II centers (Hanelt 1996). Kottuparambil et al. (2012) reported a significant decrease in the photosynthetic performance, motility and increased cellular concentration of reactive oxygen species (ROS) in the unicellular flagellated photoautotroph *Euglena agilis* (= *E. pisciformis*) following UV-B irradiation. In chloroplasts, ROS is reported to cause inactivation and degradation of Rubisco and other components of the Calvin cycle (Patsikka et al. 1998) as well as lipid peroxidation, which results in the disruption of photosynthetic pigments and ultimately in oxidative stress (Patsikka et al. 2002). UV-B radiation has also been reported to increase the net loss of D1 protein pools in natural phytoplankton (Bouchard et al. 2005a).

Severe impairment in motility, orientation and moving velocity after UV-B exposure have been reported in a number of motile microalgae, including *Chlamydomonas*, *Cryptomonas*, *Euglena*, *Gyrodinium* and *Peridinium* spp. (Hessen et al. 1997). UV-B mediated impairment of motility and/or swimming velocity would, therefore, limit the capacity of motile phytoplankton to adapt to the surrounding light environment, thus reducing photosynthesis and growth (Rai and Mallick 1998). UV-B generates reactive oxygen species (ROS) through endogenous photosensitization reactions in photosynthetic microorganisms (Rastogi et al. 2010b). Severe oxidative stress inside cells can cause DNA damage, impair photosynthetic efficiency and alter the orientation of photosynthetic microalgae (Vincent and Neale 2000; Richter et al. 2003).

UV affects the cell morphology of phytoplankton in various ways. Decreased growth rates and increased cell volume are common in diatoms under chronic UV exposure (Karentz et al. 1991a). These authors also observed elongated cells under UV radiation in various Antarctic marine diatoms, caused by arrested cell division. Behrenfeld et al. (1992) found generally smaller cell volumes in the diatom *Phaeodactylum tricornutum* grown when ambient UV-B was screened off, compared with cells exposed to UV-B. Certain diatoms (i.e. *Skeletonema costatum*) are very sensitive and did not survive for more than 3 days, whereas others (e.g. *Amphora coffeaeformis* (= *Halamphora coffeaeformis*) and *Odontella aurita*) were able to acclimate to UV stress, although through different processes when exposed to UV radiation (Fouqueray et al. 2007). The concentration of CO_2 seems to have a role in conditioning the sensitivity of a diatom (*Thalassiosira pseudonana*) as this species was more sensitive to UV radiation when acclimated to high CO_2 than under atmospheric CO_2 levels.

Studies conducted by Fritz et al. (2008) revealed that the open-ocean phytoplankton in Antarctic were more sensitive to UV radiation than coastal assemblages, the latter having higher rates of repair. UV radiation induced photoinhibition of natural post-bloom diatom-dominated phytoplankton assemblages from temperate latitudes of Patagonia. However, when samples were dominated by chlorophytes, the inhibition was less pronounced (Villafañe et al. 2008). Gao et al. (2007a) reported that the tropical phytoplankton assemblages from a coastal site of the South China Sea were significantly inhibited by UV radiation (mostly by UV-B) during sunny days. However, during cloudy days, while small cells (pico- and nanoplankton, <20 mm) were still inhibited by UV radiation, but larger cells (microplankton, >20 mm) used UV-A radiation as a source of energy for photosynthesis.

As compared with the oceanic and coastal species, estuarine diatoms are more flexible in terms of photoprotection, and thus have less photoinhibition when exposed to excess light (Lavaud et al. 2007).

UV radiation has been reported to reduce the cellular C:P (and N:P) ratios in phytoplankton, thus changing food quality

in aquatic food webs, as well as affecting biogeochemical cycling (Hessen et al. 2008). The UV sensitivity of phytoplankton to UV radiation is affected by the light history of the cells (MacDonald et al. 2003), which in turn is influenced by the mixing regime prevailing in the water column. Studies conducted by Barbieri et al. (2006) showed that the fluctuating radiation regime resulting from vertical mixing had pronounced beneficial effects on *Heterocapsa triquetra*, whereas *Dunaliella salina* was affected by both high and low solar irradiances and *Conticribra* (*Thalassiosira*) *weissflogii* was inhibited only by high solar irradiances. Helbling et al. (2008) reported the significant inhibition of photosynthesis but no DNA damage in three dinoflagellate species under static and mixing conditions during the austral spring. Increasing mixing speed increased UV-induced inhibition of carbon fixation in *Gymnodinium chlorophorum* (= *Lepidodinium chlorophorum*) and *Heterocapsa triquetra*, but not in *Prorocentrum micans*. Most of the loss in carbon fixation in *G. chlorophorum* was due to UV-B radiation, while in *H. triquetra* it was due to UV-A radiation.

UV-B radiation was found to have inhibitory effects on the growth of sub-polar phytoplankton under static and mixed conditions, but the synthesis of MAAs helped the cells to cope with UV radiation by changing the species composition towards more tolerant ones (Hernando et al. 2006). Helbling et al. (2013) suggested that under ambient nutrient conditions there is a synergistic effect between vertical mixing and UVR, increasing phytoplankton photosynthetic inhibition and excretion of organic carbon (EOC) from opaque lakes as compared to algae that received constant mean irradiance within the epilimnion. Garde and Cailiau (2000) reported the reduction in the growth rate and increased cell volume of *Emiliania huxleyi*, whereas inhibition of the electron transport and decreased maximum quantum yield of photosystem II (F_v/F_m) following UV irradiation was investigated by Marwood et al. (2000). Harada et al. (2009) found that UVR reduced chlorophyll *a* in cultures of *Amphidinium carterae* and *Thalassiosira oceanica*, reduced F_v/F_m and the growth rate of *T. oceanica*. The results of Larkum and Wood (1993) confirmed that phytoplankton are highly sensitive to UV-B radiation. Similar results have been reported by Häder and Häder (1991) while working on a marine cryptoflagellate, *Rhodomonas* (*Cryptomonas*) *maculata*. Lesser (1996) reported the significant decrease in photosynthetic performance, chlorophyll content, and Rubisco activities of dinoflagellate *Prorocentrum micans* following UV-B exposure. Ekelund (1990) observed that the growth of the motile dinoflagellates, *Gyronidium aureolum* Ba 6, and *Prorocentrum minimum* (= *P. cordatum*) Ba 12, were more sensitive to UV-B radiation than the non-motile diatoms *Ditylum brightwellii* Ba 15 and *Phaeodactylum tricornutum* Ba 16. He also reported that in addition to growth, swimming speed of the dinoflagellates *G. aureolum* and *P. minimum* were affected by UV-B radiation, while the diatoms were nearly unaffected. Prolonged irradiation time even resulted in complete loss of motility.

UV-B radiation had dramatic effects on rates of photosynthesis, motility and on absorption spectra in four studied phytoplankton species (Ekelund 1994). Photosynthesis of *Euglena gracilis* and the diatom *Phaeodactylum tricornutum* was more sensitive to UV-B inhibition than that of the dinoflagellates *Heterocapsa triquetra* and *Prorocentrum minimum* (= *P. cordatum*). It was observed that the swimming speed of *H. triquetra* decreased more after exposure to low visible light and UV-B radiation compared to high visible light and UV-B radiation. The meta-analysis conducted by Llabrés et al. (2013) demonstrated that the mortality rates in marine biota including microalgae increases rapidly in response to elevated UV-B radiation. Karentz et al. (1991a) studied twelve species of Antarctic diatoms to assess UV sensitivity in relation to cellular and molecular aspects of DNA damage and repair and concluded that a wide range of interspecific UV sensitivity was present among the diatoms, emphasizing the ecological implications of changes in natural UV regimes. Skerratt et al. (1998) studied the effects of UV-B radiation on the fatty acid, total lipid and sterol composition and content in three Antarctic marine phytoplankters and found them to be species-specific. The dinoflagellate *Gymnodinium* sp., isolated from the Mediterranean Sea was reported to suffer from oxidative stress, sustained DNA damage and to undergo cell death after less than 1 day of exposure to UVR during the photoperiod (Bouchard et al. 2013). Klisch et al. (2005) reported that solar UV-B radiation induced the formation of thymine dimers in freshwater phytoplankton assemblage exposed at the water surface.

6.1 Mitigation Strategies in Phytoplankton

The deleterious effects of UV-B have accompanied the whole evolutionary process of phytoplankton. Therefore, phytoplankton organisms have evolved certain tolerance mechanisms against UV-B radiation. These include (a) vertical migration within the water column that allows the organisms to adjust the impinging radiation to a level that is suitable for photosynthesis andavoids excessive doses of visible as well as UV radiation (Häder 1988), (b) repair of UV-induced DNA damage employing either photoreactivation, nucleotide-excision repair (NER) and/or recombinational repair and re-synthesis of damaged PS II proteins (Rastogi et al. 2010a; Singh et al. 2010d; Carreto and Carignan 2011). The capacity and kinetics of DNA repair differ greatly between phytoplankton species. This includes different relative importance of either photoreactivation and/or NER. The removal of cyclobutane dimers by photoreactivation in the presence of PAR or UV-A radiation may enhance the not

light-dependent NER of other DNA lesions (Karentz et al. 1991a, b), (c) synthesis of UV-screening compounds, such as MAAs, scytonemin-like compounds and sporollenin (Carreto et al. 1990; Vernet and Whitehead 1996; Xiong et al. 1997; Sinha et al. 1998; Singh et al. 2010d).

6.2 MAAs in Phytoplankton

The occurrence of MAAs has been reported in marine as well as freshwater phytoplankton organisms (Xiong et al. 1999; Sinha et al. 2007). MAAs have been reported to occur predominantly in members of the Dinophyceae, Bacillariophyceae and Haptophyceae (or Prymnesiophyceae) (Table 2). Lower levels were reported in diatoms, chlorophytes, euglenophytes, eustigmatophytes, rhodophytes, some dinoflagellates and some prymnesiophytes (Jeffrey et al. 1999). The genes derived from both photosynthetic and heterotrophic lineages may account for differences in the biosynthesis, accumulation and conversion of MAAs among marine algae. For instance, in the dinoflagellates *Heterocapsa triqueta* and *Oxyrrhis marina*, a dehydroquinate synthase (DHQS) similar to YP_324358 of *Trichormus* (*Anabaena*) *variabilis* PCC 7937 (Singh et al. 2010c) has been reported to be present in the chloroplast and to be fused to O-methyltransferase (Waller et al. 2006). In the dinoflagellates *H. triquetra*, *O. marina* and *Karlodinium micrum* both genes have been reported to be transferred from cyanobacteria via a prokaryote-to-eukaryote lateral/horizontal gene transfer event during evolution (Waller et al. 2006; Singh et al. 2010c). *Synechococcus* sp. and *Prochloroccocus* sp. constituting a major component of phytoplankton at the surface of the Brazil Current synthesizes several MAAs such as shinorine, palythine, porphyra-334, mycosporine-glycine, palythenic acid and shinorine-methyl ester (Carreto et al. 2005).

The occurrence of UV-absorbing compounds in marine phytoplankton was first noted in the dinoflagellates (Balch and Haxo 1984). The commonly occurring natural symbiotic dinoflagellate *Symbiodinium* sp., regardless of the clade identity, can synthesize several MAAs, namely mycosporine-glycine, shinorine, porphyra-334 and palythine, in contrast to the culturedform (Banaszak et al. 2006). Some photosynthetic free-living species of dinoflagellates such as *Prorocentrum minimun* (Sinha et al. 1998) and *Woloszynskia* sp. (Jeffrey et al. 1999) also contain several MAAs whereas in *Amphidinium carterae* only mycosporine-glycine was found (Hannach and Sigleo 1998). Higher MAA concentrations in *Prorocentrum micans*, adapted to UV radiation did not completely mitigate the detrimental effects of UV on photosynthesis (Lesser 1996). Red tide dinoflagellates such as *Gymnodinium sanguineum* (= *Akashiwo sanguinea*), *Alexandrium* sp. and *P. minimum* have been reported to contain high concentrations of MAAs (Neale et al. 1998a; Cardozo 2007; Laurion and Roy 2009). Recently, Laurion and Roy (2009) reported the presence of seven MAAs in two strains of *A. tamarense* and *H. triqueta*. They also observed two novel MAA-like compounds with distinct and previously unreported absorption maxima in the dinoflagellates *Gymnodinium galatheanum*, *Gymnodinium veneficum* (both now considered to be *Karlodinium veneficum*) (λ_{max} = 342 nm) and *Prorocentrum micans* (λ_{max} = 352 nm). Klisch and Häder (2002) reported the degradation of porphyra-334 in the marine dinoflagellate *Gyrodinium dorsum* under short wavelength UV radiation. Carignan and Carreto (2013) found that natural populations of the red tide dinoflagellate *Prorocentrum micans* showed a net dominance of M-333 together with less amounts of other MAAs. They also documented the isolation and characterization of this MAA (mycosporine-serine-glycine methyl ester, a structure confirmed by NMR) from natural dinoflagellate populations and from *Alexandrium tamarense* cultures.

High concentrations of MAAs have been reported in several prymnesiophyte species, especially *Phaeocystis pouchetti* from Antarctica (Marchant et al. 1991), which were found to be induced under PAR and PAR + UV irradiation. *Phaeocystis pouchetti* predominately contained mycosporine-glycine-valine, shinorine and mycosporine-glycine (Newman et al. 2000). The bloom-forming coccolithophorid *Emiliania huxleyi* has been reported to contain a variety of MAAs (Hannach and Sigleo 1998). Of the three species of prymnesiophytes examined by Llewellyn and Airs (2010), *Isochrysis galbana* and *E. huxleyi* contained MAAs at low concentrations, while *Phaeocystis globosa* contained no detectable MAAs.

High levels of the unusual secondary MAA asterina-330 have been reported in *Heterosigma carterae* (= *Heterosigma akashiwo*) and *Fibrocapsa* sp. (Llewellyn and Airs 2010). *Chattonella marina*, an Australian strain of the raphidophytes, has been reported to produce five times more MAAs (mycosporine-glycine; mycosporine glycine-valine and shinorine) and to grow 66 % faster than a Japanese strain of the same species under inhibiting UV-B radiation (Marshall and Newman 2002). Increased MAA production under high irradiances was also observed in other Australian strains of *Chattonella*, but was not noted in other Japanese strains suggesting ecophenotypic adaptation due to differing environmental conditions (Marshall and Newman 2002).

Generally lower levels of MAAs have been reported in cultured diatoms compared to dinoflagellates (Llewellyn and Airs 2010). Porphyra-334 with less amounts of shinorine has been reported to be present in nine bacillariophyte species (Riegger and Robinson 1997) and one additional mycosporine-glycine in *Porosira pseudodenticulata*. In Antarctica, high in vivo absorption at wavelengths indicative of MAAs was characteristic of assemblages by the

Table 2 Mycosporine-like amino acids (MAAs) in various phytoplankton species

Organisms	AS	MG	M2G	MGV	MMS	M-333	M335/360	M 328/360	M-320	MT	PNA	PE	PT	PL	PR	SH	US	SME	References
Amphidinium carterae	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	Hannach and Sigleo (1998)
Alexandrium tamarense	−	+	−	−	+	+	+		+	−	+	+	+	+	+	+	+	+	Carreto et al. (2001, 2005), Shick and Dunlap (2002)
A. catenella	−	+	−	−	+	+	+	−	+	−	+	+	+	+	+	+	+	+	Carreto et al. (2001)
Alexandrium. minutum	−	+	−	−	+	+	+	+	+	−	−	+	+	+	+	+	+	−	Carreto et al. (2001)
Chattonella marina	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−	Marshall and Newman (2002)
Corethron criophilum (=*C. pennatum*)	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	−	−	Helbling et al. (1996)
Chlorella minutissima	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	Riegger and Robinson (1997)
Chlorella sorokiniana	−	−	−	−	−	−	−	−	−	−	−	−	+		+	+	−	−	Xiong et al., (1999, 1997)
Dunaliella tertiolecta	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	Hannach and Sigleo (1998)
Emiliania huxleyi	−	−	−	−	−	−	−	−	−	−	−	−	+		+	+	−	−	Carreto et al. (2005)
Fragilariopsis cylindrus	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	Riegger and Robinson (1997)
Gyrodinium dorsum	+	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	−	−	Gröniger et al. (2000)
Gymnodinium sanguineum (= *Akashiwo sanguinea)*	−	+	−	−	−	−	−	−	−	−	−	+	+	−	+	+	−	−	Whitehead and Hedges (2002)
Gymnodinium catenatum	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	Jeffrey et al. (1999)
Heterosigma carterae (= *H. akashiwo*)	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	Jeffrey et al. (1999)
Fibrocapsa sp.	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	Llewellyn and Airs (2010)

(continued)

Table 2 (continued)

Organisms	AS	MG	M2G	MGV	MMS	M-333	M335/360	M 328/360	M-320	MT	PNA	PE	PT	PL	PR	SH	US	SME	References
Isochrysis sp.	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Hannach and Sigleo (1998)
Lingulodinium polyedra	–	–	–	+	–	–	–	–	–	–	–	+	+	+	+	–	–	–	Vernet and Whitehead (1996)
Noctiluca scintillans	–	+	–	–	–	–	–	–	–	–	+	–	+	–	+	+	–	–	Carreto et al. (2005)
Phaeocystis antarctica	–	+	–	–	–	–	–	–	–	–	+	–	+	+	+	+	–	–	Moisan and Mitchell (2001)
Phaeocystis pouchetti	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	+	–	–	Marchant et al. (1991)
Prorocentrum minimum (= *P. chordatum*)	–	+	–	–	–	–	–	–	–	–	+	+	–	–	+	+	+	+	Sinha et al. (1998)
Prorocentrum micans	+	+	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	Lesser (1996)
Scrippsiella sweeneyae	–	+	–	–	–	–	–	–		+	--	+	–	–	–	+	+	–	Taira et al. (2004)
Scenedesmus sp.	+	–	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–	Xiong et al., (1997, 1999)
Thalassiosira antarctica	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	Riegger and Robinson (1997)
Thalassiosira tumida	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	Riegger and Robinson (1997)
Conticribra weissflogii	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Hannach and Sigleo (1998)
Woloszynskia sp.	–	+	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–	Jeffrey et al. (1999)

Presence (+), Absence (–)

AS asterina-330, *M-1* mycosporine-1, *M-2* mycosporine-2, *MG* mycosporine-glycine, *M2G* mycosporine-2-glycine, *MGV* mycosporine-glycinevaline, *MMS* mycosporine-methylamine-serine, *MT* mycosporine-taurine, *PE* palythene, *PL* palythinol, *PNA* Palythenic acid, *PR* porphyra-334, *PS* palythine-serine, *PT* palythine, *SH* shinorine, *SME* shinorine methyl ester, *US* usurijene, *M-333* mycosporine-333, *M335/360* mycosporine-335/360, *M328/360* mycosporine-328/360, *M-320* mycosporine-320

chain-forming diatom *Thalassiosira gravida* (Ferreyra et al. 1994). Generally, diatoms have been reported to contain primary MAAs, but the presence of the secondary MAAs palythine and palythene has been reported in *Corethron criophilum* (= *Corethron pennatum*) (Helbling et al. 1996). Carreto et al. (2005) reported the presence of the unusual mycosporine-taurine for the first time in a diatom, *Pseudo-nitzschia multiseries*.

6.3 Scytonemin-Like Compound

A UV-absorbing compound with a similar absorption spectrum and chromatographic behavior as that of scytonemin was reported in a phytoplanktonic bloom of the Icelandic Basin (Sinha et al. 1998). However, due to the lack of chemical characterization of this pigment, there is no direct evidence for the presence of scytonemin or similar pigments in eukaryotic phytoplankton (Llewellyn and Mantoura 1997).

7 Sporopollenin

Xiong et al. (1997) have reported the occurrence of sporopollenin or sporopollenin-like substances (algenans), biopolymers of variable composition, in certain green freshwater microalgae, for example, *Scenedesmus communis* (Blokker et al. 1999) and *Chlamydomonas nivalis* (Gorton and Vogelmann 2003) that probably play an important role in screening of UV radiation. These almost undegradable macromolecules are either bound to the algal cell wall or are present in zygospores. They absorb from the ultraviolet to the blue region of the spectrum and thus, are probably responsible for the observed UV resistance of these organisms (Van Winkle-Swift and Rickoll 1997; Xiong et al. 1997; Pescheck et al. 2010). Potentially, sporopollenins may be present in green macroalgae as well. Whatever the screening compound may be, its screening ability due to absorption may be enhanced by light scattered by the macromolecular structure of the compound itself and the cell walls. It has been shown that in light-scattering suspensions the absorbance of chromophores is strongly enhanced (Butler 1962).

8 Effects of UV-B Radiation on Macroalgae

Macroalgae, mainly inhabiting the coastal areas of the world (Lüning 1985) are exposed to varying intensities of solar radiation in both the supralittoral and sublittoral zones. Algae growing in the supralittoral or in the eulittoral are exposed to solar radiation including UV, while algae growing in the sublittoral are exposed mainly to PAR. Many algae show a distinct zonation at their growing site (Franklin and Forster 1997; Bischof et al. 1998). Macroalgal populations growing at the surface or in the intertidal show a much higher degree of photoinhibition compared to macroalgae growing in the subtidal or in crevices. Several group of workers have reported the adverse effect of UV-B on photosynthetic quantum yield, oxygen evolution and respiration as well as the recovery under both in vitro and in vivo conditions (Hanelt et al. 1993; Larkum and Wood 1993; Häder et al. 1996; Häder 1997a) showed higher rates of photoinhibition in algae growing in transparent water than those in turbid water. Hanelt et al. (1997) also demonstrated the influence of stages of life cycle on the level of photoinhibition and recovery in *Laminaria saccharina* (=*Saccharina latissima*). A reduced photosynthetic activity of algae around midday was shown under field as well as laboratory conditions (Häder 1997a). Pérez-Rodríguez et al. (1998) showed that the cultures of *Dasycladus vermicularis* when exposed to PAR with or without UV radiation led to a complete loss in oxygen evolution after 24 h of PAR + UV-A + UV-B radiation. A drastic decline in the ratio of the photosynthetic quantum yield of *Mastocarpus stellatus* was observed when exposed for 3 days to a solar simulator. The decrease is mainly caused by PAR while the effect of additional UV-A or UV-B does not affect the algae as drastically as PAR. The algae showed a recovery up to about 50 % for PAR with or without UV-A, while the thalli irradiated with PAR + UV-A + UV-B recovered only to 40 % (Gröniger et al. 1999). A marked reduction in the photosynthetic pigments, chlorophyll and carotenoid content, F_v/F_m ratio in the red alga *Lemanea fluviatilis* following UV-B irradiation has been reported by Arroniz-Crespo et al. (2005). Fu et al. (2013) have reported the role of UVR in induction of mutation in tetraspores of the rhodophyte *Gracilariopsis lemaneiformis*. Doses of UV-B were reported to inhibit the photosynthetic activity and induced serious DNA damage in a green alga, *Chlorella vulgaris*. UV-B radiation at different fluence rates influenced the growth, light-harvesting pigments, photosynthesis and photosynthetic electron transport activity, phosphate uptake, acid phosphatase activity (ACP) and alkaline phosphatase (ALP) activity differentially in two studied cyanobacterial species, i.e., *Phormidium foveolarum* (= *Leptolyngbya foveolara*) and *Desmonostoc* (*Nostoc*) *muscorum* (Singh et al. 2012). Bouchard et al. (2013) studied the adverse effect of UVR on the chlorophyte *Dunaliella tertiolecta* and reported that the increased tolerance to UVR exposure may provide advantages over other more sensitive phytoplankton species within the photic zone.

The sensitivity of phycobilisome components to UVR has been reported in several red macroalgae (Schmidt et al. 2010a, b). Among the constituent of phycobiliproteins, the phycocyanin is rapidly destroyed by UV-B irradiation compared to other biliproteins (Rinalducci et al. 2006); consistent

with the observations of Roleda et al. (2012). This author showed that growth of *Gracilaria vermiculophylla* thalli under UVR was not reduced while photosynthesis (F_v/F_m) of sexual reproduction-derived carpospores was severely photoinhibited. He also demonstrated a maximum 86 % reduction in the spore's PSII function that was observed after exposure to the same UVR fluence. UV-B radiation has been found to decrease the F_v/F_m in *Pyropia* (*Porphyra*) *leucosticta* (Figueroa et al. 1997). Zhang et al. (2012) reported the adverse effect of UV-B radiation on growth and photosynthetic activity of the green microalga *Chlamydomonas microsphaera* FACHB 52. Zheng and Gao (2009) reported that UVR reduced the growth rate of *Gracilaria lemaneiformis* by 37 % when the thalli were cultured under high light level for more than 15 days. However, a study performed by Zheng (2013) showed that UVR reduced the growth rate by 20 % in the same organism. UVR was found to reduce the maximal net photosynthetic rate, apparent photosynthetic efficiency and light-saturating irradiance in the marine macroalga *G. lemaneiformis*. The uptake and metabolism of nitrogen had been reported to be depressed by UVR (Huovinen et al. 2007). Michler et al. (2002) reported the inhibition of photosynthesis in *G. lemaneiformis* following UV-B exposure. Pérez-Rodríguez et al. (1998) recorded a reduction in F_v/F_m after 72 h of exposure to PAB in the green alga *Dasycladus vermicularis*. Sinha et al. (2001a) demonstrated the role of UV-B irradiation in the formation of thymine dimers in macroalgae such as *Porphyra umbilicalis* and *Ceramium rubrum* (= *Ceramium virgatum*).

8.1 Mitigation Strategies in Macroalgae

To cope up with the deleterious effects of UV-B radiation macroalgae have developed a number of tolerance mechanisms that include avoidance by growing deeper in the water column where the level of UV-B is lower (Häder 1988). In addition, scavenging of UV-induced ROS by enzymatic and non-enzymatic antioxidant molecules, synthesis of UV-screening compounds (mycosporine-like amino acids) (Häder 1997a; Sinha et al. 1998, 2000; Carreto and Carignan 2011) and DNA repair mechanisms (Sinha and Häder 2002; Rastogi et al. 2010a) are operative in macroalgae.

8.2 Mycosporine-Like Amino Acids

Tsujino and Saito (1961) were the first to report the presence of UV-absorbing compounds in macroalgae. An qualitative and quantitative survey during the last few years showed that MAAs are distributed in macroalgae belonging to Rhodophyta, Ochrophyta and Chlorophyta from polar to tropical habitats (Häder and Figueroa 1997; Karsten et al. 1998a, b, c). Karentz et al. (1991b) reported the presence of MAAs in macroalgae from the Antarctic. Most of the MAA-producing macroalgae belong to Rhodophyceae, followed by Phaeophyceae, and only a few green algae produce MAAs (Table 3) (Karentz et al. 1991b; Hoyer et al. 2001; Kräbs et al. 2002; Sinha et al. 2007). The MAA content in algae varies between classes and with growing depths, consistent with the report of Karsten et al. (1998c). Basically three patterns of MAAs distribution are found among macroalgae; (a) high initial MAA content and no increase during light treatment, (b) low MAA content with an increase during light treatment, and (c) no initial MAA and no induction during light treatment. The red macroalga *Chondrus crispus*, harvested from the subtidal zone showed an increase in the number and amount of MAAs (Karsten et al. 1998c). Initial high levels but no significant increase in the MAAs content was found in the upper intertidal rhodophyte *Porphyra umbilicalis* (Gröniger et al. 1999). Similarly, no in vivo induction of MAAs was recorded after exposure to either UV alone or in combination with PAR in the marine red alga *Gracilaria cornea* (= *Hydropuntia cornea*), which possesses a very high amount of naturally occurring MAAs having an absorption maximum at 334 nm. In the chlorophyte *Dasycladus vermicularis* UV-absorbing substances were found, but their absorption spectra and retention times did not resemble those of known MAAs (Pérez-Rodríguez et al. 1998). Shinorine and porphyra-334 are the most common MAAs reported in macroalgae in species collected from tropical to polar waters (Karentz et al. 1991b; Karsten et al. 1998b; Helbling et al. 2004; Sinha et al. 2007). However, the MAA composition of some intertidal red macroalgae, especially from Antarctic waters (i.e., *Palmaria decipiens*, *Iridaea chordata*, *Curdiea racovitzae*) was more complex (Karentz et al. 1991b; Kräbs et al. 2002). For example, in the Antarctic red alga *P. decipiens*, palythine, asterina-330, palythinol, palythene, usujirene, and the unusual M335/360 were found in addition to shinorine and porphyra-334 (Karentz et al. 1991b; Kräbs et al. 2002). In the red alga *C. crispus*, MAA synthesis induction by PAR alone follows a pattern, starting with the synthesis of shinorine followed by asterina-330, palythinol and palythine and a decline in shinorine after 2 days (Franklin et al. 1999, 2001; Kräbs et al. 2004) showed that the synthesis of asterina-330, palythinol and palythene in the red alga *Chondrus crispus* was mainly induced by UV-B radiation whereas this radiation had a negative effect on the accumulation of the major MAAs shinorine and palythine. However, several species of red algae (e.g., *Palmaria palmata* and *P. decipiens*), including *C. crispus* collected from coastal waters of Maine (USA), contain porphyra-334, usujirene and palythene, but unlike dinoflagellates the *cis* isomer, usujirene appears to be the predominant one (Adams and Shick 2001; Conde et al. 2003).

Table 3 Mycosporine-like amino acids (MAAs) in macroalgae

Organisms	AS	MG	M2G	MGV	MMS	M-333	M335/360	M328/360	M-320	MT	PNA	PE	PT	PL	PR	SH	US	SME	M-1	M-2	References
Red algae																					
Acanthophora specifera	+	+	–	–	–	–	–	–	–	–	–	+	+	+	+	–	–	–	–	–	Karsten et al. (1998a)
Actinotrichia fragilis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	Karsten et al. (1998a)
Amphiroa sp.	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–	–	–	–	Gröniger et al. (2000)
Asparagopsis armata	+	–	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–	–	–	Karsten et al. (1998b)
Asparagopsis taxiformis	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–	–	–	Gröniger et al. (2000), Karsten et al. (1998a)
Bangia atropurpurea	+	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	–	–	–	–	Boedeker and Karsten (2005), Hoyer et al. (2002)
Bangia fuscopurpurea	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–	Boedeker and Karsten (2005)
Bangia spp.	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–	Boedeker and Karsten (2005)
Bostrychia sp.	+	+	–	–	–	–	–	–	–	–	–	–	+	+	+	+	–	–	+	–	Karsten et al. (1998a, 2000)
Caloglossa sp.	+	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	–	–	–	–	Karsten et al. (2000)
Catenella sp.	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	+	+	Karsten et al. (2000)
Callithamnion gaudichaudii	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–	–	–	Helbling et al. (2004)
Ceramium sp.	–	+	–	–	–	–	–	–	–	–	–	–	+	+	+	+	–	–	–	–	Karsten et al. (1998b)
Chondria armata	–	+	–	–	–	–	–	–	–	––	–	–	+	+	+	+	–	–	–	–	Karsten et al. (1998a)
Chondrus crispus	+	–	–	–	–	–	–	–	–	–	–	+	+	+	–	+	–	–	–	–	Karsten et al. (1998b), Sinha et al. (1998), Bischof et al. (2000), Franklin et al. (1999)
Corallina elongata (= *Ellisolandia elongata)*	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–	–	–	–	Karsten et al. (1998b)
Corallina officinalis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	Karsten et al. (1998b)
Curdiea racovitzae	+	+	–	–	–	–	–	–	–	–	–	+	+	+	–	+	–	–	–	–	Helbling et al. (2004)
Cystoclonium purpureum	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	Gröniger et al. (2000)
Devaleraea ramentacea	+	+	–	–	–	–	–	–	–	–	–	+	+	+	+	+	–	–	–	–	Karsten et al. (1998b)
Gelidiella acerosa	+	–	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–	–	–	Karsten et al. (1998a)
Gelidium sp.	+	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	–	–	–	–	Karsten et al. (1998b)
Georgiella confluens	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	–	–	–	–	Hoyer et al. (2002)
Gracilaria sp.	–	+	–	–	–	–	–	–	–	–	–	+	+	+	+	+	–	–	–	–	Karsten et al. (1998a)
Gymnogongrus sp.	+	+	–	–	–	–	–	–	–	–	–		+	–	+	+	–	–	–	–	Karsten et al. (1998b), Hoyer et al. (2002)
Iridea sp.	+	+	–	–	–	–	–	–	–	–	–	+	+	–	+	+	–	–	–	–	Carreto et al. (2005)

(continued)

Table 3 (continued)

Organisms	AS	MG	M2G	MGV	MMS	M-333	M335/360	M328/360	M-320	MT	PNA	PE	PT	PL	PR	SH	US	SME	M-1	M-2	References
Jania rubens	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	−	−	−	−	Karsten et al. (1998b), Gröniger et al. (2000)
Kallymenia antarctica (= *Trematocarpusanarcticus)*	+	+	−	−	−	−	−	−	−	−	−	−	+	+	−	+	−	−	−	−	Hoyer et al. (2002)
Laurencia sp.	+	+	−	−	−	−	−	−	−	−	−	−	+	+	−	+	−	−	−	−	Karsten et al. (1998b)
Mastocarpus stellatus	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	Karsten et al. (1998b)
Neuroglossum ligulatum	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	−	−	−	−	Hoyer et al. (2002)
Palmaria decipiens	+	+	−	−	−	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	Hoyer et al. (2002), Carreto et al. (2005)
Palmaria palmata	+	+	−	−	−	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	Karsten et al. (1998b)
Phyllophora sp.	+	+	−	−	−	−	−	−	−	−	−	+	+	−	−	+	−	−	−	−	Karentz et al. (1991b),McClintock and Karentz (1997)
Polysiphonia sp.	+	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	Gröniger et al. (2000), Karsten et al. (1998b), Helbling et al. (2002)
Porphyra umbilicalis	+	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	−	−	−	−	Korbee et al. (2005)
Porphyr/Pyropia spp.	+	+	−	−	−	−	−	−	−	−	−	+	+	−	+	+	−	−	−	−	Karsten et al. (1998b), Hoyer et al. (2002), Korbee et al. (2005)
Rhodomela virgata (=*R. confervoides)*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	Karsten et al. (1998b)
Stictosiphonia sp.	+	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	Karsten et al. (1998a, 2000)
Brown algae																					
Chorda tomentosa (= *Halosiphontometosa*)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	Karsten et al. (1998b)
Desmarestia sp.	−	+	−	−	−	−	−	−	−	−	−	−	+	−	+	+	−	−	−	−	Gröniger et al. (2000), Karentz et al. (1991b)
Dictyosiphon foeniculaceus	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	Karsten et al. (1998b)
Dictyota bartayresii (= *D. bartayresiana*)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	Karsten et al. (1998a)
Halopteris scoparia	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	−	−	−	−	Gröniger et al. (2000), Karsten et al. (1998b)
Hiamantothallusgrandifolius	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	−	Hoyer et al. (2002)
Hydroclathrus clathratus	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	Gröniger et al. (2000)
Padina crassa (= *P. gymnospora*)	+	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	Nakamura et al. (1982)
Pylaiella littoralis	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	Karsten et al. (1998b)
Sargassum oligocystum	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	−	−	−	−	Karsten et al. (1998a)

Green algae																				
Acrosiphonia sp.	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	Karsten et al. (1998b)
Boodlea composita	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Karsten et al. (1998a)
Caulerpa sp.	–	+	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–	–	–	Gröniger et al. (2000), Karsten et al. (1998a,b)
Chaetomorpha tortuosa	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	Karsten et al. (1998b)
Codium sp.	+	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	–	–	–	–	Karsten et al. (1998b), Nakamura et al. (1982)
Dictyosphaeria cavernosa	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	Karsten et al. (1998a)
Enteromorpha bulbosa	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	Hoyer et al. (2002)
Monostroma hariotii	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	––	Hoyer et al. (2002)
Ulva lactuca	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	Karsten et al. (1998b)
Valonia aegagropila	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	Karsten et al. (1998a)

Presence (+), Absence (–)

AS asterina-330, *M-1* mycosporine-1, *M-2* mycosporine-2, *MG* mycosporine-glycine, *M2G* mycosporine-2-glycine, *MGV* mycosporine-glycinevaline, *MMS* mycosporine-methylamine-serine, *MT* mycosporine-taurine, *PE* palythene, *PL* palythinol, *PNA* Palythenic acid, *PR* porphyra-334, *PS* palythine-serine, *PT* palythine, *SH* shinorine, *SME* shinorine methyl ester, *US* usurijene, *M-333* mycosporine-333, *M335/360* mycosporine-335/360, *M328/360* mycosporine-328/360, *M-320* mycosporine-320

MAAs in supralittoral *Pyropia* (*Porphyra*) *yezoensis* have been shown to block the production of thymine dimers (Misonou et al. 2003). Recently, the study of Cardozo and co-workers (2011) successfully identified five MAAs, i.e. shinorine, palythine, asterina-330, porphyra-334, and palythinol, in three species of *Gracilaria* (*G. birdiae*, *G. domingensis* and *G. tenuistipitata*). The presence of MAAs in freshly released red macroalgal spores was reported in tetraspores of *Gigartina skottsbergii* (Roleda et al. 2008) and *Iridaea cordata* (Zacher et al. 2009). The low MAA content in *Gracilaria chilensis* makes this species more susceptible to UV-B radiation (Gómez et al. 2005). Arroniz-Crespo et al. (2005) showed the presence of porphyra-334 and mycosporine-glycine in *Lemanea fluviatilis*. They also found that porphyra-334 was significantly reduced under PAB regime, whereas mycosporine-glycine showed a substantial induction under the same conditions. The accumulation of MAAs is not only induced by both UV-A and UV-B but also by blue light in the PAR spectra (Korbee et al. 2006). Likewise, no MAAs induction was detected in *Gracilaria cornea* after 5 days of exposure to ~56 µmol photons m^{-2} s^{-1} of PAR and 2.0 Wm^{-2} of UV (Sinha et al. 2000). Ha et al. (2012) utilized ^{13}C tracer and HPLC combined with an isotope ratio mass spectrometer (irMS) to demonstrate the presence of mycosporine-like amino acids of an in situ phytoplankton community of Kongsfjorden. Porphyra-334 has been found to be present in a number of *Porphyra*/*Pyropia* species (Figueroa et al. 2003). A higher content of porphyra-334 in several red algae plays an important role in photoprotection (Conde et al. 2000). Induction of mycosporine-glycine by UV-B radiation has been found in the brown alga *Laminaria saccharina* (Apprill and Lesser 2003). Helbling et al. (2004) reported the presence of MAAs in four rhodophyte species (*Ceramium* sp., *Corallina officinalis*, *Callithamnion gaudichaudii* and *Pyropia* (*Porphyra*) *columbina*) from Patagonia. The intertidal rhodophytes *Mastocarpus stellatus* and *Ceramium rubrum* exposed to a solar simulator using different cut-off filters showed a variation in the MAAs content (Sinha et al. 2001b).

In contrast to red algae, most of the green algae investigated so far lack MAAs (Hoyer et al. 2001; Karsten et al. 2005), and the occurrence of MAAs has been reported only in 13 macroalgae Chlorophyceae collected from the intertidal zone of the tropical island Hainan. Karsten et al. (1998c) found a significant concentration of photoprotective compounds, such as mycosporine-glycine and porphyra-334 only in two green algae: *Boodlea composita* and *Caulerpa racemosa*. it was found that the subaerial green macroalga *Prasiola crispa* subsp. *antarctica* contains high concentrations of a unique UV-absorbing compound with an absorption maximum at 324 nm, that was characterized as a putative MAA due to its chromatographic properties (Hoyer et al. 2001). Gröniger and Häder (2002) confirmed the occurrence of this 324 nm-MAA in the closely related *Prasiola stipitata* from the supralittoral zone of the rocky island Helgoland (North Sea).

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Part V

Secondary Metabolites

Lipid Metabolism in Microalgae

Inna Khozin-Goldberg

1 Introduction

This chapter covers the biochemistry and physiology of lipid metabolism in microalgae. In it we summarize the current knowledge in the field acquired over the last two decades, noting some of the earlier seminal works. The first aspect to be covered is the diversity of fatty acid and lipid classes in microalgae; then we will outline the major lipid-biosynthesis routes and pathways of fatty acid modification. In the last part of the chapter we address the effects of environmental and nutritional factors, as well as stressful conditions, on lipid metabolism in microalgae. The reader is referred to several earlier and more recent comprehensive reviews on the subject (Thomson 1996; Harwood 1998; Guschina and Harwood 2006, 2009a, b; Hu et al. 2008), in which earlier sources of information can be found.

A striking feature in the last decade has been the continuously increasing availability of genomic and transcriptomic information on a large number of microalgae species. Microalgae have gained the attention of the scientific community, particularly lipid biochemists and microalgal biotechnologists, as a source of valuable nutritional ingredients, such as long-chain polyunsaturated fatty acids (LC-PUFA) and carotenoids, as well as precursors for biodiesel production. The field of microalgal lipids, particularly with respect to the identification of gene functions and regulation of lipid-biosynthetic pathways, is in its infancy. However, a wealth of previous biochemical and physiological data had been previously acquired. The last few years have witnessed substantial progress toward understanding the biochemical reactions of lipid biosynthesis in microalgae, supported by comprehensive knowledge acquired in the field of plant lipid biochemistry. Importantly, intensive investigations focusing on the biochemistry and enzymology of triacylglycerol formation in microalgal cells and vast systems biology studies have already revealed some novel gene functions and features, indicating that lipid metabolism in microalgae might differ in some aspects from that in higher plants (Liu and Benning 2013). A thorough understanding of the lipid-biosynthesis pathways in different groups of microalgae is a prerequisite for genetic engineering of microalgae toward enhanced lipid production and modifications in fatty acid composition.

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2 Fatty Acids and Their Diversity in Microalgae

Fatty acids are long aliphatic carbon chains that vary in length, degree of unsaturation, and structure. Given the huge diversity of microalgae and their habitats in nature (Guschina and Harwood 2006), and their distinct evolutionary history, these organisms feature a wider variety of fatty acids, comprising fatty acyl components of different lipid classes, than that found in higher plants. In contrast to the plant acyl lipids, where straight-chain 16-carbon (C16) and C18 saturated and unsaturated fatty acids are most common, without and with up to three double bonds—the long-chain polyunsaturated fatty acids (LC-PUFA) with chains of C20 and C22 and a higher number of double bonds (up to six) are abundant in certain microalgal phyla. In fact, microalgal fatty acid composition varies substantially among the representatives of the different phylogenetic groups (Borowitzka 1988; Hu et al. 2008; Khozin-Goldberg et al. 2011; Lang et al. 2011). Recent research has provided comprehensive information on the fatty acid profiles of over 2,000 microalgal strains, comprising almost all phyla and classes of eukaryotic microalgae but mainly freshwater terrestrial species and cyanobacterial strains (Lang et al. 2011). As an outcome of this research, 76 different fatty acids with different chain lengths and regio-stereochemistries of double-bond positions have been

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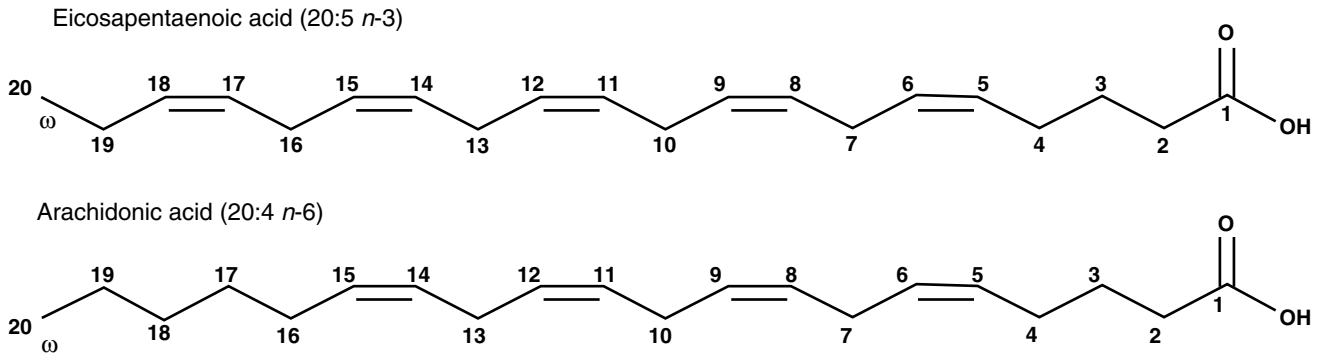

Fig. 1 The chemical structures of the LC-PUFA arachidonic acid (*ARA*) and eicosapentaenoic acid (*EPA*)

identified by gas chromatography (GC) and GC coupled with mass spectroscopy (GC-MS). Fatty acids usually contain even numbers of carbons, but fatty acids with odd-numbered chains have also been reported to exist in microalgae (Lang et al. 2011).

In systematic nomenclature, the number of carbon atoms and double bonds, and their positions in the fatty acid molecules are specified; for example, saturated palmitic acid is designated 16:0 (16 carbons, no double bonds), and monounsaturated oleic acid is designated 18:1 (18 carbons, one double bond). For example, the LC-PUFA eicosapentaenoic acid (EPA) is designated $20{:}5^{\Delta 5,8,11,14,17}$, where $x{:}y^{\Delta}$ stands for the number of carbons, the number of double bonds in *cis* configuration, and the bonds' positions (Δ) counting from the carboxyl end of the fatty acid molecule (Fig. 1). On the other hand, EPA is regarded as an omega-3 (ω-3) fatty acid (note that the ω-3 designation is identical to *n*-3), when the position of the last double bond is counted from the terminal methyl group (the ω end). As depicted in Fig. 1, arachidonic acid (ARA, $20{:}4^{\Delta 5,8,11,14}$ *n*-6) is a ω-6 C20 LC-PUFA with four double bonds. This nomenclature, specifying the position of the first double bond from the methyl end along with a common rule that the sequential double bonds in the unsaturated fatty acid molecule occur at 3-C intervals enables a depiction of the biosynthetic pathways of various PUFA and LC-PUFA. The enzymology of LC-PUFA biosynthesis will be further detailed in Sect. 7 of this chapter. The sequential double bonds in the unsaturated fatty acid molecule are as a rule separated by methylene groups (so-called methylene-interrupted). However, some examples of the non-methylene-interrupted fatty acids are known in plants and in microalgae, such as, for example, octadecatrienoic acid (common name picolinic acid, $18{:}3^{\Delta 5,9,12}$) and octadecatetraenoic acid (common name coniferonic acid, $18{:}4^{\Delta 5,9,12,15}$) in the green alga *Chlamydomonas reinhardtii* (Kajikawa et al. 2006). Systematic and common names, as well as the shorthand designations, of the most commonly encountered fatty acids, including those that have been detected in algae, are available at http://lipidlibrary.aocs.org. This site contains a wealth of useful information and the reader is encouraged to visit this valuable resource.

C16 and C18 saturated and monounsaturated fatty acids demonstrate different abundances in the different microalgal groups (Borowitzka 1988; Hu et al. 2008). Fatty acids C14:0, C16:0 and C16:1 *n*-7, for instance, are common in Bacillariophyta and Eustigmatophyta, whereas C16:0 and C18:1 *n*-9 are abundant in the Chlorophyta. The fatty acid composition in Cyanobacteria is generally simpler, consisting of C16 and C18 fatty acids; among the most common C18 PUFA in this phylum are linoleic acid (LA, $18{:}2^{\Delta 9,12}$), α-linolenic acid (ALA, $18{:}3^{\Delta 9,12,15}$) and γ-linolenic acid (GLA, $18{:}3^{\Delta 6,9,12}$) (Cohen et al. 1995). ALA and GLA are present in microalgae of the class Chlorophyceae (Chlorophyta), which is most closely related to the higher plants, whereas LC-PUFA are quite rare in this group. However, ARA, EPA and docosahexaenoic acid (DHA, $22{:}6^{\Delta 4,7,10,13,16,19}$) were detected at 5 % frequency in ca. 900 strains of examined representatives of the Chlorophyceae (Lang et al. 2011). The microalgae belonging to the classes Trebouxiophyceae and Ulvophyceae (Chlorophyta) appeared to be more enriched with species able to synthesize ARA and EPA (Lang et al. 2011). The microalga *Lobosphaera incisa* (formerly *Parietochloris incisa*),[1] a member of the Trebouxiophyceae (Karsten et al. 2005), is known as the best producer of ARA (Bigogno et al. 2002b). The ability of marine species of Prasinophyceae (Chlorophyta), such as *Ostreococcus tauri* (Wagner et al. 2010), *Micromonas pusilla* (Dunstan et al. 1992) and *Pyramimonas cordata* (Petrie et al. 2010a) to produce *n*-3 LC-PUFA has been described.

Diverse species of microalgae, mainly marine planktonic species of Bacillariophyta, Dinophyta, Eustigmatophyta, Haptophyta, and Rhodophyta, produce LC-PUFA of the ω-3 family—EPA and/or DHA (Borowitzka 1988; Volkman et al.

[1]Wherever possible the currently accepted names for species are used. The name used in the paper cited is also indicated. For details of names see chapter "Systematics, Taxonomy and Species Names: Do They Matter?" of this book (Borowitzka 2016).

1991,1993; Cohen 1999; Molina Grima et al. 1992, 1999; Sukenik 1999). Given that microalgae are the primary natural producers of LC-PUFA, microalgae-derived LC-PUFA play an important role in nature by enriching aquatic organisms in these essential fatty acid components. These fatty acids are of high physiological significance due to their importance as membrane components and precursors to biologically active eicosanoids (reviewed in Gill and Valivety 1997; Simopoulos 2002, 2008; Le et al. 2009; Khozin-Goldberg et al. 2011; Haslam et al. 2013 among others). This is especially important in the marine aquaculture because of the marine fish's limited capacity to synthesize LC-PUFA de novo from the essential C18 PUFA LA and ALA, therefore, the dietary value of microalgal LC-PUFA in aquaculture is now widely appreciated (Benemann 1992; Lavens and Sorgeloos 1996; Reitan et al. 1997; Sukenik et al. 2009). The exploitation of microalgal LC-PUFA holds promise for human nutrition as well (Kagan et al. 2013; Leu and Boussiba 2014).

ARA and EPA are abundant in members of the red algal lineage (Rhodophyta), as exemplified by the well-studied *Porphyridium cruentum* (Porphyridiophyceae) (Khozin et al. 1997; Cohen 1999). Members of a heterokont clade (Stramenopiles)—a broad group of unicellular eukaryotic organisms comprising some microalgal groups, such as diatoms and eustigmatophytes—are often rich in EPA and DHA, and contain low levels of C18 fatty acids. EPA is abundant in species of Eustigmatophyta, such as marine and freshwater species of the genus *Nannochloropsis* (Eustigmatophyceae); both EPA and DHA are encountered in members of the Bacillariophyta such as the pennate diatom microalgae *Phaeodactylum tricornutum* (Bacillariophyceae) and the centric diatom *Thalassiosira pseudonana* (Coscinodiscophyceae), and in Haptophyta such as *Isochrysis galbana* (Coccolithophyceae) and *Dicranema* (*Pavlova*) *lutheri* (Pavlovophyceae). Many of these species are cultivated in mariculture to supply marine fish with essential *n*-3 LC-PUFA. The precursor of DHA, docosapentaenoic acid (DPA, 22:5$^{\Delta7,10,13,16,19}$, *n*-3), occurs in DHA-producing microalgae and is found in particularly high proportions in representatives of the class Chlorarachniophyceae (Leblond et al. 2005). Discovery of two highly unsaturated fatty acids—octacosaoctaenoic acid (28:8 *n*-3) and octacosaheptaenoic acid (28:7 *n*-6)—in the toxic dinoflagellate *Karenia brevis* (Dinophyceae) further increases the array of LC-PUFA in microalgae-like organisms (Leblond et al. 2003).

C18 PUFA with more than three double bonds, such as octadecatetraenoic acid (18:4$^{\Delta6,9,12,15}$, *n*-3), common name stearidonic acid (SDA), are often encountered in the Chlorophyta. C18 PUFA with four to five double bonds are ubiquitous in dinoflagellates (Dinophyceae), which have been shown to contain the unusual octadecapentaenoic acid (OPA, 18:5 $^{\Delta3,6,9,12,15}$, *n*-3) (Leblond et al. 2003, 2010a). The coccolithophore *Emiliania huxleyi* (Coccolithophyceae)—a marine haptophyte microalga found ubiquitously in oceans—also contains OPA along with DHA (Sayanova et al. 2011). Note that *E. huxleyi* also produces unique long-chain (C37–39) lipids as neutral storage lipids, including alkenes, alkenones, and alkenoates, that are unlike the *cis*-PUFA described above in that they have two to four unusual *trans*-alkene bonds that occur at 7-C intervals (Eltgroth et al. 2005).

C16 PUFA with unusual for the higher plant number and location of double bonds may also occur in microalgae; for instance, in contrast to the hexadecatrienoic acid 16:3$^{\Delta7,10,13}$ that is abundant in higher plants, the diatom *P. tricornutum* contains its isomer 16:3$^{\Delta6,9,12}$ (Domergue et al. 2003), and *C. reinhardtii*, for example, contains the hexadecatetraenoic acid 16:4$^{\Delta4,7,10,13}$ (Zäuner et al. 2012).

3 Diversity of Lipid Classes in Microalgae

Two major classes of glycerolipids—glycoglycerolipids and phosphoglycerolipids—are the main membrane acyl lipid constituents of microalgal cells. They are composed of a 1,2-*sn*-diacylglycerol (DAG) moiety linked to a polar group in the *sn*-3 position. In glycoglycerolipids, the polar group is a sugar group, and in phosphoglycerolipids, it is a phosphate residue linked to an amino-alcohol, amino acid, carbohydrate or other functional moiety. The non-phosphorous ether-linked glycerolipids (termed betaine lipids), which are unusual in higher plants but abundant in some microalgal classes, contain a betaine moiety linked to DAG by an ether bond. Different membrane systems in microalgal cells are characterized by distinct compositions of acyl lipid classes (Thompson 1996; Harwood 1998). Galactolipids, with the predominant structures 1,2-di-O-acyl-3-O-β-D-galactopyranosyl-*sn*-glycerol and 1,2-di-O-acyl-3-O-(6′-O-α-D-galactopyranosyl-β-D-galactopyranosyl)-*sn*-glycerol prevail among the plastidial lipids, whereas phospholipids and betaine lipids generally constitute the largest proportion of extraplastidial lipids.

The predominant chloroplast lipids are the two galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG) (Fig. 2). The plastid lipids also include the phospholipid phosphatidylglycerol (PG). The composition of the chloroplast lipids in microalgae is assumed to resemble that in higher plant cells, but this has been merely inferred from whole-cell lipid analyses (Harwood 1998). Organelle isolation at a sufficient level of purity and intactness is a challenge in many microalgae because of their multilayered and rigid cell wall as in the green algae, or, for example, the siliceous material in skeletons of some diatoms. Furthermore, additional membranes

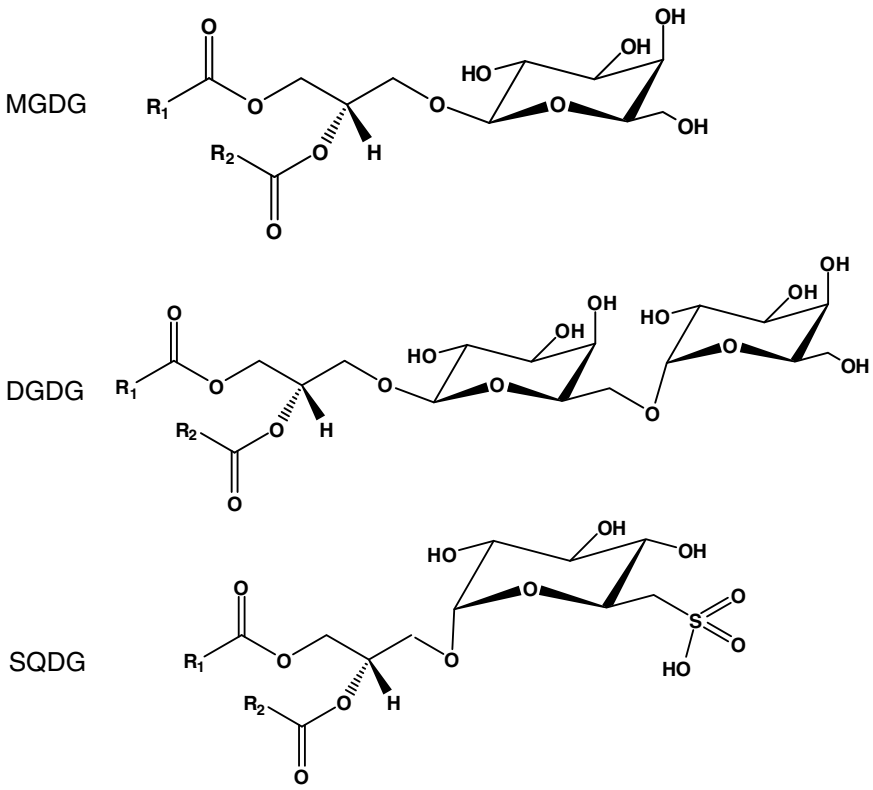

Fig. 2 The structures of the chloroplast membrane glycoglycerolipids. *MGDG* monogalactosyldiacylglycerol, *DGDG* digalactosyldiacylglycerol, *SQDG* sulfoquinovosyldiacylglycerol; R_1 and R_2 are fatty acyl chains

envelop the plastid in cells of those microalgae that evolved in the course of secondary and tertiary symbiosis. Due to the complexity of the cell structure and mechanical strength of the cell wall, cellular fractionation and organelle isolation have only been performed in a few instances, such as with the cell-wall-less species of the microalgae of the genus *Dunaliella* and the green alga *C. reinhardtii* (Mendiola-Morgenthaler et al. 1985; Sheffer et al. 1986; Peeler et al. 1989; Thompson 1996; Harwood 1998; Fan et al. 2011; Terashima et al. 2011). The isolated chloroplasts of *D. salina* were reported to have a 5.5 times higher molar content of glycolipids than phospholipids (Peeler et al. 1989). MGDG may constitute up to 40 or 50 % of polar acyl lipids while the percentage of DGDG amounts to up to 20 % (Thompson 1996; Harwood 1998). The ratio between the two major galactolipids, MGDG and DGDG, is variable in microalgae since it is strongly influenced by responses to environmental and nutritional cues; thus comparisons of, or generalizations about different microalgal species grown in different nutrient media and cultivation conditions can be made with caution. The major phospholipids are phosphatidylcholine (PC), phosphatidylethanolamine (PE), PG, and phosphatidylinositol (PI) (Fig. 3). These lipids are major constituents of the extraplastidial membranes. In analogy with higher plants, it is assumed that PC occurs in the outer layer of the chloroplast envelope membrane, at least in cells of the green algae. PI and PI phosphates (PIP) play important roles in lipid signaling and signal transduction. PI levels from 1 to 6 M % of total acyl lipids have been reported in microalgae (Harwood 1998). PI amounted to 6 M % in *Chlamydomonas moewusii* (Arisz et al. 2000) with a ratio of 100:1.7:1.3 determined for PI:PIP:PI-biphosphate in this alga. Phosphatidic acid (PA) is normally present in minor amounts, being an important lipid biosynthesis intermediate and a secondary messenger molecule (Munnik 2001). PA accounted for 0.67 M % of the phospholipids in *Chlamydomonas moewusii* (Arisz et al. 2000); when higher amounts of PA determined in lipid extracts it might be an indication of phospholipid degradation during homogenization and extraction.

As noted above, cellular fractionation coupled with lipid class analyses is rarely performed in microalgae and has only been carried out in a few algal species, mostly of those for which gentle cell breakage is feasible. Several earlier studies have dealt with the cell-wall-less microalga *D. salina* (Thompson 1996 and references therein). Sterols and the phospholipids PE and PC were most prevalent in the plasma membrane-enriched fraction. PIs also constituted a significant component of the *D. salina* plasma membrane fraction; PI-4-phosphate and PI 4,5-bisphosphate accounted for 5.2 % and 1.5 % of the plasma membrane phospholipids, respectively (Peeler et al. 1989). Another substantial component of the plasma membrane fraction appeared to be a betaine lipid, 1,2-diacylglyceryl-*O*-4-(*N*,*N*,*N*-trimethyl)homoserine (DGTS) (Sheffer et al. 1986; Peeler et al. 1989).

Many microalgae contain non-phosphorous betaine lipids which are not present in higher plants, but occur in lower

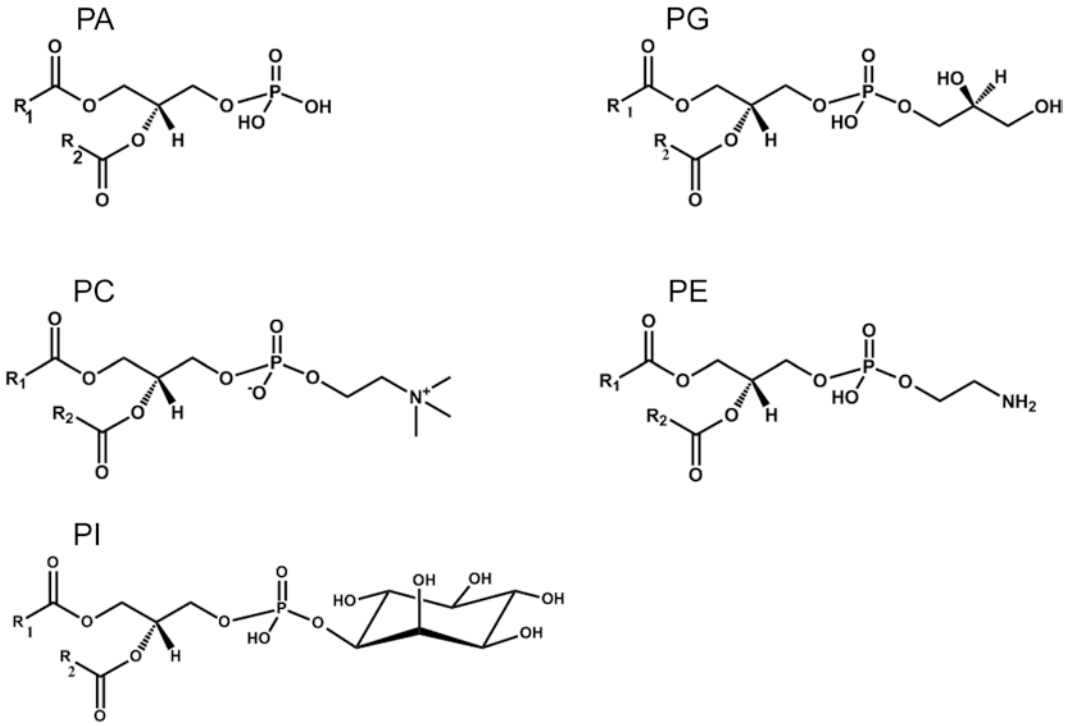

Fig. 3 The structures of major phospholipids. *PA* phosphatidic acid, *PC* phosphatidylcholine, *PE* phosphatidylethanolamine, *PG* phosphatidylglycerol, *PI* phosphatidylinosytol

plants, fungi, and some prokaryotes (Dembitsky 1996) (Fig. 4). The betaine lipids occur in many representatives of distant evolutionary groups, for example: DGTS is abundant in many representatives of the green microalgae (Chlorophyta), and in those evolved by secondary endosymbiosis: the haptophytes, the eustigmatophytes, the diatoms, etc. Occurrence, evolutionary and taxonomic significance of the betaine lipids are thoroughly addressed in Dembitsky (1996) and Kato et al. (1995; 1996). DGTS does not seem to be ubiquitous in diatoms, as recent lipid analysis by thin-layer chromatography (TLC) coupled with matrix-assisted laser desorption/ionization time-off-flight (MALDI-TOF) mass spectrometry (MS) did not identify DGTS in the freshwater diatom microalga *Cyclotella meneghiniana* (Vieler et al. 2007). Recently, the occurrence of DGTS has been questioned in the diatom *Phaeodactylum tricornutum* (Abida et al. 2015). DGTS and PC are zwitterionic lipids with a quaternary ammonium group giving them similar biophysical properties. PC is absent in *C. reinhardtii* and is assumed to be functionally substituted by DGTS (Giroud et al. 1988; Giroud and Eichenberger 1989; Riekhof et al. 2005a). In microalgae containing both DGTS and PC, DGTS may replace PC under conditions of phosphorus limitation (Khozin-Goldberg and Cohen 2006). Remarkably, a non-methylene-interrupted fatty acid, picolinic acid, is attached almost exclusively to DGTS in *C. reinhardtii* (Giroud et al. 1988; Giroud and Eichenberger 1989). The alanine analog of DGTS, 1,2-diacylglyceryl-3-O-2′-(hydroxymethyl)-(N,N,N-trimethyl)-β-alanine (DGTA), was initially discovered in brown algae (Phaeophyceae) (Harwood 1998). *Rhodomonas* (*Chroomonas*) *salina* (Cryptophyceae) (Eichenberger et al. 1996) and *Ochromonas danica* (Chrysophyceae) (Vogel and Eichenberger 1992) that contain both DGTS and DGTA. 1,2-diacylglyceryl-3-O-carboxy-(hydroxymethyl)-choline (DGCC) was identified in the haptophyte *Dicranema* (*Pavlova*) *lutheri* (Eichenberger and Gribi 1997); analysis of subcellular membrane fractions demonstrated that DGCC, DGTA and diacylglycerylglucuronide (DGGA) accumulate in non-plastid membranes in *D. lutheri*. Earlier information on betaine lipid distribution in microalgae can be found in several research papers and reviews (Araki et al. 1991; Sato 1991; Eichenberger 1993; Dembitsky 1996; Harwood 1998).

Non-phosphorous lipids were found to be dominant in the polar lipid fraction of chlorarachniophytes (Chlorarachniophyceae) of the genera *Bigelowiella*, *Gymnochlora* and *Lotharella* characterized by unusual amoeboid morphology (Leblond et al. 2005). Further study identified DGTS and DGTA present at approximately equal proportions with exception of *Lotharella globosa*, whereas DGTA was dominated (Roche and Leblond 2010). A recent comparative study by Armada et al. (2013) of two novel haptophyte isolates–*Pseudoisochrysis paradoxa* (Isochrysidaceae) and *Diacronema vlkianum* (Pavlovaceae)–identified and characterized the composition of betaine lipids

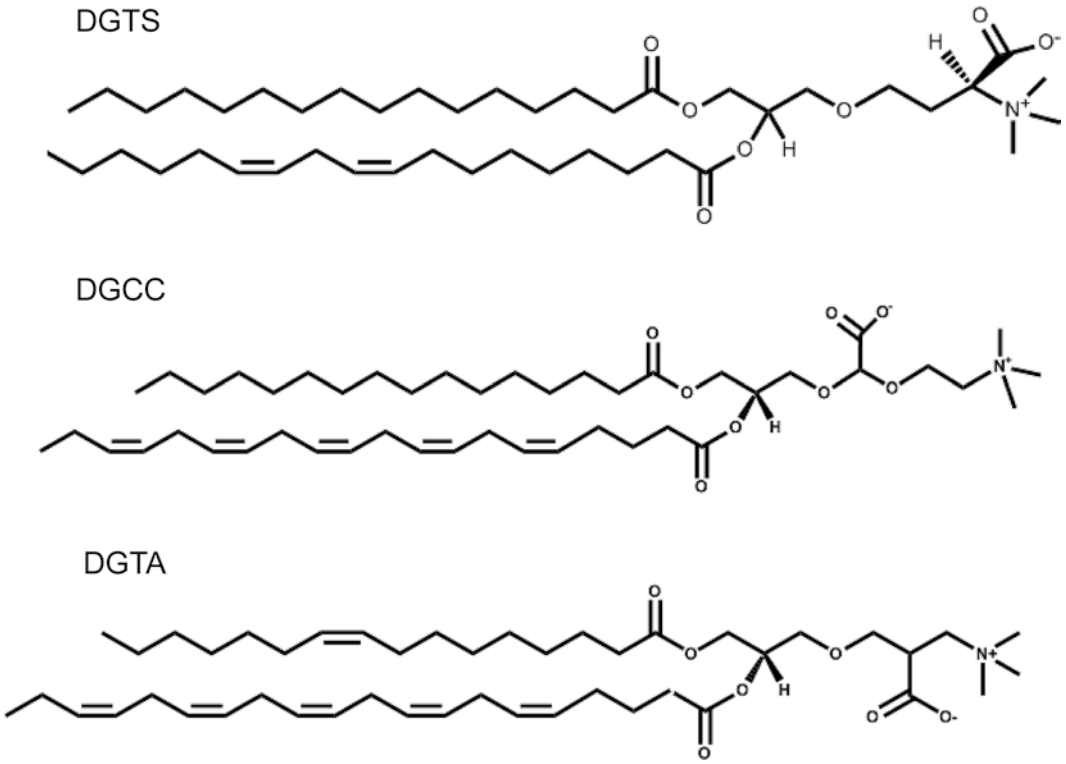

Fig. 4 The structures of the different types of betaine lipids in microalgae

by HPLC/electrospray ionization(ESI)-TOF-MS operating in multiple reaction monitoring (MRM) mode. This technique is now widely used in lipidomic studies with microalgae as well and allows for the identification of molecular species composition of different lipid classes, e.g. species with different compositions of acyl groups. The authors demonstrated that these haptophyte isolates have PG as their only phospholipid, whereas *D. vlkianum* exceptionally contained three different betaine lipids—DGTS, DGTA and DGCC—while *P. paradoxa* lacked DGCC.

The unusual chlorosulfolipids were reported as abundant in *Ochromonas danica*, along with some arsenic-containing lipids present in marine algal species (Harwood 1998; Dembitsky and Srebnik 2002; Dembitsky and Levitsky 2004 and references therein). Chlorosulfolipids seemed abundant in species of both freshwater and marine red, green and brown algae, including unicellular species (Dembitsky and Levitsky 2004). Identification of arseno-lipids, which are present in tiny amounts, requires the most sophisticated methods of analysis and derivatization; a combination of advanced methods was used to identify lipophilic arsenic species in the macrophyte brown alga (Raab et al. 2013). Recent advances in methods for the isolation of novel lipids and their characterization from microalgae by modern analytical methods are described by Guschina and Harwood (2006).

Neutral lipids are represented in microalgae predominantly by triacylglycerols (TAG) (Fig. 5); diacylglycerols (DAG), sterols and sterol esters are also present (Leblond and Chapman 2000). Some microalgae are able to produce hydrocarbons and ether lipids, such as the freshwater colonial chlorophyte *Botryococcus braunii*. Three races of *B. braunii* are distinguished based on the types of hydrocarbons accumulated by each (Metzger and Largeau 2005). The transcriptome of *B. braunii* race B has been sequenced (Molnar et al. 2012) and a unique mechanism for the biosynthesis of the major triterpene botryococcene in *B. braunii* has been recently elucidated (Neihaus et al. 2011). Sterol and hydrocarbon production by microalgae is beyond the scope of this chapter and will be described elsewhere. This chapter focuses on the pathways of TAG biosynthesis and the role of TAG accumulation in microalgal cells.

4 De Novo Fatty Acid Biosynthesis and Carbon Supply

4.1 Carbon Supply to Fatty Acids Synthesis

Fatty acid biosynthesis is one of the primary pathways of cellular metabolism (Harwood 2005) because it provides fatty acid moieties (acyl groups) for the assembly of major

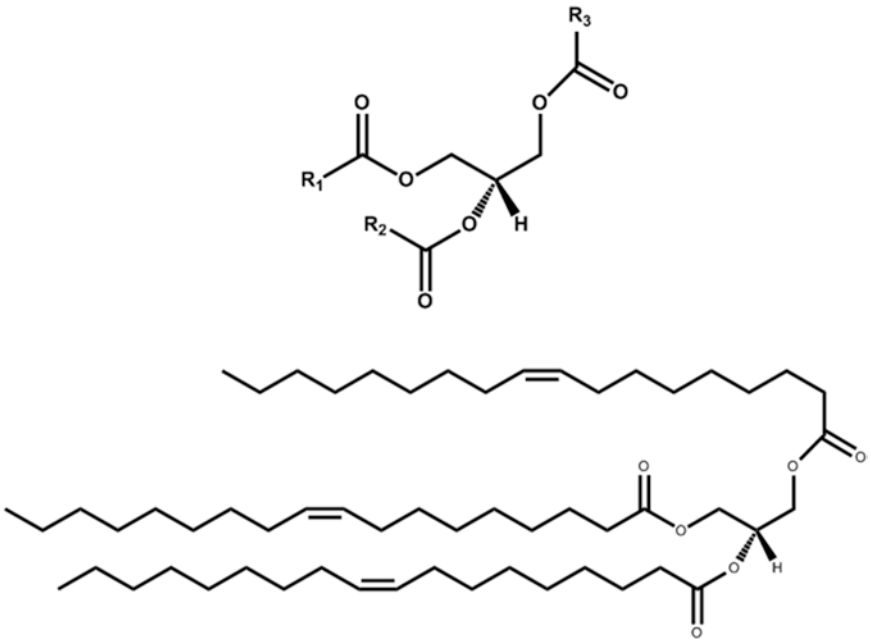

Fig. 5 Triacylglycerol structure as exemplified by trioleoylglycerol. *R1*, *R2* and *R3* are fatty acyl chains

membrane acyl lipids such as glycosylglycerides and phosphoglycerides, and storage lipids such as TAG.

It is assumed that similar to higher plant cells, de novo production of fatty acids takes place in the plastid of microalgal cell and requires carbon in the form of acetyl-coenzyme A (acetyl-CoA), energy (ATP) and reducing power (NADPH), as well as a certain set of predominantly nuclear-encoded proteins. The term "microalgae" encompasses a broad group of evolutionarily distinct microorganisms belonging to different clades that have very different evolutionary histories and cellular organization and compartmentalization of the central carbon pathways (reviewed in Hildebrand et al. 2013). Therefore, some generalized assumptions might be revisited as further insights into the biochemistry and molecular biology of lipid biosynthesis in microalgae of different phylogenetic groups are gained. One example is a recent and thorough comparative analysis of central carbon metabolism, carbon-fixation organization and carbohydrate storage in different algal groups which showed high variation in the cellular organization of core metabolic networks across these groups, evolving from primary and secondary endosymbiosis and evolutionary selection (Hildebrand et al. 2013). Specifically, the major difference between the green algal lineage (Chlorophyta), which is most closely related to higher plants and has a primary chloroplast (evolved as result of primary endosymbiosis), and stramenopiles, haptophytes, cryptophytes, and chlorarachniophytes that evolved via secondary endosymbiotic event, arises from the fact that the complex plastid of these latter microalgae is enclosed by three to four membranes and not by two as in the cells of the green algae, with the outermost envelope membrane forming an intrinsic connection to the ER. The cells of these organisms contain an additional biochemically active compartment—the periplastid space (Hildebrand et al. 2013; Smith et al. 2012). Differences in cellular organization, central carbon metabolism and compartmentalization of storage carbohydrates imply possible variations in carbon partitioning, protein import and lipid trafficking, and the involvement of versatile transporters metabolically connecting different cellular compartments. It has been recently demonstrated that N-glycosylated nuclear-encoded proteins in diatoms destined to the plastid are transported across up to three additional membranes into the plastid stroma (Peschke et al. 2013).

Fatty acid synthesis in the plastid stroma is initiated by acetyl-CoA carboxylase which requires acetyl-CoA and bicarbonate (Harwood 2005). Little is known about the primary players in plastidial acetyl-CoA production in microalgae, but the involvement of different routes can be speculated (Shtaida et al. 2015). It is assumed that the bulky acetyl-CoA molecule cannot enter the plastid by crossing the chloroplast envelope because of its limited permeability through biological membranes and lack of transporters (Oliver et al. 2009), unlike the precursors to acetyl-CoA synthesis, such as, for example, acetate and malate. It is therefore commonly accepted that acetyl-CoA is produced in each cellular compartment that requires it as an important primer in various biochemical reactions, such as the plastid which requires acetyl-CoA to support fatty acid production (Oliver et al. 2009). The biosynthetic origin of acetyl-CoA in the plastid has been intensively investigated by plant lipid biochemists (Ke et al. 2000; Harwood 2005; Oliver et al. 2009), because several biochemical routes can potentially contribute to acetyl-CoA production in plastids—such as reactions

mediated by plastid-localized acetyl-CoA synthase (ACS), pyruvate dehydrogenase (PDH) complex, acetyl-carnitine, and ATP-citrate lyase (ACL).

The chloroplastic PDH multienzyme complex (cpPDH complex) is considered to be the main source of acetyl-CoA formation in the plastids of plants and is composed of multiple copies of three subunits: a PDH (E1, composed of subunits E1α and E1β), a dihydrolipoamide acetyltransferase (E2), and a dihydrolipoyl dehydrogenase (E3). It mediates pyruvate decarboxylation to acetyl-CoA and requires CoA, NAD^+ and lipoic acid as a cofactor, regulated by light, Mg^{2+}, and feedback inhibition by acetyl-CoA and NADH. The dedicated PDH complex in mitochondria (mtPDH complex) is involved in acetyl-CoA production in this organelle. Investigations in higher plants have suggested that cpPDH complex is the main source of acetyl-CoA formation and is primarily responsible for acetyl-CoA synthesis in the plastids (Ke et al. 2000; Lin et al. 2003). The expression pattern of the genes encoding the E1β and the E2 subunits were correlated with the pattern of oil accumulation (Ke et al. 2000; Lin et al. 2003) and disruption of the gene for the E2 subunit by T-DNA insertion caused an early embryo lethal phenotype in *Arabidopsis* (Lin et al. 2003). Flux analyses of lipid biosynthesis in plant oil-seed embryos further confirmed that most of the carbon is directed to fatty acid synthesis in plastid channels to the acetyl-CoA pool via cpPDH complex (Schwender et al. 2003). Further evidence came from comparative analyses of gene expression data: the genes encoding cpPDH complex subunits were co-expressed with genes encoding enzymes of fatty acid synthesis (Mentzen et al. 2008).

Direct molecular evidence for the role of cpPDH complex in providing acetyl-CoA for fatty acid synthesis in microalgae is limited; however, genomic data and patterns of gene expression under conditions triggering TAG accumulation indicate that it may also play an essential role mainly in photoautotrophic oleaginous microalgae that are able to accumulate large amounts of storage lipids, as well as in general for chloroplast-membrane lipid biosynthesis. For example, in the oleaginous eustigmatophyte *Nannochloropsis gaditana*, the number of genes encoding enzymes involved in pyruvate metabolism, as well as many other lipid-biosynthesis-related genes, appeared to be higher than in *C. reinhardtii*, *P. tricornutum* and *Ectocarpus siliculosus* (Jinkerson et al. 2013). Subunits of the cpPDH complex were annotated in the genomes of microalgae of the genus *Nannochloropsis* as clearly distinct from those of the mtPDH complex (Vieler et al. 2012b; Jinkerson et al. 2013). Gene-expression analysis of *Nannochloropsis* IMET I further revealed an important role for the cpPDH complex in directing carbon flow toward fatty acid biosynthesis and TAG formation (Li et al. 2014; Jia et al. 2015). Genes predicted to encode subunits of the cpPDH complex were upregulated (from 1 to 1.5 log2 fold-change) in concert with genes encoding some steps in fatty acid synthesis in the oleaginous microalga *Ettlia* (*Neochloris*) *oleoabundans* (Chlorophyta) under conditions of nitrogen depletion, leading to an increase in total lipid content from 22 to 36 % of dry weight (Rismani-Yazdi et al. 2012). The transcript encoding the PDH subunit E1α (CHLREDRAFT_127786; annotated, however, as the mitochondrial isoform) increased in abundance following nitrogen deprivation in *C. reinhardtii* grown mixotrophically in Tris-acetate-phosphate (TAP) medium (Miller et al. 2010). The genes putatively encoding the cpPDH complex were shown to be upregulated in studies with *C. reinhardtii* in the stationary phase grown in photoautotrophic medium (Lv et al. 2013). Two transcripts of the genes related to pyruvate metabolism were claimed to be strongly increased in the green microalga *Micractinium pusillum* under nitrogen starvation, as determined by suppression subtractive hybridization and real-time PCR (Li et al. 2012c). However, the full length sequences of the genes had to be cloned to enable their curation with any degree of certainty. It should be emphasized that isoforms of many enzymes involved in carbon-precursor partitioning to fatty acid synthesis exist in different cell compartments, and experimental functional verification and cellular localization have to be performed for microalgae species. Prediction of cellular localization by the different algorithms available for microalgae is not straightforward, because these algorithms were trained with higher plant organelle-targeting presequences (transit peptides) (Tardif et al. 2012). The recently developed PredAlgo program, a new multisubcellular localization-prediction tool, is dedicated to microalgae, particularly to *C. reinhardtii* (Tardif et al. 2012). Note that dual protein targeting to mitochondria and plastids has been reported in *Chlamydomonas*.

Fascinating results on the reciprocal function of the E2 subunit of cpPDH complex in *C. reinhardtii* in regulating chloroplast protein synthesis and carbon metabolism was recently obtained by Bohne et al. (2013); they found that this subunit possesses unexpected RNA-binding properties, as confirmed by in vitro RNA-binding assays that affects the chloroplast-translation machinery. Chloroplast localization was confirmed by two methods: targeting the N-terminal presequence fused to GFP to the chloroplast, and cellular fractionation. Analyses of the transformant lines with significantly reduced gene expression and enzymatic activity [generated by RNA interference (RNAi)] revealed that E2 of the chloroplast complex is involved in the synthesis of both the major chloroplast protein D1 and acetyl-CoA, implying that this regulation may function to coordinate the synthesis of lipids and proteins for the biogenesis of chloroplast membranes (Bohne et al. 2013). Notably, reduced enzyme activity did not lead to dramatic changes in overall lipid formation by the RNAi lines, implying that there is still sufficient acetyl-CoA production by the remaining enzymatic activity.

A recent study documented, for the first time, a critical role for cpPDH complex in supplying precursors for fatty acid synthesis under conditions of nitrogen starvation (Shtaida et al. 2014). Artificial microRNA (amiRNA) *pdh* mutants with decreased expression of a E1α subunit of cpPDH complex showed an impaired growth phenotype, significantly reduced TAG content and altered fatty acid composition under conditions of photoautotrophy and nitrogen deprivation. At the same time, no considerable differences were observed between mutant and control lines cultivated mixotrophically in the presence of acetate (Shtaida et al. 2014). Furthermore, comparative study of three green algal species revealed that elevated Acetyl-CoA production and increased expression of cpPDH correlate with a greater extent of TAG accumulation under nitrogen deprivation (Avidan et al. 2015).

Pyruvate, the substrate for cpPDH complex, is provided by pyruvate kinase (EC 2.7.1.40; often abbreviated either PYK or PK) activity in the final and ATP-forming step of glycolysis; PK is one of four glycolytic enzymes—responsible for conversion of glycerate-3-phosphate derived from the Calvin Cycle to acetyl-CoA in the plastid. A low-payoff part of glycolysis is assumed to operate in the plastid of the green microalgae (chlorophytes); however, the plastid of *C. reinhardtii* seems to possess glyceraldehyde phosphate dehydrogenase (GAPDH) as the only glycolytic enzyme (Mitchell et al. 2005). Enolase catalyzing conversion of 2-phosphoglycerate to phosphoenolpyruvate (PEP) seems to be absent in plastid of the mature leaves of higher plants, signifying importance of PEP specific transporters in the envelope membrane (Chapman et al. 2013 and ref. therein) (Fig. 6). Our search for the plastid isoforms in the green microalgae genomes also did not reveal a potent candidate for enolase encoding gene. Numerous transporters [the maltose transporter (MEX1), the triose phosphate translocators (TPT), glucose 6-phosphate (GPT)/xylulose 5-phosphate (XPT) translocators, and phosphoenolpyruvate translocators (PPT)] on the chloroplast envelope suggested to play a pivotal role linking plastidial and cytoplasmic metabolism in the photosynthetic cells of higher plants and microalgae by connecting the carbon flow between the extraplastidial compartment and the plastid (please refer to for more details to Weber and Linka 2011; Chapman et al. 2013; Hildebrand et al. 2013). Microalgae evolved via the secondary endosymbiotic event lost some plastidial transporters during evolution (Weber and Linka 2011). Detailed bioinformatics survey of three genomes of divergent representatives of diatoms revealed some significant differences in organization of the carbon partitioning pathways in the cytosol and the chloroplast; particularly the pennate diatoms seem to have a more complete plastid-localized pathway than that of the centric diatom *T. pseudonana* (Smith et al. 2012). Remarkably, mitochondrion of the diatom *P. tricornutum* was ascribed to harbor a complete ancient Entner-Doudoroff glycolytic pathway, presumably enabling production of pyruvate in the organelle (Fabris et al. 2012).

Pyruvate is a central "hub" molecule that plays a pivotal role in the biosynthesis of sugars, amino acids, and lipids. The plastid PK (EC 2.7.1.40) converts PEP to pyruvate and appeared to catalyze a crucial step in the conversion of photosynthate into oil (Andre et al. 2007). The importance of the plastid PK in providing precursors for fatty acid production has been well documented in developing oil seeds (Andre et al. 2007; Baud et al. 2007). This situation presumably mirrors that in the cells of the oleaginous green algae (Chlorophyta), where the plastid PK may be of critical importance in carbon supply to fatty acid synthesis. Such as, PK gene expression was slightly increased, along with the subunits of the cpPDH complex in the oleaginous microalga *Ettlia* (*Neochloris*) *oleoabundans* under nitrogen starvation (Rismani-Yazdi et al. 2012). Several PK isoforms, including the plastid isoform, have been tentatively annotated in the genome of sequenced microalgae. In the genome of *C. reinhardtii*, however, only one putative PK isoform showed a weak organelle-targeting signal. According to studies performed in our laboratory, this isoform likely encodes the mitochondrial PK given the cellular localization pattern following expression of the N terminus fused to GFP in *A. thaliana* protoplasts (N. Shtaida, I. Khozin-Goldberg, personal communication). Furthermore, the plastid PK seems to be absent in *C. reinhardtii* as inferred earlier by biochemical approach (Klein 1986) and further re-affirmed in a proteomics study on isolated chloroplasts (Terashima et al. 2011), suggesting strong dependency on the supply of PEP from the cytoplasm or an alternative route for its production in this alga. PEP transport across the chloroplast envelope membranes, provided by the parallel glycolytic pathway in the cytosol, is feasible and may occur simultaneously with 2-phosphoglycerate and inorganic phosphate Pi to the cytosol (Flügge et al. 2011; Chapman et al. 2013) (Fig. 6). Furthermore, there is recent evidence for the presence of pyruvate transporters in the chloroplast inner envelope in C4 plants (Furumoto et al. 2011).

Fatty acid synthesis, and subsequently TAG production, in *C. reinhardtii* is enhanced by added acetate (Fan et al. 2012; Goodenough et al. 2014) and thus the pathway via acetyl-CoA synthase is a likely route in this case. Acetyl-CoA formation via CoA activation of acetate is achieved by acetyl-CoA synthase (ACS, EC 6.2.1.1), catalyzing the following reaction:

$$\text{acetate} + \text{ATP} + \text{CoA} \Longleftrightarrow \text{acetyl-CoA} + \text{AMP} + \text{PPi}.$$

The plastid ACS isoform in developing seeds does not appear to play a considerable role in directing carbon flow toward fatty acid synthesis, because a *acs*-knockout mutant did not demonstrate altered lipid levels (Lin and Oliver 2008). Furthermore, there was no correlation between ACS gene expression and patterns of lipid accumulation (Ke et al. 2000). To address the function of the plastid ACS in plants,

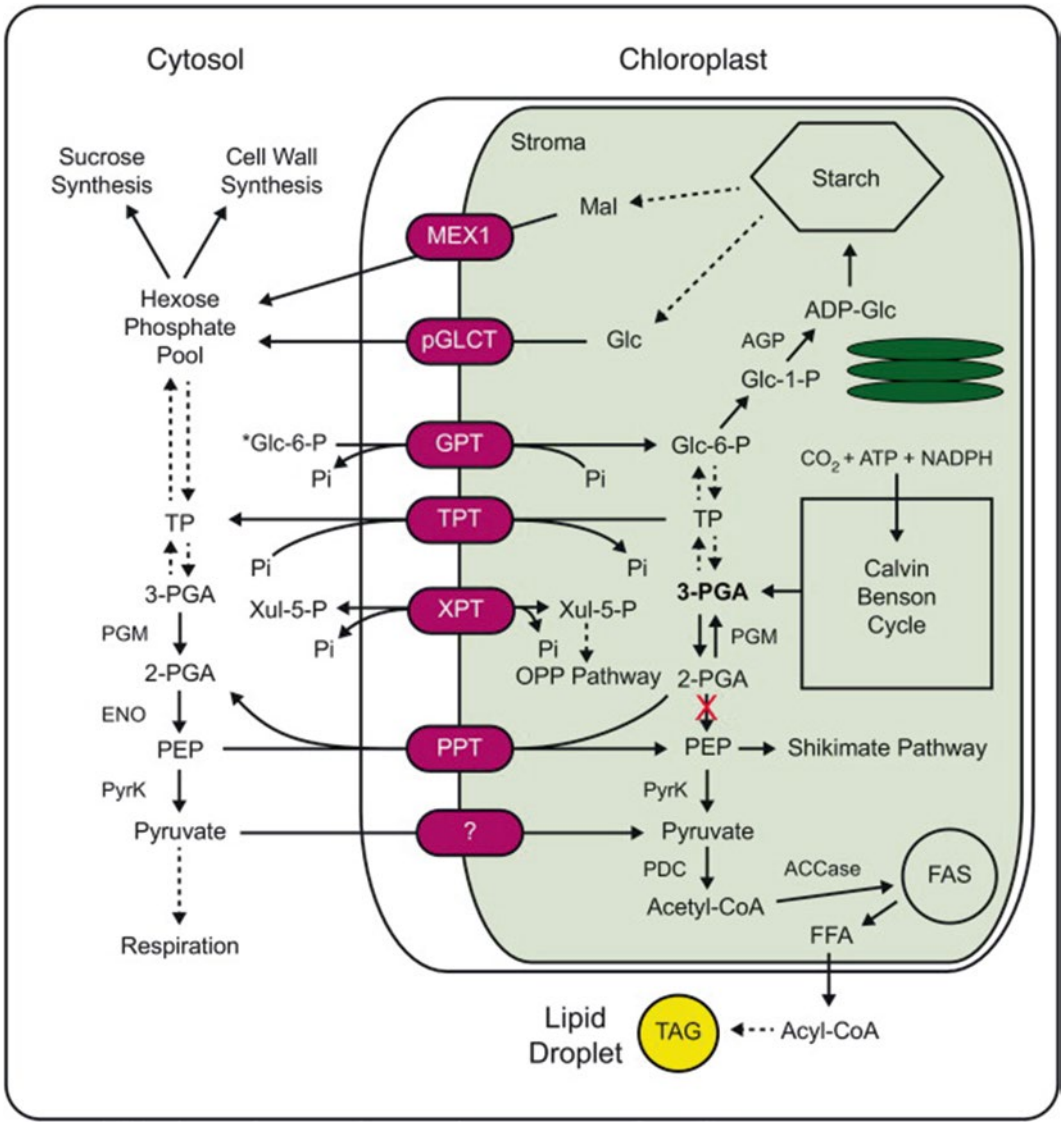

Fig. 6 Suggested pathways for the transport of glycolytic intermediates to and out of chloroplast as exemplified by a photosynthetic leaf cell (adapted) (Reproduced from Chapman et al. (2013); courtesy of Elsevier. Import of Glc-6-P to the stroma via GPT transporter was shown to occur in heterotrophic plant tissues)

it was speculated that the pathway employing ACS serves to bypass the cpPDH complex via the so-called "aerobic fermentation pathway" under conditions requiring active metabolic load and oxygen consumption; the enzymes involved in this pathway are pyruvate decarboxylase (PDC), and aldehyde dehydrogenase, and the terminal step is catalyzed by ACS (Fig. 7).

The anaerobic fermentation pathway, which is dependent on pyruvate production in the plastid, has been well studied in *C. reinhardtii* (Atteia et al. 2006; Catalanotti et al. 2012, 2013) (Fig. 7). *Chlamydomonas* harbors an anaerobic fermentation pathway that is governed by several pyruvate-utilizing plastidial enzymatic routes and contributes to acetyl-CoA production under anoxic conditions. Two enzymes convert pyruvate into acetyl-CoA—pyruvate formate lyase (PFL) with dual localization to the chloroplast and mitochondria, forming formate (Atteia et al. 2006), as well as the chloroplast-localized pyruvate ferredoxin oxidoreductase, which decarboxylates pyruvate and generates CO_2 and does not require (NAD) (van Lis et al. 2013).

$$\text{pyruvate} + \text{Acetyl-CoA} \Longleftrightarrow \text{formate} + \text{CoA}$$

Acetyl-CoA produced by these reactions is further converted to acetate in the plastid by the action of phosphate acetyltransferase (PAT) and acetate kinase (ACK); the ACK/PAT pathway may operate under mixotrophic conditions to produce acetyl-CoA along with ACS. Pyruvate can also be decarboxylated to acetaldehyde by PDC (Fig. 7).

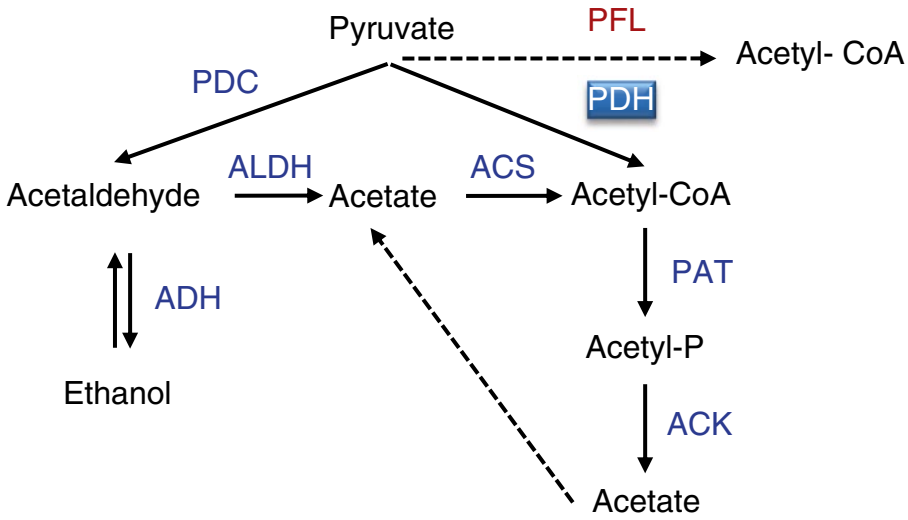

Fig. 7 Schematic representation of alternative plastidial pathways of pyruvate utilization and acetyl-CoA formation. The reactions shown to exist in chloroplast of *Chlamydomonas reinhardtii* are indicated with *dashed arrows*

Acetate did not seem to be a good carbon source for fatty acid synthesis in oil seeds, while it enhanced fatty acid synthesis and storage lipid accumulation in *Chlamydomonas* under mixotrophic conditions (Fan et al. 2012). In contrast to *Arabidopsis*, which has a single ACS, multiple ACS were found in the genome of *Chlamydomonas* in line with the high capacity for acetate assimilation and metabolism in this alga (Shockey and Browse 2011). Several ACS-encoding genes are differentially expressed under conditions of nitrogen starvation, including the plastid isoform which is upregulated in mixotrophic nitrogen-depleted cultures (Miller et al. 2010). The full repertoire of central carbon metabolism genes was annotated in *N. oceanica* in agreement with this alga's ability to utilize organic carbon sources for lipid production (Vieler et al. 2012b). However, there is a report of acetate supplementation negatively affecting lipid production in this alga (Hu and Gao 2003).

Radiolabeled [1-^{14}C]acetate has been utilized in numerous studies as a lipogenic precursor in microalgae and it was readily incorporated into various lipid classes through de novo fatty acid synthesis (Sukenik and Carmeli 1990; Henderson and Mackinlay 1992; Schneider and Roessler 1994; Eichenberger and Gribi 1997; Bigogno et al. 2002a; Guschina et al. 2003; Guihéneuf et al. 2011), implying existence of ACS activity in microalgal cells. Furthermore, in mixotrophically grown *C. reinhardtii* (in the presence of acetate), it was recently proposed that acetyl-CoA produced by ACS and ACK/PAT signals substrate availability for fatty acid synthesis, which may cause product inhibition of the cpPDH complex and, putatively, partial disassembly of the functional mega-Dalton complex that is present under photoautotrophic conditions (Bohne et al. 2013). These novel and intriguing results suggest a regulatory function for the E2 subunit of cpPDH complex in response to growth on reduced-carbon energy sources.

Storage carbohydrates, such as starch in Chlorophytes, are one of the major carbon reserves in microalgal cells and a sink for photosynthates. Starch metabolism in green algae plastid may provide a carbon source for fatty acid production under conditions triggering intensive oil formation. Furthermore, inhibited or compromised starch formation via mutagenesis, such as a mutation in the key regulatory enzyme of starch synthesis ADP-glucose pyrophosphorylase (AGPase), brought about a significant increase in the rate of TAG production and lipid-droplet accumulation in starchless mutants of *C. reinhardtii* (Li et al. 2010a; Goodson et al. 2011; Fan et al. 2012). Fan et al. (2012) demonstrated that TAG accumulation lags behind that of starch and a rapid increase in TAG production occurs in mixotrophic cultures of *C. reinhardtii* when carbon supply via acetate feeding exceeds the capacity of starch synthesis. A comparative time-course study with the starch-less mutant bafJ5 and the wild-type strain dw15 cultured with or without acetate in nitrogen-depleted medium revealed significant increases in the extent and rate of oil accumulation with acetate supplementation, suggesting that TAG accumulation in *Chlamydomonas* under nitrogen starvation is to a large extent limited by the carbon supply (Fan et al. 2012).

Carbohydrate production often precedes that of neutral lipid accumulation in the green algae. This phenomenon was described, for example, in photoautotrophic cultures of the green microalga *Haematococcus pluvialis* induced to produce the keto-carotenoid astaxanthin within TAG-rich lipid droplets (LD): a rapid and substantial increase in the content of total carbohydrates preceded that of total fatty acid production (Recht et al. 2012). Inhibition of starch degradation by the amylase inhibitor methoxybenzoxazolinone (MBOA) resulted in decreased lipid production in the green oleaginous microalgae *Pseudochlorococcum* sp. (Li et al. 2011) and *Lobosphaera incisa* (Shtaida and Khozin-Goldberg unpublished). In the oleaginous eustigmatophyte

Nannochloropsis however, carbohydrates did not seem to contribute much to fatty acid formation, suggesting that in this microalga, carbon supply for fatty acid production de novo is mainly supported by photosynthate assimilation (Recht et al. 2012; Jia et al. 2015). *Nannochloropsis* algae seem to rely on TAG as the major carbon storage product (Vieler et al. 2012b). Metabolomics studies documented mannitol, galactose and glucose as major monosaccharides in *Nannochloropsis* (Pal et al. 2013; Jia et al. 2015). Transcriptional expression analysis ascertained a major role for the pyruvate dehydrogenase pathway in driving carbon flow to fatty acid and TAG biosynthesis, while β-1,3-glucan degradation and mannitol metabolism were also involved, albeit to a lesser extent (Jia et al. 2015).

Acetyl-CoA in extraplastidial compartment can be derived from β-oxidation of fatty acids in the special organelles peroxisomes (alternatively referred to as glyoxisomes in higher plants, a site of glyoxylate shunt) as well as in TCA cycle in mitochondria. Catabolism of membrane and storage lipids, such as TAG, may be constitutively functioning in the cells of oleaginous algae. The genes coding for the enzymes of β-oxidation and of the glyoxylate pathway, including isocitrate lyase and malate synthase were annotated in the genome of the oleaginous microalga *N. oceanica* CCMP1779 (Vieler et al. 2012b). Recent metabolomics studies in microalgae imply a complex network operating in central carbon and nitrogen metabolism in microalgae cells to provide carbon skeletons (such as malate and citrate) and reducing equivalents under conditions triggering intensive lipid accumulation (e.g. Blaby et al. 2013; Recht et al. 2014; Schmollinger et al. 2014; Shtaida et al. 2015). Review of these studies is beyond the scope of this chapter. The involvement of polar membrane turnover in providing fatty acid moieties for TAG formation will be noted later on.

4.2 Acetyl-CoA Carboxylase

Acetyl-CoA carboxylase (ACCase; acetyl-CoA:carbon-dioxide ligase (ADP-forming); EC 6.4.1.2) is a biotin-containing enzyme that catalyzes an initial and key regulatory step in fatty acid biosynthesis—conversion of acetyl-CoA to malonyl-CoA by ATP-dependent carboxylation. The plastid-localized heteromeric ACCase is a fully dissociable multi-subunit protein composed of three domains and four subunits: three subunits [biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP), α-subunit of carboxyltransferase (CT)] are encoded in the nucleus, while subunit CT-β is encoded by the chloroplast genome (Nikolau et al. 2003; Harwood 2005).

The reaction catalyzed by ACCase is performed in two steps: BC catalyzes the initial partial reaction mediating carboxylation of a biotin cofactor, covalently bound to the central BCC domain, using bicarbonate (Fig. 8). The second partial reaction is catalyzed by CT, transferring the carboxyl group to acetyl-CoA and yielding malonyl-CoA (Nikolau et al. 2003; Harwood 2005). Prior to entering the fatty acid synthase (FAS) complex in the plastid, malonyl-CoA is converted by malonyl-CoA:acyl-carrier protein (ACP) transacylase) MAT (EC 2.3.1.39) to malonyl-ACP; it actually donates two carbons in each of the consecutive condensation steps in fatty acid synthesis.

In the cytosol of plant and algal cells, malonyl-CoA production is presumed to be mediated by a different ACCase isoform, a multidomain homomeric ACCase (often referred to as multifunctional) that is required for the biosynthesis of various secondary metabolites as well for fatty acid elongation (Harwood 2005); in LC-PUFA-producing microalgae, malonyl-CoA produced in cytosol is used for C18–C20/C22 LC-PUFA elongation. A critical role of homomeric ACCase for C18–C20 fatty acid elongation in the ER was demonstrated in planta by a reverse genetic approach (Baud et al. 2003). Worth noting is a recently characterized gene encoding malonyl-CoA synthase (EC 6.2.1.14), which mediates the formation of malonyl-CoA from malonate and CoA in both plants and mammals; cellular localization studies localized the protein to the nucleus as well as the cytoplasm in plant cells (Chen et al. 2011).

In most higher plants, a heteromeric ACCase resides in the chloroplast; however, some weeds and monocotyledonous plants also have the homomeric ACCase in their plastids (Konishi et al. 1996; Harwood 2005). Remarkably, the first ACCase gene cloned from microalgae, namely the diatom *Cyclotella cryptica*, appeared to encode a multidomain ACCase harboring the chloroplast-targeting pre-sequence in its N terminus (Roessler and Ohlrogge 1993). In this microalga, the significant increase in the activity of ACCase was shown under storage-lipid-inducing conditions of silicon-deficiency (Roessler 1988). However, attempts to overexpress the ACCase gene in the diatoms *C. cryptica* and *Fistulifera* (*Navicula*) *saprophila* to increase lipid accumulation did not result in a significant change in lipid content (Sheehan et al. 1998), implying existence of complex regulation over this enzymatic step.

A genome-wide survey of currently available proteome data of sequenced algae from taxonomically divergent microalgal groups was performed by Huerlimann and Heimann (2013). As already mentioned, microalgae of evolutionarily distinct groups feature distinctive metabolic and structural differences. Using bioinformatics tools for comparisons to available ACCase amino acid sequences and predictions of organellar localization, the authors concluded that the presence of heteromeric or homomeric ACCase depends on the evolutionary origin of the plastid. In the cells of chlorophytes (with the exception of the early-divergent class Prasinophyceae), glaucocystophytes and rhodophytes

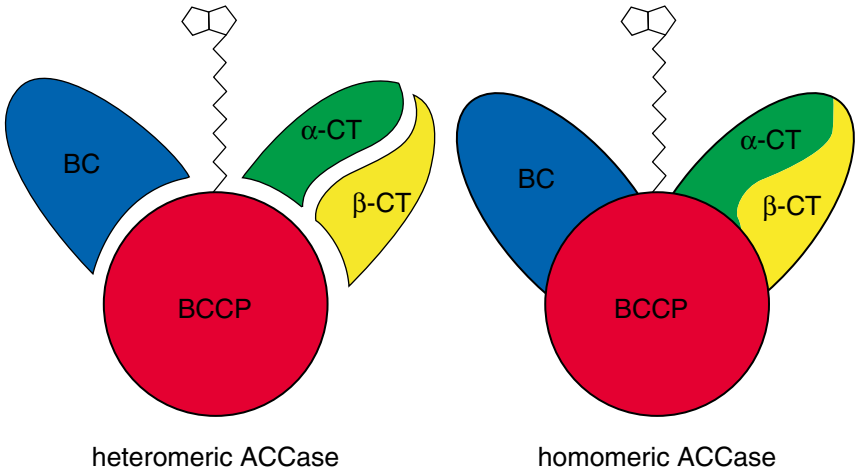

Fig. 8 Simplified schematic representation of the organization of two types of ACCases. Dissociable subunits of the heteromeric ACCase are shown as separated by a gap (Reproduced from Nikolau et al. 2003; courtesy of Elsevier)

that evolved by primary endosymbiosis, heteromeric ACCase is predicted to be located in the plastid. The authors identified the acetyl-CoA-binding region which displayed distinct differences in the heteromeric CT-β subunit and the homomeric CT-β domain. In contrast, the plastids of the Heterokontophyta and Haptophyta, evolved via secondary endosymbiosis, as well as the apicoplast-containing Apicomplexa, harbor a homomeric ACCase as the major plastid form, in line with previously cloned and isolated ACCase from a diatom (Roessler and Ohlrogge 1993). On the other hand, all components of the plastidial multimeric ACCase were identified in the genome of the heterokont eustigmatophyte *N. oceanica* CCMP1779, while the homomeric ACCase was also annotated as a cytosolic enzyme in the CCMP1779 genome (Vieler et al. 2012b).

There is much evidence of an important regulatory role for ACCase in controlling carbon flux to lipids in plants (summarized and discussed in detail in Harwood 2005 and references therein). Based on flux-control analysis, ACCase may exert up to 60 % of total flux control to lipids (Page et al. 1994). These measurements were performed following administration of homomeric ACCase-specific inhibitors to barley and maize leaves harboring this isoform in the plastid. A large body of research has been conducted with purified ACCases from several grasses to elucidate the mechanism governing the catalytic reaction and the action of graminicide herbicides (for detailed information, please refer to Harwood 2005). A number of herbicides targeting homomeric ACCase were administered to the microalgal cultures to determine the importance of carbon flow via ACCase for neutral lipid formation, and to correlate that with an influence on lipid-droplet formation. The herbicide sethoxydim, for example, which belongs to the cyclohexanedione class of ACCase inhibitors, was shown to inhibit β-carotene accumulation in plastidial lipid droplets in high-light-induced cultures of the green alga *Dunaliella salina* (as *Dunaliella bardawil*), and an up to 45 % decrease in ACCase activity was measured in vitro (Rabbani et al. 1998). TAG formation was significantly decreased at 25–50 μM sethoxydim and was completely abolished at 100–200 μM of this herbicide in *D. salina* under nitrogen starvation (Davidi et al. 2012). Which ACCase isoform is responsible for this inhibition remains to be elucidated. Four graminicide herbicides of the cyclohexanedione and aryloxyphenoxypropionate classes, namely quizalofop, fenoxaprop, sethoxydim and haloxyfop, were effective at inhibiting the growth of *Nannochloropsis oculata* (Chaturvedi et al. 2004) with quizalofop exhibiting the strongest effect and the lowest killing concentration.

A growing number of publications are reporting quantitative information on alterations in the expression levels of lipid biosynthesis-related genes, primarily during intensified neutral lipid (TAG) accumulation induced by nitrogen depletion, based on the powerful next generation RNA-sequencing (RNA-Seq) methods; more recently, reports of protein abundance have also been published, as determined by global proteomics of microalgal cells.

Given the important rate-limiting role of plastidial ACCase in lipid biosynthesis, we review in more detail some of the information available on the patterns of expression of ACCase-encoding genes in microalgae. As can be seen, there are variations in these genes' expression patterns that are not always correlated with patterns of lipid accumulation. It should also be mentioned that only a few studies have investigated the temporal patterns of change in detail. The transcripts encoding ACCase appeared to be very abundant in the transcriptome of an oil-rich race A strain of B. *braunii* (Baba et al. 2012). Only the BC subunit of the plastid ACCase isoform was significantly upregulated in response to nitrogen starvation in the photosynthetic oleaginous green microalga *Ettlia* (*Neochloris*) *oleoabundans*, whereas expression of other subunits was repressed (Rismani-Yazdi et al. 2012). Studies in our laboratory with the green microalga *L. incisa* showed significant upregulation of the gene for the BC subunit in early stages of nitrogen starvation, while expression

of the homomeric ACCase isoform followed a different trend, increasing with time of nitrogen starvation; this is consistent with its function in extraplastidial fatty acid elongation and production of LC-PUFA in this alga (Shtaida and Khozin-Goldberg unpublished results). In the diatom *P. tricornutum*, the plastidial ACCase showed greater changes in expression (threefold increase) than the cytoplasmic ACCase during the growth stage associated with gradual nitrate and phosphate depletion, whereas neither form was strongly upregulated at the transcriptional level during prolonged lipid accumulation in the absence of nitrogen (Valenzuela et al. 2012). Significant changes in plastidial ACCase expression (at the transcript level) were determined in the course of oil accumulation in the oleaginous diatom *Fistulifera solaris* JPCC DA0580 concurrent with enhanced de novo generation of fatty acids and their deposition in TAG (Tanaka et al. 2015). On the other hand, only modest changes in transcript levels of genes encoding fatty acid metabolism, including ACCase, were reported within 48 h of nitrogen deprivation in *C. reinhardtii* cultivated under photoheterotrophic conditions supplemented with acetate (Miller et al. 2010). Similar results were obtained in another detailed transcriptomic study with *Chlamydomonas* (Boyle et al. 2012) investigating temporal alterations in gene expression, including shorter time points, upon transfer to nitrogen starvation. Downregulation of genes encoding ACCase subunits was already evident within 60 min and continued up to 48 h. The most prominent decrease was exhibited by the gene encoding malonyl-ACP acting downstream of ACCase (up to about 15 % of its expression level at time 0). Under these conditions, strong evidence for upregulation of membrane lipid turnover and the final steps of TAG assembly were obtained, suggesting recycling of membrane lipids to TAG as an important source of fatty acids (Miller et al. 2010; Boyle et al. 2012) (this will be further discussed in the section on TAG biosynthesis). Another study investigated transcriptomic changes in batch photoautotrophic cultures of *C. reinhardtii* at different growth stages; ACCase transcripts were abundant at all growth stages, showing some downregulation along with MAT-encoding gene upon nitrogen depletion from the nutrient medium (Lv et al. 2013). Wan et al. (2011) found increased expression levels of *ACCD* encoding heteromeric CT-β subunit and low levels of a gene encoding homomeric ACCase in the green alga *Chlorella sorokiniana* under mixotrophic (glucose-fed) conditions leading to enhanced lipid production. Remarkably, the oleaginous photoautotrophic eustigmatophytes *N. gaditana* and *N. oceanica* IMET I did not show any significant alterations in expression level of mRNA coding for proteins involved in the de novo FA biosynthetic pathway (Corteggiani Carpinelli et al. 2014; Li et al. 2014), unlike the chloroplast PDH complex whose expression was significantly enhanced in response to nitrogen scarcity (Jia et al. 2015). Boyle et al. (2012) performed RNA-Seq transcriptome analysis in *C. reinhardtii* at 1 h, 8 h, and 48 h after transferring the cells to nitrogen-deficient TAP medium hypothesizing that changes in mRNA abundance should precede changes in TAG accumulation. Indeed, the study revealed genes whose temporal expression patterns were associated with an increase in TAG accumulation, almost none of them related to de novo fatty acid synthesis. In agreement, another global comparative transcriptome analysis of the response to nitrogen deprivation of three widely researched laboratory strains of *C. reinhardtii* [the cell wall-containing strain CC4532, the parental STA6 strain CC4349, and the mutant strain CC4348 (*sta*6)], all grown in the presence of acetate, revealed only a temporal decrease in the mRNA abundance of genes encoding enzymes implicated in de novo fatty acid biosynthesis, including ACCase (Schmollinger et al. 2014); mRNA levels of the latter recovered between 12 and 24 h after onset of nitrogen depletion and remained almost unaltered thereafter. However, at the protein level, two subunits of the chloroplast-localized ACCase [β-carboxyltransferase (BCX1) and biotin carboxyl carrier protein (BCC1)] were strongly reduced upon nitrogen deprivation.

Obviously, it is quite challenging to provide a general picture of ACCase transcriptional regulation under conditions triggering lipid accumulation given the diversity of microalgae and the variety of cultivation and lipid-accumulation-inducing conditions used in the many different studies. Nevertheless, there are a number of important factors known to modify the rate of neutral lipid accumulation in microalgae—particularly under physiologically stressful conditions that have severe consequences for the cell cycle and cellular metabolism functions, such as nutrient deprivation and increased light intensity (Roessler 1990; Hu et al. 2008; Solovchenko 2012)—and hence affect gene-expression patterns. Furthermore, the supply and build-up of precursors for the ACCase, rather than the actual levels of these enzymes per se, might play a significant role in TAG formation (Valenzuela et al. 2012). Elucidation of protein abundance may provide better insight into the regulation of ACCase levels under these conditions.

Taking this into account, Guarnieri et al. (2011, 2013) investigated ACCase protein abundance (quantified by means of a comparative de novo transcriptomics-proteomics pipeline under nitrogen-replete and nitrogen-depleted conditions in *Chlorella vulgaris* UTEX 395) and determined an approximately twofold increase under nitrogen-depleted conditions. Remarkably, the MAT protein abundance, acting downstream of ACCase, was similarly upregulated approximately 1.4-fold, as were some components of FAS (Guarnieri et al. 2011); this revealed a concerted pattern of proteins involved in fatty acid synthesis and the significance of de novo protein synthesis in oleaginous microalgae under conditions triggering intensive TAG accumulation. This pattern

of change is dissimilar to the above described patterns of gene expression in mixotrophic cultures of *C. reinhardtii*. In their recent work, Guarnieri et al. (2013) examined the temporal alterations in the *C. vulgaris* proteome in more detail following 24, 72, and 144 h of nitrogen deprivation. Under nitrogen-replete conditions, the heteromeric ACCase isoform was about twice as abundant as the homomeric isoform, but under nitrogen depletion. The protein abundance of the homomeric isoform dropped at as early as 24 h and further decreased below detection limits at 72 h following nitrogen depletion, whereas the protein abundance of the heteromeric ACCase continued to increase. Opposed trends of change were found for the abundances of the heteromeric plastidial ACCase isoform and, as identified by proteomics, AMP-activated kinase (Guarnieri et al. 2011, 2013); this led the authors to suggest that this kinase is an important regulatory switch affecting activity of ACCase, as shown in mammalian cells. To exert its action, AMP-activated kinase should be localized to the same compartment as the target ACCase, and therefore further investigation is obviously needed. Remarkably, subunits of a heteromeric plastidial ACCase were among proteins identified by a quantitative proteomics approaches as significantly differentially up-expressed in a previously mentioned starch-less mutant sta6 of *C. reinhardtii* as compared to its parental strain cw15 (Wang et al. 2012a).

Regulation of plastidial ACCase activity is under complex control by light, redox status through thioredoxin reduction, post-transcriptional and post-translational modifications, and feedback inhibition (Sasaki et al. 1997; Sasaki and Nagano 2004; Harwood 2005; Chapman and Ohlrogge 2012). Feedback inhibition of the heteromeric ACCase by the primary product of fatty acid synthesis was recently demonstrated in experiments with cell-suspension culture derived from embryos of the oil-seed plant *Brassica napus* supplied with tween esters of fatty acids, predominantly 18:1-containing ones (Andre et al. 2012). The results of the study suggested that the amount of 18:1-ACP controls plant fatty acid synthesis through its biochemical inhibition of plastidial homomeric ACCase in oleaginous tissues. Whether these mechanisms hold true in oleaginous microalgae remains to be investigated.

4.3 Reactions of Fatty Acid Synthesis

De novo fatty acid biosynthesis is initiated by condensation of acetyl-CoA (the primer) and malonyl-ACP (the donor) and involves the sequential addition of a two-carbon unit from malonyl-ACP to the growing acyl chain in the form of acyl-ACP. Fatty acid biosynthesis is performed by stromal (type II) FAS, an easily dissociable multisubunit consisting of four monofunctional enzymes. In mammalian-type cytosolic type I FAS, the constituent seven catalytic components are covalently linked in a multifunctional megasynthase that shares many structural and biochemical similarities with polyketide synthases (PKS) (Smith and Tsai 2007). PKS contain multiple copies of the same domains as type I FAS arranged in a different manner and may participate in a variety of biosynthetic reactions. Different types of putative PKS can be identified in genomes of various groups of microalgae. Whether there is type I FAS in the cytoplasm of microalgal cells remains uncertain; however, the evolutionary history of secondary and tertiary endosymbionts suggests that this is possible. Remarkably, five different systems of fatty acid synthesis have been reported in the euglenoid *Euglena gracilis*, among them a cytosolic multifunctional FAS I whose main products are C16 fatty acids (Hoffmeister et al. 2005 and earlier references therein). A putative type I FAS homolog of that from animals was tentatively annotated in *N. oceanica* CCMP1779 (Vieler et al. 2012b).

The reactions catalyzed by plant FAS are described in several sources (Harwood 2005; Li-Beisson et al. 2010). Here we provide some basic information that is relevant to each step. Each two-carbon elongation involves four enzymatic steps: condensation, reduction, dehydration and a second reduction consuming two molecules of NADPH and releasing one molecule of CO_2 from malonyl-ACP. The condensation reactions are catalyzed by condensing enzymes termed β-ketoacyl-ACP synthases (KAS) (EC 2.3.1.41), forming a C-C bond in Claisen-type condensation. Three types of KAS are known to participate in the different steps of acyl-chain elongation. The KAS III isoform catalyzes the initial condensation reaction of acetyl-CoA and malonyl-ACP yielding a 4-C β-ketobutyryl-ACP. KAS I catalyzes the six following subsequent iterative steps leading to biosynthesis of the saturated palmitoyl 16:0-ACP; a third condensing enzyme, KAS II, catalyzes elongation of 16:0-ACP to stearoyl-ACP (18:0-ACP) (Fig. 9). The activities of the different types of KAS may be differentiated by sensitivity to different antibiotics; for example, KAS I is sensitive to cerulenin, whereas KAS II is less sensitive to cerulenin but is inhibited by arsenite; KAS III is inhibited by thiolactomycin-type antibiotics (Harwood 2005). After condensation, the intermediate is reduced by NADPH-requiring β-ketoacyl-ACP reductase (KAR) (EC 1.1.1.100). The enzyme β-hydroxyacyl-ACP dehydratase (HAD) (EC 4.2.1.59) generates enoyl-ACP which is reduced by the second reductase, enoyl-ACP reductase (ENR); the latter may use both NADH and NADPH to form a saturated acyl-ACP. The major products of FAS in the plastid are C16 and C18 saturated acyl-ACPs; 18:0-ACP is successively and efficiently desaturated by a soluble Δ9 stearoyl-ACP desaturase (SAD) (EC 1.14.99.6) in the chloroplast stroma.

SAD represents a unique type of soluble desaturase in plants with no structural similarity to mammalian or fungal SAD, whose soluble nature allowed for crystallographic

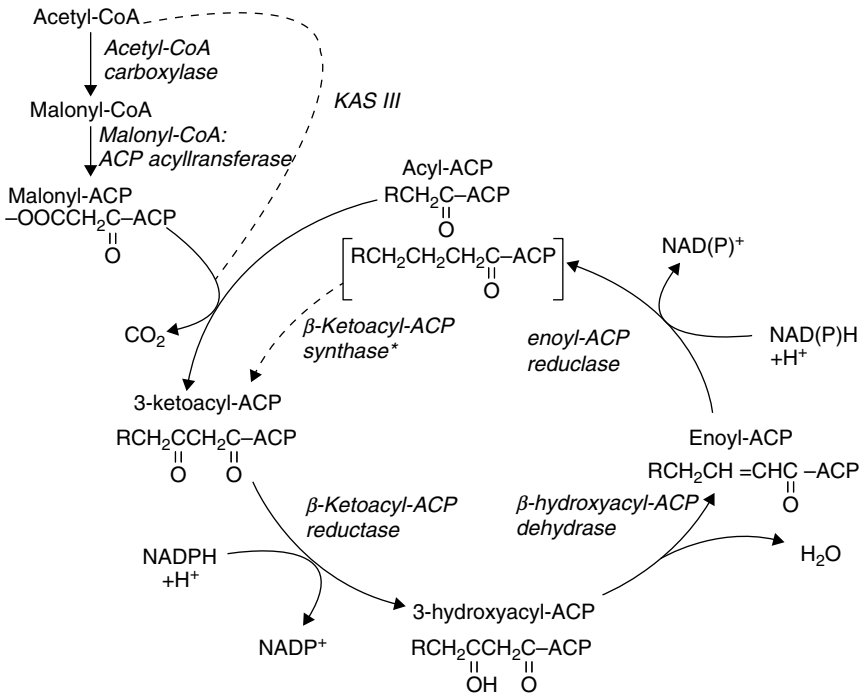

Fig. 9 Schematic representation of the reaction of fatty acid synthase (*FAS*) (Harwood 2010 Reproduced from the AOCS Lipid Library site: http://lipidlibrary.aocs.org/plantbio/fa_biosynth/index.htm. Permission granted by the American Oil Chemists' Society (AOCS) Lipid Library)

studies of the purified protein (Shanklin and Cahoon 1998). In *Arabidopsis*, several SAD isoforms are known, with some family members showing a preference for 16:0-ACP (Kachroo et al. 2007). In the genome of the diatom *P. tricornutum*, a single gene encodes a SAD-like homolog featuring the diatom-characteristic N-terminal chloroplast bipartite targeting peptide (Dolch and Maréchal 2015). Given that 16:1Δ9 constitutes a major monounsaturated fatty acid in this diatom, the authors suggested that this acyl-ACP desaturase acts predominantly on 16:0. SAD from the green oleaginous microalga *Chromochloris* (*Chlorella*) *zofingiensis* showed similarity to the putative green algal SAD and was predicted to have a chloroplast-targeting signal; this SAD was cloned and functionally expressed in *Escherichia coli* (Liu et al. 2012b). The level of gene expression was examined under a variety of stressful conditions known to induce oleoyl-rich TAG accumulation in this alga, such as high light, nitrogen starvation and their combination. Both the BC subunit of the plastid ACCase and SAD-encoding genes demonstrated significant and coordinated upregulation under stress conditions, as was determined by real-time PCR, which preceded TAG accumulation. An RNA-Seq study of nitrogen-starved *C. reinhardtii* under mixotrophic conditions revealed downregulation of SAD gene within several hours of nitrogen depletion (Boyle et al. 2012). Immunoblot analysis of protein abundance revealed a decrease in the acyl-ACP desaturase protein encoded by *Chlamydomonas SAD* (Cre17.g701700; FAB2) in response to iron starvation; this was correlated with decreases in some other iron-containing proteins, such as the plastidial ferredoxin required for the desaturase activity, as well as an increase in saturated fatty acids after 24–48 h of iron starvation (Urzica et al. 2013). Transcriptional regulation of SAD under iron starvation appeared to be more complex and opposed to protein abundance, displaying increased transcript abundance throughout the entire time course (48 h).

4.4 Termination of Fatty Acid Synthesis and Export from the Plastid

Fatty acid synthesis in the plastid is terminated by the action of acyl-ACP thioesterases (TE) generating ACP and free fatty acids from the acyl-ACP complexes with 16:0-ACP, 18:0-ACP, 18:1Δ9-ACP and sometimes 14:0-ACP. A certain proportion of the fatty acids remain in the plastid and are utilized in the prokaryotic pathway of glycerolipid synthesis, while most of them are released to the ER to be used in the eukaryotic pathway (Sect. 5.1). Fatty acyl-ACP thioesterase FATA and FATB enzymes reside in the inner chloroplast envelope in plant cells and have been shown to be responsible for the release of unsaturated and saturated C16 and C18 fatty acids, respectively, from their acyl-ACP forms, to be further re-esterified to acyl-CoA and used in the extraplastidial compartment (Salas and Ohlrogge 2002). FAT enzymes compete with the plastidial glycerol-3-phosphate acyltransferase (GPAT) for their common substrate, the acyl-ACP pool, with a fraction which is utilized by GPAT being destined for glycerolipid biosynthesis in the plastid. The recombinant FATA protein of *Arabidopsis thaliana* displayed very

high TE activity toward 18:1$^{\Delta 9}$-ACP in in vitro assays, whereas FATB displayed its highest activity toward 16:0-ACP and only 75 % of that activity toward 18:1$^{\Delta 9}$-ACP (Salas and Ohlrogge 2002). In some plants producing unusual short-chain or monounsaturated fatty acids, acyl-ACP TE specificity may be responsible for their incorporation into extraplastidial lipids (Dörmann et al. 1994; Voelker et al. 1992; Salas and Ohlrogge 2002). In many marine microalgae, 14:0 and 16:1 are present in the extraplastidial lipids, suggesting that the export of these fatty acids from the chloroplast might be initiated by the action of acyl-ACP TE, another option is desaturation of 16:0 in the ER.

The role of FATA and FATB isoforms in *Arabidopsis* in controlling termination of FAS and distribution of de novo-produced fatty acids in the extraplastidial lipids was elucidated by a reverse-engineering approach (Bonaventure et al. 2003, 2004; Moreno-Pérez et al. 2012). Remarkably, analysis of slow-growing FATB-knockouts with decreased palmitate content in leaf phospholipids but not in plastidial galactolipids revealed the homeostatic mechanisms that allow membrane lipids to preserve their fatty acid composition to compensate for substantial changes in the rates of fatty acid and glycerolipid metabolism. The mutants seemed to show increased rates of FAS, increased protein expression levels (BCCP, ACP, and SAD) and accelerated rates of lipid turnover and fatty acid degradation. FATA-knockout mutants of *Arabidopsis* featured lower seed oil contents and altered fatty acid composition as well as reduced metabolic flow of the different glycerolipid species into the seed oil (Moreno-Pérez et al. 2012).

The microalgal research community has shown strong interest in this group of enzymes given their potential for manipulation of microalgal TAG fatty acid composition and thus for improving oil quality for biofuel feedstock production (Radakovits et al. 2010, 2011; Blatti et al. 2012). Given that short fatty acid chains are more suitable for gasoline and jet fuel production, genetic engineering of microalgae for expression of the short chain-specific higher plant TE is seen as a potent means of improving the quality of microalgal oils for the purposes of biodiesel production (Radakovits et al. 2010). Indeed, the substrate preference of acyl-ACP TE is an important determinant of the fatty acid composition of TAG as inferred from studies performed with oil-seed plants with TAG fatty acids of uncommon chain lengths (e.g. C8:0, C10:0, C12:0). Furthermore, molecular engineering efforts using acyl-ACP TE from such plants in higher plants resulted in a dramatic increase in the content of short-chain fatty acids in the seed oil (Voelker et al. 1992; Voelker and Kinney 2001). The expression of genes encoding two codon-optimized land plant ACP-TE with preference toward 12:0-ACP and 14:0-ACP in the diatom microalga *P. tricornutum* indeed resulted in increased accumulation of shorter chain length fatty acids in TAG (Radakovits et al. 2011). However, the transformed lines demonstrated decreased growth rates associated with higher cellular lipid content, indicating that overexpression of plant TE resulting in alterations in the acyl composition of cellular lipids has a negative and stressful impact on microalgal cells. It should be noted that this microalga naturally contains 14:0 in its lipid classes. With the onset of the stationary phase, the relative proportion of short fatty acids decreased due to an overall increase in the endogenously produced 16:0 and 16:1 fatty acids—the major acyl component of TAG in this alga.

Phylogenetic analysis reported by Blatti et al. (2012) separated a distinct green algal group of acyl-ACP TE composed of three members (*Chlamydomonas*, *Chlorella*, *Volvox*) from higher plant FATA and FATB. A TE candidate gene similar to *Arabidopsis* FATA or FATB was not found in the genome of *N. oceanica*, but an ortholog similar to a putative TE of *Ectocarpus siliculosus* was identified (Vieler et al. 2012b). Interestingly, no homologs for the genes encoding the acyl-ACP TE were identified in the newly assembled genome of the oleaginous diatom *Fistulifera solaris* (Tanaka et al. 2015). On the other hand, a large-scale analysis of five oleaginous *Nannochloropsis* species (a total of six strains) identified five acyl-ACP TE which comprised one of several expanded gene families that were related to the oleaginicity of *Nannochloropsis* (Wang et al. 2014). More studies are obviously needed to investigate the diversity of acyl-ACP TE in microalgae involved in the export of the de novo synthetized fatty acids from the plastid. A large variation in those mechanisms can be envisioned in the cells of microalgae from distinct evolutionary groups, in particular from groups whose extraplastidial lipid synthesis is tightly linked to chloroplast lipid metabolism.

Two plant TE genes and one nuclear-encoded endogenous TE of *C. reinhardtii*, all biased for efficient expression in *C. reinhardtii* chloroplasts, were expressed at high levels in the chloroplast of this microalga (Blatti et al. 2012). Overexpression of endogenous CrTE seemed to result in an increased level of short-chain fatty acids, whereas no alteration in the fatty acid profile was determined upon expression of the plant TE. It is likely that *C. reinhardtii* acyl-ACP TE encoded by *FAT1* (Cre06.g256750) can hydrolyze both saturated and unsaturated substrates. These results were projected by an in vitro activity-based crosslinking probe assay designed to selectively trap transient protein-protein interactions between the TE and ACP; plant TE did not seem to show any mechanistic crosslinking to ACP of *C. reinhardtii* (Blatti et al. 2012).

As already mentioned, transcription-profiling studies have been performed for several microalgal species but only a few of them have dealt with the temporal changes that are important for assigning a role for a certain step in lipid metabolism. All KAS-encoding genes were concertedly upregulated along with genes putatively encoding the PDH

complex, the BC subunit of ACCase and acyl-ACP TE, while the KAR-encoding gene was repressed in the nitrogen-starved green alga *N. oleoabundans* under photoautotrophic conditions (Rismani-Yazdi et al. 2012). Similar expression patterns of BC- and KAS-encoding genes were determined in our studies with the green alga *L. incisa* (Trebouxiophyceae) (N. Shtaida, I. Khozin-Goldberg, personal communication). This pattern is reminiscent of the typical transcriptional alterations in developing oil seeds (Baud and Lepiniec 2010; Troncoso-Ponce et al. 2013). Significant alterations in gene expression of acyl-ACP TE (upregulation) were determined in the mixotrophically grown *C. reinhardtii* under nitrogen deprivation (Miller et al. 2010). The results of significant upregulation of FAT1 with time of nitrogen starvation were confirmed in another gene-expression study of this alga (Boyle et al. 2012), indicating the enhanced export of fatty acids from the chloroplast. Genes encoding KAR and ENR were upregulated by 2- to 2.5-fold (log2) in photoautotrophic batch cultures of *C. reinhardtii* (Lv et al. 2013). However, KAS I and KAR were shown to be downregulated in another study with *C. reinhardtii* CC-125 subjected to nitrogen deprivation under strictly photoautotrophic conditions (Msanne et al. 2012). Gong et al. (2011) characterized a TE from *P. tricornutum* (PHATR_33198), which showed no similarity in its amino acid sequence to plant acyl-ACP TE or bacterial TE. The authors demonstrated that the level of gene expression increases under N-starvation and claimed that overexpression of this protein in *P. tricornutum* increases total lipid content.

The expression patterns of fatty acid synthesis-related genes, at least in the green oleaginous algae under photoautotrophic conditions, suggest the importance of transcriptional regulation upon setting up the nitrogen-depleted conditions conducive to intensive TAG accumulation in the long-term. In plants, a ubiquitous transcription factor WRINKLED 1 (WR 1) coordinates the expression of genes functioning in fatty acid synthesis, and the provision of pyruvate, acetyl-CoA and malonyl-ACP (Baud and Lepiniec 2010; Chapman and Ohlrogge 2012; Ma et al. 2013). Little is known about transcriptional factors that might be involved in the control of the fatty acid synthesis machinery in microalgae. The inducible transcription factor NRR was revealed by RNA-Seq of *C. reinhardtii* after short-term exposure to nitrogen depletion, and the increase in its mRNA appeared to be highly specific to the nitrogen-starvation response and to precede that of TAG-assembly genes (Boyle et al. 2012). A proposed transcriptional regulatory network, possibly engaged in the regulation of fatty acid synthesis and TAG assembly, was identified in *Nannochloropsis* by a comparative genome-scale bioinformatics search (Hu et al. 2014); those predictions require further experimental validation. Isolation and detailed characterization of the transcriptional responses of a Compromised Hydrolysis of Triacylglycerols 7 mutant (cht7) of *C. reinhardtii*, which is affected in the reversal of nitrogen-starvation-induced quiescence, provided novel insight into the regulation of specific transcriptional processes that require resuppression of cell proliferation upon nutrient replenishment and growth recovery (Tsai et al. 2014). The mutant was isolated by screening a mutant collection for compromised degradation of major LD protein and TAG, and growth recovery upon nitrogen replenishment. A putative CXC DNA-binding motif was identified in the deduced CHT7 protein sequence; further studies using EGFP-tagged CHT7 suggested a nuclear localization. Several lines of evidence are consistent with CHT7's possible role as a regulator of gene expression, acting at least as a repressor of a subfraction of the transcriptional program associated with cell quiescence (Tsai et al. 2014). It was proposed that CHT7 can represent a potent target for the rational engineering of TAG accumulation in microalgal cells.

Long-chain acyl-CoA synthases (LACS) are thought to participate in the export of de novo-produced fatty acids from the plastid to the ER by activating free fatty acids (16:0, 18:0 and the predominantly exported 18:1) in the plastid envelope. LACS belong to the superfamily acyl-CoA-activating enzymes (acyl-CoA ligases) (EC 6.2.1.3) and catalyze the formation of long-chain acyl-CoA (Kornberg and Pricer 1953). The mechanism of the enzymatic reaction is described in detail by Shockey and Browse (2011), who provide a genome-wide survey of acyl-CoA-activating enzymes in a variety of organisms, including *C. reinhardtii*. Briefly, in the first step, free fatty acid and ATP form an enzyme-bound acyl-AMP intermediate accompanied by pyrophosphate (PPi) release; the thioester bond is formed in the second step accompanied by AMP release. Accordingly, the amino acid sequences of LACS feature putative AMP- and CoA-binding sites in a predicted catalytic domain.

LACS are essential enzymes since they provide activated fatty acids for both catabolic and anabolic purposes in cells. Numerous LACS isoforms have been suggested to play a pivotal role in regulating the size, composition and availability of the cytoplasmic acyl-CoA pool required for various enzymatic activities, including de novo glycerolipid assembly in the ER, acyl-CoA-dependent membrane lipid and TAG biosynthesis pathways, as well as β-oxidation of fatty acids in peroxisomes (termed glyoxisomes in oil seeds) (Chapman and Ohlrogge 2012). Multiple LACS isoforms are generally present in the genomes of eukaryotic organisms from different lineages and can be localized to different cellular compartments (Shockey and Browse 2011). In cyanobacteria, acyl-ACP synthases fulfill the role of exogenous fatty acid activation for the various metabolic purposes in the prokaryotic cell (Kaczmarzyk and Fulda 2010). In yeast and mammals, LACS are essential in activating the exogenous fatty acids. Furthermore, a recent study by Poppelreuther et al. (2012) demonstrated that the mammalian isoform

ACSL3 was is effectively translocated from the ER to nascent LD upon fatty acids supplementation and manipulation of ACSL 3 gene expression can affect fatty acid uptake and formation of LD.

Nine LACS isoforms have been identified in *A. thaliana* (Fulda et al. 2002; Schnurr et al. 2002); among them, only LACS9 was localized to the plastid envelope (the outer membrane), and was proposed to participate in the export of nascent fatty acids produced by the chloroplast FAS to the extraplastidial compartment (Schnurr et al. 2002). However, the lacs9 mutant did not show any significant lipid phenotype but demonstrated a significant reduction in chloroplast LACS activity as compared to the wild type; this activity was nevertheless sufficient to support the calculated rate of fatty acid synthesis (Schnurr et al. 2002). Further research using a lacs1 lacs9 double mutant suggested that the ER-localized LACS1 and LACS9 may have overlapping functions in directing fatty acids to TAG biosynthesis in *Arabidopsis* seeds (Zhao et al. 2010).

The genes encoding the plastidial activity responsible for the postulated massive flux of de novo-produced fatty acids remain unknown. Nevertheless, the evolutionarily conserved role of AtLACS9 orthologs in oil-seed plants was supported by comparative EST analysis of developing seeds of four oil-seed plants: it was the most abundant LACS isoform in all oil seeds examined in the transcriptomic study (Troncoso-Ponce et al. 2013). Comparative transcriptomics has become a valuable tool for revealing the regulation and role of certain enzymatic steps in lipid biosynthesis in land plants and holds promise in microalgae.

A genome-wide search for acyl-activating enzyme diversity in different organisms, including *C. reinhardtii*, did not reveal the plastid LACS9 ortholog in this alga (Shockey and Browse 2011) but proteins showing similarity to *Arabidopsis* LACS4 (ER-localized) and LACS5 were found by bioinformatics means (Shockey and Browse 2011). Two putative LACS-encoding genes of *Chamydomonas* (Cre03.g182050 and Cre13.g566650; http://www.phytozome.net/) showed altered expression patterns upon transfer to nitrogen-depleted TAP medium, namely increased transcript (RPKM) number at certain time points (Miller et al. 2010; Boyle et al. 2012); however, cellular localization could not be predicted for these putative proteins using the currently available prediction algorithms. Putative LACS with higher maximum scores than those for *Chlamydomonas* can now be identified in diverse sequenced microalgae by similarity search, showing 35–42 % identity to AtLACS9.

Cellular localization and biochemical characterization of the putative LACS is essential for their functional assignment. Peroxisomal LACS participating in oxidative catabolism of fatty acids can be differentiated by peroxisome-targeting signals PST1 and PST2 (Fulda et al. 2002), but they might not be easily recognized in algae. The peroxisomal localization of LACS isoforms 6 and 7 in *Arabidopsis* was experimentally confirmed (Fulda et al. 2002) and their role in providing carbon from TAG reserves for seedling establishment was verified using double mutants (Fulda et al. 2004). Correlation of the gene-expression patterns of the putative LACS candidates with other players in the β-oxidation pathway may shed some light on the role of the putative LACS in lipid metabolism.

A putative acyl-CoA synthase (HO757597) and acyl-CoA binding protein (HO757953) were induced by transfer to nitrogen-depleted conditions as revealed by transcriptomic study of the microalga *Micractinium pusillum* (Li et al. 2012c). Multiple LACS (11–12) were found in the genome of each of six *Nannochloropsis* strains (Wang et al. 2014) and in the centric diatom *Thalassiosira pseudonana* (Tonon et al. 2005a). A putative LACS from *N. oculata* that was reported capable of restoring growth of the LACS-deficient yeast strain *Saccharomyces cerevisiae* YB525 fed with fatty acids as a carbon source (Zhang et al. 2012b). Only one isoform LACS3 showed significant upregulation in *P. tricornutum* during active nitrogen depletion with no change at later stages (Venezuela et al. 2012). One of the putative LACS genes of *T. pseudonana* (*TplacsA*) was characterized in detail and appeared to encode a LC-PUFA-specific acyl-CoA synthase. According to its functional characterization, it was postulated that LACS with the ability to activate LC-PUFA might be involved in the LC-PUFA-biosynthetic pathway rather than in the export of de novo-produced fatty acids.

Two *Chlamydomonas* LACS that were suggested to be involved in fatty export from the plastid were upregulated under conditions of nitrogen starvation, while candidate genes encoding enzymes of β-oxidation were repressed (Miller et al. 2010). Repression of β-oxidation was predicted to occur in *Ettlia* (*Neochloris*) *oleoabundans* under nitrogen starvation based on downregulation of most of the genes involved in β-oxidation or peroxisomal function; remarkably, only a LACS gene assigned, however, as being involved in β-oxidation showed some upregulation (Rismani-Yazdi et al. 2012). Interestingly, a gene pair putatively encoding mitochondrial LACS was upregulated in the oleaginous diatom *Fistulifera solaris* (Tanaka et al. 2015). An exceptionally high level of expression of the β-oxidation pathway, predicted to occur in the mitochondria (and not in the peroxisome), was identified in this diatom during neutral lipid accumulation; this was corroborated by the concerted expression of genes encoding several mitochondrial components implicated in the fatty acid-degradation pathway. It seems likely that the capacity for simultaneous carbon acquisition for fatty acid synthesis, on the one hand, and carbon mobilization through the oxidative catabolism of fatty acids, on the other hand, comprises the important mechanism that reciprocally governs the ability of *F. solaris* to survive and sustain lipid biosynthesis under conditions of nitrogen depletion (Tanaka et al. 2015).

5 Biosynthesis of Plastid Lipids

Biosynthesis of plastid lipids at molecular level is best studied in cyanobacteria—such of the genera *Synechococcus* and *Synechocystis*—and in higher plants, due to the availability of mutants and versatile forward and reverse genetic tools. Considerably less is known about the enzymatic steps and their spatial localization, or the trafficking of lipid intermediates in microalgal cells in general and, in those with complex plastids enveloped in three or four membranes in particular. Genes encoding glycerolipid-biosynthesis enzymes can now be identified in the genomes of various eukaryotic microalgae. However, an examination of the predicted proteins at the biochemical and molecular levels is a prerequisite for assigning their function. We will therefore refer to the knowledge on lipid metabolism accumulated in higher plants, while highlighting available information in microalgae.

5.1 Origin and Biosynthesis of Diacylglycerol Backbone

Most of the thylakoid membrane lipids are assembled in the chloroplast envelope membranes and thus necessitates interorganellar lipid trafficking (Benning 2009; Hurlock et al. 2014); furthermore the biosynthesis of chloroplast lipids requires tight coordination between the chloroplast and the ER and import of the precursors (Somerville and Browse 1991; Wang and Benning 2012). Diacylglycerol (DAG) serves as a backbone for the biosynthesis of galactolipids (MGDG and DGDG) and sulfolipid (SQDG), while cytidine diphosphate diacylglycerol (CDP-DAG) is used for the biosynthesis of PG (Sect. 6). In the so-called prokaryotic pathway, so-named to designate the evolutionary origin of the organelle, DAG and PG are formed in the chloroplast. The eukaryotic pathway involves extraplastidial lipids, and the precursors for galactolipid assembly in the chloroplast originate in the endoplasmic reticulum (ER) (Heinz and Roughan 1983; Somerville and Browse 1991; Ohlrogge and Browse 1995; Benning 2009).

First, we will describe the major routes involved in the prokaryotic pathway. This pathway leads to the formation of chloroplast glycerolipids containing saturated and unsaturated C16 fatty acids exclusively in the *sn*-2 position of the glycerol backbone. A typical higher plant example is *Arabidopsis thaliana*, referred to as a 16:3 plant, with 18:3 *n*-3/16:3 *n*-3 MGDG being the major molecular species produced entirely via the prokaryotic pathway. In the eukaryotic pathway, the predominant MGDG species is 18:3 *n*-3/18:3 *n*-3 and in the so-called 18:3 plants, 18:3 *n*-3 comprises up to 80–90 % of the fatty acids in MGDG (Somerwille and Browse 1991). The distribution with C16 PUFA located at the *sn*-2 position of the glycerol backbone that is characteristic for the prokaryotic MGDG has been shown in the green algae, such as *Chlorella*, *Chlamydomonas* and *Trebouxia*, among others. MGDG of green algae contains C16 PUFA 16:3 and 16:4 at the *sn*-2 position, whereas DGDG and SQDG are characteristically rich in 16:0 at this position. In some microalgal groups (the red algae and the eustigmatophytes), 16:0 and 16:1 *n*-7 are most abundant at the *sn*-2 position of MGDG generated by the prokaryotic-like pathway, whereas C16 PUFA occur rarely (Sukenik et al. 1993; Adlerstein et al. 1997; Sukenik 1999; Khozin-Goldberg et al. 2002b). MGDG molecular species with shorter fatty acids, such as 12:0 and 14:0, have been detected in chlorarachniophytes (Leblond and Roche 2009).

To produce a backbone for plastid lipid synthesis in cells of the photosynthetic microalgae, dihydroxyacetone phosphate (DHAP), formed from the central metabolite glyceraldehyde-3-phosphate generated in the reductive pentose phosphate pathway (Calvin cycle) and/or in plastid glycolysis, is reduced to glycerol-3-phosphate (G-3-P) by the action of glycerol-3-NAD+-dependent phosphate dehydrogenase (G3PDH). In this reaction, a glycerol backbone is formed for the sequential acylation of the *sn*-1 and *sn*-2 positions of G-3-P. A functionally active, likely chloroplast isoform of G3PDH has been recently reported in the green alga *Dunaliella* (Cai et al. 2013). Two out of three isoforms of glycerol-3-phosphate dehydrogenase were induced at the transcript (mRNA) level in three strains of *C. reinhardtii* [CC4349, CC4532and CC4348 (sta6)] exposed to nitrogen starvation under mixotrophic conditions in the presence of acetate (Blaby et al. 2013; Schmollinger et al. 2014). Moreover, these genes were among the exceptional lipid-biosynthesis-associated genes whose expression was upregulated in response to the absence of nitrogen; the expression of many others was either stable or decreased in the three strains, despite their different capacities to produce starch and TAG. This large-scale study again emphasized that in the presence of acetate, respiratory carbon metabolism is prioritized over photosynthesis, so recycling fatty acids from the progressively degrading membrane lipids rather than de novo fatty acid synthesis provides acyl groups for TAG biosynthesis (Schmollinger et al. 2014).

The plastid isoform of G-3-P acyltransferase (GPAT, EC 2.3.1.15) catalyzes the first step of de novo glycerolipid biosynthesis—acylation of the *sn*-1 position of G-3-P predominantly with 18:1-ACP (acyl carrier protein), generating lyso-phosphatidic acid (LPA). The significance of this enzymatic step in glycerolipid biosynthesis in the *A. thaliana* chloroplast was reinforced by evidence that mutation in the gene encoding the soluble plastid GPAT isoform (*ATS1*)

nearly abolishes the prokaryotic pathway of thylakoid lipid biosynthesis (Xu et al. 2006).

The next step is catalyzed by lyso-phosphatidic acid acyltransferase (LPAAT, EC 2.3.1.51), which exclusively utilizes 16:0-ACP to esterify the *sn*-2 position of LPA, forming phosphatidic acid (PA). The gene encoding the plastidial LPAAT isoform was cloned from *A. thaliana* (*AST*2), the chloroplast localization of the encoded protein was confirmed by expression of the GFP-fusion, and the proteome analysis of the chloroplast envelope membranes (Ferro et al. 2003); the function was affirmed by expression in a LPAAT-deficient strain of *E. coli* (Yu et al. 2004). Plastidic LPAAT appeared to be essential for embryo development as disruption of *AST*2 resulted in embryo lethality (Yu et al. 2004). Given the strict acyl specificity of chloroplast-localized LPAAT, the chloroplast glycerolipids produced via the prokaryotic pathway will carry C16 acyl groups at the *sn*-2 position—a diagnostic feature that enables distinguishing chloroplast glycerolipids produced via the prokaryotic route from those derived from the eukaryotic one in higher plants (Somerwille and Browse 1991; Li-Beisson et al. 2010). The recently discussed ramifications for the accepted biochemical routes in microalgae will be noted later in this section. In the next step, the phospatidic acid phosphatase/phosphohydrolase (PAP, EC 3.1.3.4), exclusively localized to the inner chloroplast envelope in *Arabidopsis*, removes the phosphate group from PA yielding DAG (Andrews et al. 1985; Nakamura et al. 2007; Li-Beisson et al. 2010), which is utilized in the reactions of galactolipids and sulfolipid assembly as shown on the scheme.

$$\mathrm{DHAP} \rightarrow \mathrm{G-3-P} \rightarrow \mathrm{LPA} \rightarrow \mathrm{PA} \rightarrow \mathrm{DAG} + \mathrm{Pi}$$

Alternatively, PA produced in the plastid is used to form CDP-DAG, a precursor for the anionic chloroplast lipid PG (Sect. 6). Putative chloroplast GPAT and LPAAT have, for example, been identified in the genomes of both sequenced *Nannochloropsis* species based on their predicted localization and protein domain organization (Radakovits et al. 2011; Vieler et al. 2012b). The genes encoding the homologs of *A. thaliana ATS1* and *ATS2* in the oleaginous diatom *Fistulifera solaris* were upregulated in the stationary phase along with enhancement of de novo fatty acid synthesis (Tanaka et al. 2015).

The pool of DAG moieties for the eukaryotic pathway of chloroplast lipid assembly is thought to be formed in the ER via activities of the ER-localized GPAT and LPAAT that mediate the *sn*-1 and *sn*-2 specific esterification, respectively, and PAP in the reactions similar to shown on the above scheme. It is widely recognized that GPAT and LPAAT's preference for acyl substrates contributes to the positional distribution of acyl groups in extraplastidial lipids. Accordingly, molecular lipid species of chloroplast lipids originated from the ER-localized eukaryotic pathway can generally be distinguished based on the fatty acid composition and positional distribution of acyl chains. As already noted, a distinct feature of the chloroplast lipids derived from precursors supplied via the eukaryotic pathway is the absence of C16 fatty acid at the *sn*-2 position of the glycerol backbone; instead, C18 PUFA or almost solely LC-PUFA (in the case of some LC-PUFA-producing microalgae) acyl chains are localized to both the *sn*-1 and *sn*-2 positions of the glycerol backbone (Schneider et al. 1995; Khozin et al. 1997; Adlerstein et al. 1997; Leblond et al. 2010a, b). For example, molecular species of MGDG 20:4 *n*-6/20:4 *n*-6, 20:4 *n*-6/20:5 *n*-3 and 20:5 *n*-3/20:5 *n*-3 have been suggested to be analogous to eukaryotic MGDG molecular species 18:2 *n*-6/18:2 *n*-6, 18:2 *n*-6/18:3 *n*-3 and 18:3 *n*-3/18:3 *n*-3 of higher plants (Khozin et al. 1997) (Fig. 10). However, the stereospecific positional distribution of the acyl groups in the extraplastidial glycerolipids in some microalgae may vary from the conventional models that are accepted for higher plants (Abida et al. 2014; Davidi et al. 2014). In particular, the lipidomics analysis of *Phaeodactylum tricornutum* revealed extraplastidial polar lipids carrying C16 PUFA at the *sn*-2 position.

The ER-localized GPAT pathway initiates biosynthesis of both polar lipids and TAG, utilizing the G-3-P backbone produced in the extraplastidial compartment either from DHAP or from glycerol by the action of GK. As deduced from transcriptome information on *Dunaliella tertiolecta*, the activity of glycerol kinase (GK), also yielding G-3-P, might be associated with the glycerolipid lipid biosynthesis (Rismani-Yazdi et al. 2011). Muto et al. (2015) explored the genomic information on the oleaginous diatom *Fistulifera solaris* to identify two candidate genes, *GK1* and *GK2*. The deduced amino acid sequences showed more than 50 % percent similarity to those of the annotated *GK* genes in the diatom *Thalassiosira pseudonana* and featured the characteristic FGGY carbohydrate kinase domain. Overexpression of *GK2* in *F. solaris* resulted in only moderate changes in lipid content in both the presence and absence of glycerol in the nutrient medium, despite the elevated level of expression in the transformant lines.

Little molecular genetic data exist on the properties and substrate preferences of the ER-localized GPAT with *sn*-1 regio-specificity that catalyzes the transfer of acyl group from acyl-CoA to G-3-P (EC 2.3.1.15) and involved in glycerolipid biosynthesis in algae and higher plants. More information is available on human and yeast GPAT and LPAAT enzymes involved in glycerolipid biosynthesis (Lewin et al. 1999; Wendel et al. 2009; Pagac et al. 2011, 2012). However, activities of the membrane-bound GPAT were earlier characterized at biochemical level in homogenates of many plant species (reviewed in Murata and Tasaka 1997). The available data on biochemical activities that do exist suggest that in plants and mammals, the ER-localized GPAT isoforms

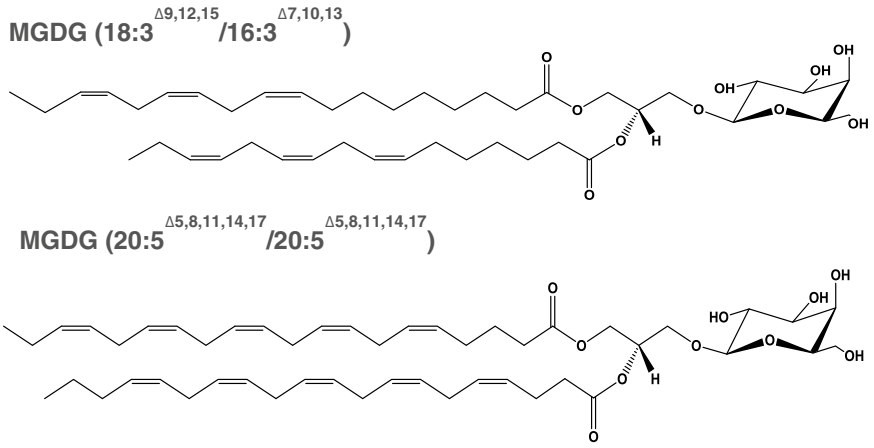

Fig. 10 Two types of MGDG: the prokaryotic $18{:}3^{\Delta 9,12,15}/16{:}3^{\Delta 7,10,13}$ and the eukaryotic-like $20{:}5^{\Delta 5,8,11,14,17}/20{:}5^{\Delta 5,8,11,14,17}$

involved in glycerolipid biosynthesis may have broad substrate specificity that is not restricted to de novo produced 18:1 and 16:0 in the form of acyl-CoA. Such as, the preferred substrates for purified sunflower enzyme were palmitoyl-CoA and linoleoyl-CoA, and to a lesser extent, oleoyl-CoA (Ruiz-López et al. 2010). While, the purified and solubilized microsomal GPATs from oil palm tissues showed a strong preference for palmitoyl-CoA as compared to other acyl-CoA substrates, in line with the high content of palmitate (16:0) in this oil plant (Manaf and Harwood 2000).

A GPAT family consisting from land plant-specific eight members were annotated in *A. thaliana* and assumed to participate in different reactions in the extraplastidial compartment, but not in membrane or neutral glycerolipid biosynthesis (Yang et al. 2012b; Chapman and Ohlrogge 2012). Accordingly, no significant similarity to GPAP 1-8 has been identified in microalgae (Yang et al. 2012b). An ER-localized GPAT isoform (GPAT9) from *A. thaliana* (Gidda et al. 2009) was shown to be most similar to the mammalian GPAT3 affirmed to have role in TAG biosynthesis (Cao et al. 2006). Homologs of the ER-localized isoform GPAT9 have been tentatively annotated in the genomes of sequenced microalgal species, furthermore the heterologous expression of the green algal GPAT led to the enhancement in TAG formation in a model alga (Iskandarov et al. 2015). The amino acid sequence of the cloned and biochemically characterized GPAT of the centric diatom microalga *T. pseudonana* showed 24 and 23 % identity to yeast GPATs and little homology to bacterial, mammalian or *Arabidopsis* GPATs (Xu et al. 2009). In in vitro assays, TpGPAT accepted quite a range of acyl-CoA substrates, including unsaturated ones, with a preference for 16:0-CoA.

LPAAT (EC 2.3.1.51) activity in the ER catalyzes the *sn*-2 specific transfer of the acyl group, due to its substrate selectivity discriminating C16 acyl-CoA, accordingly C16 acyl groups are generally absent at the *sn*-2 position of the glycerolipids produced through the eukaryotic pathway in higher plants. Molecular evidence for the role of LPAAT family proteins in glycerolipid biosynthesis is limiting (Chapman and Ohlrogge 2012). Two LPAATs of *Brassica napus* expressed in a mutant strain of *E. coli* displayed a remarkable preference for oleoyl-CoA over palmitoyl-CoA when 1-oleoyl LPA was used as the acceptor, consistent with a cytoplasm-localized LPAAT associated with the eukaryotic pathway (Maisonneuve et al. 2010). Genes putatively encoding LPAAT-like proteins have been tentatively annotated in microalgal genomes, but none of them have yet been characterized. Since GPATs and LPAATs participate in TAG biosynthesis, we will refer to these enzymes again in the section on that topic.

The dephosphorylation of PA is catalyzed by PA phosphatase (EC 3.1.3.4). PA and DAG that are produced from LPA are central lipid metabolites and are linked to different enzymatic steps of polar membrane lipid biosynthesis and acyl editing in the ER, along with several additional enzymatic activities involving phospholipids that may enrich the DAG pool with nascent and polyunsaturated fatty acids (Bates et al. 2009; Bates and Browse 2012). PA and DAG formation in the ER appeared to be substantially more complex than it was previously assumed (Chapman and Ohlrogge 2012). For example, short-term acetate-labeling experiments with developing soybean (*Glycine max*) embryos showed that almost 60 % of the de novo-synthesized 18:1 is actually incorporated directly into the *sn*-2 position of PC via an acyl-editing mechanism (acyl group exchange between acyl-CoA pool and existing lipids), rather than through the sequential acylation of G-3-P (Bates et al. 2009), and perhaps through the metabolism of PC mediated by the activity of lyso-PC acyltransferase (LPCAT; EC 2.3.1.23) at the chloroplast envelope (Mongrand et al. 2000; Andersson et al. 2004). The ability of microalgal enzymes to incorporate CoA-activated acyl groups to PC, likely via the so-called acyl-editing mechanism involving LPCAT, was previously demonstrated in several microalgae in pulse-chase experiments with radiolabeled fatty acids (Khozin et al. 1997; Bigogno et al. 2002a; Khozin-Goldberg et al. 2002b). PC and particularly its *sn*-2

position were predominantly labeled (5 and 95 % of total counts at *sn*-1/*sn*-2) in the red microalga *Porphyridium cruentum* following 30 min pulse-labeling with [1-^{14}C]18:2 *n*-6 (Khozin et al. 1997); while [1-^{14}C]20:4 *n*-6 incorporated into both positions of PC, implying either rapid metabolism through the GPAT or activity *sn*-1 specific LPCAT (Khozin et al. 1997).

It should be noted that acetate labeling has been widely used in studies of lipid metabolism in microalgae (e.g. Giroud and Eichenberger 1988; Sukenik and Carmeli 1990; Schneider and Roessler 1994; Eichenberger and Gribi 1997; Bigogno et al. 2002a; Guschina et al. 2003; Guihéneuf et al. 2011). In pulse-chase experiments, [^{14}C]acetate is incorporated into fatty acids via plastid-localized fatty acid synthesis and de novo-produced fatty acids are subsequently utilized in the prokaryotic pathway faster than in the eukaryotic pathway, thus the labeling kinetics with [^{14}C]acetate pinpoints the consecutive operation of two pathways (Heinz and Roughan 1983). However, in the LC-PUFA-producing algae, a clear picture of the label incorporation via the de novo pathway was misrepresented by rapid label incorporation into LC-PUFA, likely due to formation of malonoyl-CoA and elongase activity (Schneider and Roessler 1994; Bigogno et al. 2002a). In *Nannochloropsis* sp., more than half of the [1-^{14}C]acetate was incorporated into longer and more unsaturated fatty acids (Schneider and Roessler 1994), while [^{14}C] bicarbonate was incorporated into the primary products of chloroplast fatty acid synthesis, 16:0, 16:1, and 14:0. In *C. reinhardtii*, lacking PC and the eukaryotic-type chloroplast glycerolipids, acetate was incorporated into the prokaryotic chloroplast lipids (MGDG, DGDG and PG) (Giroud and Eichenberger 1989). In the haptophyte *Diacronema* (*Pavlova*) *lutheri*, synthesis of galactolipids and phospholipids was less sensitive to differences in light intensity when incubated with [1-^{14}C]acetate vs. bicarbonate (Guihéneuf et al. 2010); the authors suggested that acetate was used as a heterotrophic carbon source.

The export of precursors from the ER to the plastid envelope—providing DAG backbones for eukaryotic galactolipid assembly—and the exact nature of the lipid precursor molecules have recently become the subject of intensive research effort. Several lipid molecules seen as potential candidates, such as DAG, lyso-PC, PA, and various enzymatic activities potentially involved in the delivery of eukaryotic DAG backbone are being investigated in higher plants (Mongrand et al. 2000; Xu et al. 2003; Nakamura et al. 2009; Wang et al. 2013). Based on lipids radiolabeling patterns, Eichenberger and Gribi (1997) suggested that in the haptophyte *P. lutheri*, C18 and C20 fatty acids are imported from the cytoplasm to the chloroplast, allowing for the synthesis of eukaryotic MGDG without the import of DAG.

Substantial progress toward untangling the nature of the exported lipids has been achieved in studies in *Arabidopsis* (Wang and Benning 2012). The trigalactosyldiacylglycerol (TGD) proteins have been shown to be required for lipid transfer from the ER to the inner envelope, with PA being involved in the transfer process (Wang et al. 2013). The existence of this route was supported by the discovery of chloroplast envelope-ER contact sites and seems feasible in microalgae, particularly those possessing the chloroplast ER. In this route, TGD1, 2, and 3 proteins form a putative ATP-binding cassette (ABC) transporter carrying ER-derived lipids through the inner envelope membrane of the chloroplast. The most recently characterized component, TGD4, is a lipid transfer protein that resides in the outer chloroplast envelope with an integral dimeric β-barrel that binds PA with its N terminus where two amino acid sequences required for PA binding have been identified (Wang et al. 2013).

DAG produced in the extraplastidial compartment was predicted to be a possible precursor for eukaryotic galactolipid synthesis. However, there was no direct experimental proof. Several enzymatic activities generating DAG from PC, such as phospholipase C and phospholipase D along with PAP activity, are inferred to play role in DAG formation. Two PAH (phosphatidic acid hydrolase) genes encoding the lipin homologs in *Arabidopsis*—*At*PAH1 and *At*PAH2—with Mg^{2+}-dependent PA phosphatase activity were proposed to be involved in the eukaryotic pathway of galactolipid biosynthesis (Nakamura et al. 2009). Lipins were recently identified as the ubiquitous proteins with PA phosphatase activity (Péterfy et al. 2001; Csaki and Reue 2010). *Arabidopsis* PAHs appeared to be cytoplasmic proteins (Nakamura et al. 2009; Eastmond et al. 2010), distantly related to yeast or mammalian lipins and more closely related to putative lipins identified in algae and protists (Nakamura et al. 2009). Indeed, hypothetical lipin-like proteins with different degrees of similarity to AtPAHs and the typical lipin N- and C-terminal domains are tentatively annotated in many phylogenetically divergent sequenced algal species, such as *Ostreococcus lucimarinus* and *O. tauri*, *Micromonas pusilla*, *C. reinhardtii*, *Guillardia theta*, *Cyanidioschyzon merolae*, *E. huxleyi*, and *Ectocarpus siliculosus*.

It was proposed that PAH1 and PAH2 are involved in the ER-localized eukaryotic pathway of membrane lipid metabolism in *Arabidopsis* and that the produced DAG serves as a substrate for eukaryotic MGDG synthesis given that double mutant pah1pah2 plants demonstrated decreased PA hydrolysis, impaired biosynthesis of the eukaryotic type MGDG and an abnormal pattern of PC-to-MGDG conversion in pulse-chase experiments with [^{14}C]acetate. Furthermore, a severe reduction in the eukaryotic-type galactolipids occurred in the double mutant plants concomitant with a drastic increase in the level of phospholipids, along with expansion of the ER membrane, implying that increased production of phospholipids diverts flux away from the eukaryotic pathway (Eastmond et al. 2010).

As can be seen, molecular studies in microalgae lag behind the biochemical and molecular genetic studies with the land plants. However, numerous radiolabeling studies coupled with a positional analysis of chloroplast lipids have indicated the existence of prokaryotic- and eukaryotic-like pathways of chloroplast lipid biosynthesis in many microalgae species, including those that produce LC-PUFA (Eichenberger and Gribi 1997; Khozin et al. 1997; Khozin-Goldberg et al. 2002a, b; Bigogno et al. 2002a, b; among others). For example, the prokaryotic-like and eukaryotic-like pathways were predicted to operate in the red alga *P. cruentum* (Khozin et al. 1997; Adlerstein et al. 1997) and the eustigmatophyte *Monodus subterraneus* (= *Monodopsis subterranea*) (Khozin-Goldberg et al. 2002b). The kinetics of the labeling pattern of different lipid classes suggested a flux of fatty acids from cytoplasmic to chloroplastic lipids, mainly from PC but also from TAG to galactolipids, characteristic of the eukaryotic pathway. Similar results were obtained by Bigogno et al. (2002a) in pulse-chase experiments with radiolabeled 18:1 in the green microalga *L. incisa*. The labeling pattern of C18/C16 molecular species of MGDG exhibited typical kinetics of the prokaryotic pathway. According to genome annotation of *N. oceanica* CCMP1779, both pathways are likely to operate in this eustigmatophyte (Vieler et al. 2012b), in line with earlier radiolabeling studies (Schneider and Roessler 1994), contributing to biosynthesis of plastidic glycerolipids.

5.2 MGDG and DGDG

The galactolipids MGDG and DGDG are the predominant structural lipids of thylakoids, and indispensable for the proper functioning of photosynthetic complexes, chloroplast development and photoprotection (Jarvis et al. 2000; Aronsson et al. 2008; (Kobayashi et al. 2007, 2013). Crystal-structure analysis of a homodimeric PSII from the cyanobacterium *Thermosynechococcus elongatus* revealed that each PSII monomer can position 25 lipid molecules, including 11 MGDG and 7 DGDG molecules, revealing their importance for PSII function (Guskov et al. 2010; Kern and Guskov 2011). Lipid analysis of the isolated membranes of the chloroplast envelope in plants showed that MDGD is enriched in the inner leaflet envelope while DGDG is dominant in the outer leaflet (Block et al. 1983, 2007). Later it was confirmed at the biochemical and molecular levels that MGDG is primarily assembled in the inner-envelope membrane, whereas DGDG is formed entirely in the outer envelope. Tri- and tetragalactosyldiacylglycerols could also be formed under certain physiological conditions or in isolated chloroplasts due to processive galactosyltransferase activity (Benning and Ohta 2005). Therefore, intensive trafficking of lipid precursors appears to occur in the plastid. Several reviews provide a comprehensive summary of galactolipid biosynthesis and trafficking in higher plant cells (Dörmann and Benning 2002; Kelly and Dörmann 2004; Benning and Ohta 2005; Benning 2009; Breuers et al. 2011). An excellent historical overview of the discovery and role of galactolipids in higher plants (Dörmann and Heinz 2011), which also highlights the most up-to-date information on the subject, is available at http://web.lipidlibrary.aocs.org.

MGDG biosynthesis is mediated by a MGDG synthase (MGD) [UDP-galactose (UDP-Gal):1,2-diacylglycerol (DAG) 3-β-D-galactosyltransferase; EC 2.4.1.46] that utilizes the sugar-group donor molecule UDP-Gal and the acceptor molecule DAG (Awai et al. 2001). Three isoforms of MDG catalyze MGDG synthesis in *A. thaliana*: the MGD1 isoform (type A) is responsible for the bulk of galactolipid synthesis in chloroplasts under phosphorus-sufficient conditions, whereas the MGD2 and MGD3 isoforms (type B), located in the outer envelope, prefer eukaryotic-type DAG species and are required for DGDG production from precursors derived from the ER and their consequent accumulation in extraplastidial membranes under phosphorus starvation (Kelly and Dörmann 2004; Benning and Ohta 2005). Phylogenetic analysis of MGD in land plants led to the conclusion that two types of MGD were acquired in the common ancestor of Spermatophyta ~323 MYA (Yuzawa et al. 2012). MGD2 and MGD3 are induced under phosphate starvation and their deficiencies do not exert substantial phenotypic changes under nutrient-sufficient conditions (Kobayashi et al. 2009a, b).

MGDG synthesis in cyanobacteria requires an additional enzymatic step mediating the formation of monoglucosyl-DAG (MGlcDG) by a glucosyltransferase using UDP-glucose (UDP-Glc) followed by epimerization of MGlcDG to MGDG (Awai et al. 2006). MGD belong to the glycosyltransferase 28 (GT28) family, whereas MGlcDG synthase (MGlcD) belongs to the GT2 family (Awai et al. 2006). Plant lipid scientists have looked into the physiological significance of the preference for galactose as a head group in thylakoid lipids (Hölzl et al. 2006). For example, expression of glucosyltransferase from the photosynthetic bacterium *Chloroflexus aurantiacus* in the galactolipid-deficient mutant of *A. thaliana* led to accumulation of galactose-containing lipids and complemented a growth-defective phenotype; however, the degree of trimerization of light-harvesting complex (LHC) II and the photosynthetic quantum yield of transformed plants were only partially restored, implying that a galactose-bearing head group is required for proper functioning of the photosynthetic machinery (Hölzl et al. 2006). Comprehensive phylogenetic analyses of MGD homologs from prokaryotic and eukaryotic organisms revealed that eukaryotic MGD, including those of Viridiplanta, Rhodophyta, and Heterokontophyta, probably have a single ancestor in the ancient Chloroflexi (Yuzawa et al. 2012). Horizontal transfer of a bacterial galactosyltransferase into the nuclear genomes of

the green and red algal lineages, glaucophytes and higher plants has been proposed (Botté et al. 2011).

Putative *MGD* from Heterokontophyta formed a single group on a phylogenetic tree, suggesting that the common ancestor acquired MGD, likely from Rhodophyta, through a secondary endosymbiotic event. In a heterokont MGD group that was assembled in this phylogenetic study (Yuzawa et al. 2012), a number of genes putatively encoding MGD were annotated, such as in two sequenced marine diatoms, *Phaeodactylum tricornutum* and *Thalassiosira pseudonana*, and in *Aureococcus anophagefferens* (Pelagophyceae), a cause for harmful brown tide blooms. The genomes of the green microalgae *C. reinhardtii* and *Volvox carteri* (Chlorophyceae), and the marine planktonic *Micromonas pusilla* and *Ostreococcus lucimarinus* (Prasinophyceae), have a single candidate for MGD, indicating that different mechanisms might be recruited in these algae to cope with phosphate starvation. A similarity search of the recently available microalgal genome data revealed multiple genes in the genomes of *Ectocarpus siliculosus* (Phaeophyceae), but a single candidate in *Nannochloropsis gaditana* (Eustigmatophyceae). Multiple genes putatively encoding MGD suggests more complex regulation of galactolipid biosynthesis and raises the possibility of different isoforms participating in lipid remodeling under different nutrient statuses. Furthermore, extensive genome variability might occur in evolutionarily different phyla of microalgae, as was recently reported for the coccolithophore *Emiliania huxleyi* CCMP1516 (Haptophyta) by pan genome sequencing of the core genome and more than a decade of different isolates (Read et al. 2013). The revealed expansive gene repertoire has been associated with the extremely high abundance of this organism in oceans in different climatic zones. Searching the recently published *E. huxleyi* genome revealed four putative MGD candidate, all belonging to the GT28 family typical of this enzyme. A more thorough study is required to examine the cellular localization, functionality and evolutionary origin of the putative MGD in these evolutionarily distinct groups of microalgae. One transcriptomic study reported that three genes encoding putative MGD in the *P. tricornutum* genome showed increased expression 2 days after transfer to nitrogen starvation (Phatrdraft_9619, Phatrdraft_54168 and Phatrdraft_14125) (Yang et al. 2013), leading the authors to suggest that the gene products might be involved in membrane lipid reorganization under these conditions.

MGDG deficiency revealed a prominent effect on photosynthesis-related parameters in higher plants and severe defects in the structural organization of the chloroplast (Jarvis et al. 2000; Aronsson et al. 2008; Schaller et al. 2010; Wu et al. 2013). The *mgd1*-knockdown mutant could not tolerate high light intensities and demonstrated an impaired violaxanthin cycle and pH-dependent activation of violaxanthin de-epoxidase and the PsbS protein necessary for photoprotective thermal dissipation (Aronsson et al. 2008). Recent characterization of the tobacco *mdg1* mutant indicated that MGDG plays important roles in maintaining both the linear electron transport process and photostability of the PSII apparatus (Wu et al. 2013). So far, MGDG-deficient mutants have not been reported in microalgae; however, aberrant MGDG synthesis has been documented as a consequence of manipulating chloroplast desaturase expression (Zäuner et al. 2012).

DGDG biosynthesis is governed by the action of DGDG synthase (DGD) (EC 2.4.1.241), utilizing MGDG and UDP-Gal (Dörmann et al. 1999). DGDG synthase are encoded by two gene members in higher plants (*DGD1* and *DGD2*) and likely by several genes in microalgae based on a similarity search with the *Arabidopsis DGD1* gene. Phosphate starvation in higher plants leads to induction in *DGD2* expression and substantial accumulation of DGDG in extraplastidial membranes that substitute for dismantling phospholipids under such conditions (Härtel et al. 2000; Li et al. 2006; Kelly et al. 2002). A DGDG-deficient mutant disrupted in the *dgdA* gene was constructed in the cyanobacterium *Synechocystis sp*PCC6803 (Sakurai et al. 2007). DGDG did not appear to be essential for growth of the cyanobacteria, but it was important for PSII function, likely through the binding of intrinsic proteins required for stabilization of the oxygen-evolving complex.

Formation of extraplastidial DGDG with positional distribution of fatty acids and molecular species composition similar to phospholipids was shown to occur under phosphate starvation in land plants (Kelly et al. 2003). The synthesis of DGDG takes place in the outer envelope of the chloroplast and under phosphate starvation, DGDG is exported and accumulated in the extraplastidial membranes (Kelly and Dörmann 2004; Jouhet et al. 2007), such as the plasma membrane (PM) (Andersson et al. 2003, 2005), tonoplast (Andersson et al. 2005), and mitochondria (Jouhet et al. 2004). Double *dgd1 dgd2* mutants of *Arabidopsis* were not able to increase DGDG content under phosphate deprivation, while the mutant plants were able to exhibit some galactolipid:galactolipidgalactosyl transferase activity (GGGT) and produce small amounts of s tri- and tetragalactosyldiacylglycerols (Kelly et al. 2003). GGGT activity, which is localized to the outer envelope and utilizes chloroplast-derived lipid precursors for oligogalactolipid production concomitantly with DAG production, is activated in isolated chloroplasts and in *Arabidopsis tgd* mutants (Benning and Ohta 2005). It was suggested that GGGT might be involved in the turnover of chloroplast lipids under stress conditions and during senescence (Benning and Ohta 2005), simultaneously providing DAG moieties for TAG formation (Sakaki et al. 1990). Recently, the gene encoding the enigmatic GGGT was identified as essential for freezing tolerance in *Arabidopsis* leaves (Moellering et al. 2010). A set of experimentally supported evidence allowed proposing that under conditions induced by freezing, GGGT partially converts MGDG to DGDG and oligogalactolipids. DAG is sub-

sequently removed from the membranes by TAG-synthesizing activity. There are indications of the presence of oligogalactolipids in microalgae (Harwood 1998; Gray et al. 2009a; Leblond et al. 2010b).

Substitution of phospholipids by non-phosphorous lipids under phosphate limitation also occurs in microalgae, providing a globally relevant biochemical mechanism driving the ability of phytoplankton to survive and proliferate in oceans where phosphorus is limiting (Van Mooy et al. 2009). Various phytoplanktonic microalgae have been shown to involve sulfolipids and betaine lipids in this response. The impact of phosphate starvation on acyl lipid class distribution was studied in the freshwater eustigmatophyte *Monodus subterraneus* (=*Monodopsis subterranea*) (Khozin-Goldberg and Cohen 2006). Cellular contents of DGDG increased under phosphate starvation as opposed to MGDG, the cellular content of which did not change. The major increase was documented in TAG under phosphate starvation and limitation. With an increase in the proportion of TAG (out of total acyl lipids), those of DGDG and the betaine lipid DGTS decreased substantially less than the proportion of MGDG. DGDG that was accumulated under phosphate deprivation in *M. subterraneus* did not resemble PC, and the C20/C16 structure of DGDG molecular species (20:5/16:1 > 20:5/16:0) was preserved. The results of the fatty acid analysis of lipid classes suggested that DGTS is a likely source of C20 acyl groups that can be exported to the *sn*-1 position of DGDG, perhaps as a prokaryotic-type MGDG.

Little is known about the regulation of galactolipid composition at the molecular level in microalgae. However, a fairly large body of experimental evidence exists regarding alterations in content and fatty acid composition of galactolipid classes as a consequence of variations in environmental conditions. Microalgae alter their MGDG-to-DGDG ratio in response to nutrient availability (sufficiency, limitation or deprivation), osmotic downshift or increased salinity, light availability, etc. In this context, it is important to emphasize that MGDG is a non-bilayer-forming lipid whereas DGDG is a bilayer-forming lipid, hence the ratio of the two major galactolipids is important for stabilization of the bilayer structure and functioning of the photosynthetic membrane. A conical MGDG molecule with a relatively small head group and frequently two PUFA acyl groups has a tendency to pack into the so-called hexagonal H_{II} structures disrupting the bilayer, as opposed to DGDG with its larger head group which forms a bilayer even when PUFA are attached to the glycerol backbone (Lee 2000). In photosynthetic membranes, MGDG is suggested to adopt a bilayer structure in complex with LHCII (Lee 2000).

A MGDG-to-DGDG ratio of more than 2 is typical for microalgae grown under balanced growth and light-limiting conditions, when cells display high cellular contents of photosynthetic pigments. The MGDG-to-DGDG ratio was drastically augmented (reaching 5.8) in the cells of chemostat cultures of three species of ice diatoms (*Navicula gelida* var. *antarctica*, *Fragilariopsis curta* and *Nitzschia mediocon-stricta*) grown at –1 °C under very low light (2 µmol photons m^{-2} s^{-1} as compared to 15 µmol photons m^{-2} s^{-1}) (Mock and Kroon 2002). The major fatty acid of MGDG (EPA, 20:5*n*-3) increased by ca. 50 % (out of total fatty acids) under 2 µmol photons m^{-2} s^{-1} in correlation with the increase in Chl *a*/*c* and increased rate constants for the electron transport. The eustigmatophyte *Nannochloropsis oceanica* increases its MGDG-to-DGDG ratio in response to osmotic downshift, relieving the consequences of salt stress in conjunction with increased Chl content, decreased production of osmoprotectants and enhanced growth (Pal et al. 2013). The opposite trend is common in algae grown at increased salinities (Huflejt et al. 1990; Stefanov et al. 1994); in general, the ratio of MDGD to DGDG decreases under conditions that are not favorable for growth (such as nutrient starvation, high-light intensities).

The fatty acid composition of MGDG is typically highly unsaturated in eukaryotic microalgae, as in the land plant plastid, in both C18 PUFA- and LC-PUFA-producing algae. An exception is the unicellular rhodophyte *Cyanidioschyzon merolae* which lacks the plastidial desaturation pathway, with 16:0 and 18:2 *n*-6 being the major fatty acids of both MGDG and DGDG (Sato and Moriyama 2007). Highly unsaturated MGDG, especially in the LC-PUFA-producing algae, may possibly provide increased fluidity and curvature to the thylakoid membrane in microalgal cells under variable environmental conditions. A large body of earlier data exists on fatty acid composition and molecular species of major plastidial galactolipids (e.g. Yongmanitchai and Ward 1993; Adlerstein et al. 1997; Khozin et al. 1997; Makewicz et al. 1997; Khozin-Goldberg et al. 2002b; Sato et al. 2003c among others); this has been complemented and advanced by recent progress in modern lipidomics MS-based techniques. A summary of previous and of some recent results is provided below.

The green algae are typically rich in C18 PUFA and may comprise both prokaryotic- and eukaryotic-type MGDG, such as in *Parachlorella* (*Chlorella*) *kessleri* 11h (Sato et al. 2003c). The solely prokaryotic-type C18/C16 galactolipids present in *C. reinhardtii* lacking PC support the ultimate role of this phospholipid in the eukaryotic pathway of MGDG biosynthesis (Benning 2009). In the green microalga *L. incisa*, which is exceptionally rich in ARA (20:4 *n*-6), both C18 PUFA- (higher plant-like) and C20 LC-PUFA-containing molecular species of MGDG and DGDG were identified (Khozin-Goldberg et al. 2005). The increase in the relative proportion of the AA-containing molecular species C20/C20 and C20/C18 MGDG was shown to occur when cells from nitrogen-starved cultures rich in ARA-containing TAG were replenished with a nitrogen source at optimal and

low temperatures. This study provided evidence of the microalga's ability to mobilize TAG reserves for membrane-lipid remodeling and build-up under conditions requiring a fast lipid-metabolism response, such as a sudden replenishment of nutrients.

MGDG and DGDG composition was examined in two representatives of the freshwater glaucocystophytes, *Cyanophora paradoxa* and *Glaucocystis nostochinearum* (Leblond et al. 2010b). The Glaucophyta lineage evolved via primary endosymbiosis and its representatives possess a cyanelle (an almost-intact cyanobacterium). These organisms appeared to have 20:5/16:0 MGDG, 20:5/20:5 MGDG, 20:5/16:0 DGDG, and 20:5/20:5 DGDG (*sn*-1/*sn*-2) as the major forms of MGDG and DGDG, resembling the major molecular species typical of the rhodophyte microalgae (Khozin et al. 1997). C18 and C16, which are abundant in cyanobacteria, were present only as minor components in the examined glaucocystophytes, indicating that cyanobacterial-type lipid biosynthetic capabilities were lost during evolution (Leblond et al. 2010b).

The alterations in molecular species of galactolipids rich in 20:4 *n*-6 and 20:5 *n*-3 in the red microalga *Porphyridium cruentum* in response to changes in growth temperature and biomass concentrations were studied by Adlerstein et al. (1997). The eukaryotic-like 20:5 *n*-3/20:5 *n*-3 MGDG increased its relative proportion at the expense of the prokaryotic-like 20:5 *n*-3/16:0 with a decrease in temperature in the range of 30, 25 and 20 °C, indicating the important role of the highly unsaturated eukaryotic-type MGDG in the adaptation of cells to low growth temperatures. EPA decreased in MGDG and DGDG molecular species at the expense of AA with increasing availability of light per cell, in line with the proposed plastidial localization of the desaturation step. The pathway of EPA biosynthesis in this microalga was elucidated utilizing radiolabeled fatty acid precursors (Khozin et al. 1997). The kinetics of label redistribution suggested that 20:4 *n*-6 is formed in the lipid-linked desaturation pathway involving the extraplastidial lipids, followed by transfer to the chloroplast where 20:5 *n*-3/20:5 *n*-3 and 20:5 *n*-3/16:0 MGDG and 20:5 *n*-3/16:0 DGDG are formed by ω-3 (Δ17) desaturation.

Positive-ion electrospray/mass spectrometry (ESI/MS) and ESI/MS/MS were utilized to analyze the fatty acid composition and positional distribution of MGDG and DGDG in various classes of dinoflagellates (Leblond and Lasiter 2009; Roche and Leblond 2010; Dahmen et al. 2013), with the aim of underscoring the evolutionary origin of the plastid in this broad group of organisms, containing both photosynthetic and heterotrophic species, and of delineating their galactolipid-biosynthetic pathways. The photosynthetic dinoflagellates that contain the carotenoid pigment peridinin possess secondary plastids of red algal origin (Leblond and Lasiter 2009). The galactolipids of the brown-pigmented strains from the genera *Chattonella*, *Fibrocapsa* and *Heterosigma* (Raphidophyceae) consist primarily of 20:5/18:4 (*sn*-1/*sn*-2) MGDG and 20:5/18:4 DGDG molecular species. Isolates of the green-pigmented raphidophyte *Gonyostomum semen* also contained 18:3/18:4 MGDG and DGDG; the presence of 18:3 in the *sn*-1 position was interpreted as indicating the origin of the plastid in the green algal lineage (Leblond and Lasiter 2009). *Lingulodinium polyedrum* (a peridinin-containing dinoflagellate, formerly termed *Gymnodinium*) produces 20:5/18:5 and 20:5/18:4 (*sn*-1/*sn*-2) as the major forms of MGDG and DGDG (Dahmen et al. 2013). A survey of 35 species of peridinin-containing warm-adapted dinoflagellates allowed separation of the examined strains into two groups, the first with C18/C18 fatty acids (18:5/18:4, 18:5/18:5 MGDG) and the second with C20/C18 (20:5/18:4, 20:5/18:5 MGDG and DGDG) (Gray et al. 2009b). In a study of cold-adapted growth at 4 °C, only *Gymnodinium* sp. C5 contained the C20/C18 combination (20:5/18:5) of DGDG. The only difference was the prevalence of 18:5/18:5 DGDG in the cold-adapted species as compared to the warm-adapted dinoflagellates, potentially affecting membrane fluidity. Remarkably, an oligogalactolipid trigalactosyldiacylglycerol (TGDG) was reported for the first time in dinoflagellates in this study and appeared as the major galactolipid with the following molecular species composition: 18:1/14:0, 18:1/16:0, 18:1/18:1 (Gray et al. 2009b). A major form of TGDG did not mirror any of the MGDG or DGDG molecular species, implying the different and unidentified biosynthetic origin of this lipid. The authors suggested that a third galactose in the head group enhances membrane fluidity at low temperatures by decreasing the packing ability of the galactolipids.

Most photosynthetic dinoflagellates have a secondary plastid of red algal origin, containing the carotenoid pigment peridinin. MGDG and DGDG composition was analyzed in three dinoflagellates lacking the carotenoid peridinin and possessing a plastid of alternative origin predicted to be acquired via a tertiary endosymbiotic event (Leblond and Lasiter 2009, and ref. therein). *Lepidodinium chlorophorum*, *Karenia brevis* and *Kryptoperidinium foliaceum* were suggested to have acquired a plastid of prasinophyte, haptophyte and pennate diatom origin, respectively. Accordingly, the free-living microalga *Tetraselmis* sp. (the prasinophyte), *E. huxleyi* (the haptophyte) and *Navicula perminuta* (the diatom) were also included in the study. Only 18:5/18:5 MGDG and 14:0/14:0 and 18:1/16:0 DGDG appeared to be common among *K. brevis* and *E. huxleyi*, indicative of the endosymbiont origin of the ability to produce 18:5/18:5. MGDG (20:5/16:3) and DGDG (20:5/16:2) were the major species in both *K. foliaceum* and *N. perminuta. L. chlorophorum* and *Tetraselmis* sp. shared 18:5/16:4 MGDG; 18:5/16:3 and 18:3/16:4 MGDG were more abundant in *Tetraselmis* sp., whereas 20:5/16:4 DGDG was not detected in this free-

living prasinophyte. The authors concluded that that *L. chlorophorum*'s ability to produce 16:4 at the *sn*-2 position of MGDG likely traces back in evolution to the green algal ancestor. The origin of 18:5/18:5 MGDG in *L. chlorophorum* could not be established by analytical methods of galactolipid species analysis and requires further in-depth elucidation at the molecular level.

Different groups have reported molecular species compositions of the diatom microalgae. Sixteen molecular species of MGDG and nine of DGDG were identified in the marine diatom *Stephanodiscus* sp. by lipidomics study employing UPLC-ESI-Q-TOF-MS (Xu et al. 2010); all were of the prokaryotic type, containing C16 acyl groups with up to four double bonds at the *sn*-1 and *sn*-2 positions, and 18:4 and 20:5 located exclusively at the *sn*-1 position. A similar pattern was detected in the DGDG of this diatom. In two centric diatoms, *Skeletonema marinoi* and *Conticribra* (*Thalassiosira*) *weissflogii*, and the pennate diatom *P. tricornutum*, MGDG and DGDG were also composed primarily of C20/C16 and C18/C16 (*sn*-1/*sn*-2) combinations (Dodson et al. 2013), indicating that galactolipids are likely assembled in the prokaryotic-like pathway using highly unsaturated C18 or C20 PUFA produced in the extraplastidial compartment. Two pennate diatoms, *Haslea ostrearia* and *Navicula perminuta*, contained C18/C16 as well as C18/C18 forms of MGDG and DGDG, indicating the different biosynthetic origins of DAG for their assembly. In *P. tricornutum*, 16:3 and 16:4 were almost exclusively restricted to the *sn*-2 position of MGDG, with MGDG 20:5/16:3 being a major species whereas DGDG was more saturated (Abida et al. 2015). There were no 20:5/20:5 or 20:5/C18 MGDG forms identified in that study. Similarly, the *sn*-2 position of MGDG and DGDG is occupied exclusively by C16 acyl groups in the diatom *Fistulifera solaris* JPCC DA0580 (Liang et al. 2014). Based on a lipidomics study that enabled the simultaneous characterization of MGDG molecular species composition combined with a stereospecific analysis of acyl groups, Abida et al. (2015) postulated that a low affinity of the plastid GPAT for C18 acyl groups and the overall availability of acyl groups of different chain lengths predispose the positional distribution of C16, C18 and C20 to the *sn*-1 position of MGDG in *P. tricornutum*. We and others have shown that C20 LC-PUFA for the build-up of prokaryotic-like molecular species likely originate in the extraplastidial compartment (Schneider and Roessler 1994; Khozin et al. 1997; Khozin-Goldberg et al. 2002b).

A lipidomics study of the centric marine diatom *T. pseudonana* exposed to short-term alterations in environmental conditions revealed significant changes in the distribution of MGDG and DGDG species. Galactolipids, which were detected and quantified by liquid chromatography (LC) coupled to a high-resolution MS-based lipidomics platform, were represented by a range of molecular species from 30:0 to 38:6 (number of carbon atoms : number of double bonds); within 24 h after the transfer to nitrogen starvation, MGDG and DGDG species 34:1, 34:2, 34:2 (C18/C16 combinations) increased markedly, in line with the increased abundance of 16:1 fatty acid under stress conditions (Bromke et al. 2013). The dominant species of MGDG in *Nannochloropsis* IMET 1, detected by lipidomics, were EPA-containing species MGDG 40:10 (20:5/20:5) and MGDG 34:5 (20:5/14:0), and those composed of saturated and monounsaturated acyl groups, such as MGDG 32:1 (Li et al. 2014).

Several isolated mutants of *C. reinhardtii* impaired in certain steps of plastidial fatty acid desaturation showed differential distribution of MGDG and DGDG and alterations in molecular species. The RNAi-silenced line of the chloroplast-localized Δ4 desaturase acting on $16{:}3^{\Delta 7,10,13}$ displayed a decreased level of MGDG (Zäuner et al. 2012). In contrast, the overexpressing line demonstrated higher total MGDG levels with no impact on DGDG, consequently raising the MGDG-to-DGDG ratio and cellular Chl content. The results of this study raised the possibility of a direct relationship between PUFA-containing molecular species composition and MGDG abundance in *C. reinhardtii*. The most recent communication is on a mutant defective in the promoter region of the putative ω-3 fatty acid desaturase, featuring reduced proportions of all ω-3 fatty acids (both C18 and C16) in all lipid classes, including the extraplastidial ones (Nguyen et al. 2013). Based on the cellular localization and impact of the mutation on fatty acid composition of chloroplast and extraplastidial lipids, the authors postulated that a single ω-3 desaturase exists in *C. reinhardtii*, probably located in proximity to the ER-chloroplast envelope junctions (contact sites). The mutant did not demonstrate any growth phenotype as compared to the wild type at 15 °C, but showed enhanced short-term thermotolerance to 45 °C as indicated by slower reduction in the F_v/F_m ratio, a measure of PSII activity. Molecular species analysis of polar lipids in the complemented lines with chloroplast-codon-biased desaturase gene was performed by UPLC coupled with LC-MS/MS showing, for example, recovery of the molecular species of MGDG (18:3/6:4) to the level of the wild type.

Another mutant of *C. reinhardtii* linking plastidial galactolipid metabolism with TAG accumulation was recently isolated by Li et al. (2012a). The mutant, deficient in TAG accumulation, appeared to have a lesion in a gene encoding a galactolipid lipase, designated plastid galactoglycerolipid degradation 1 (*pgd1*). The recombinant PGD1 protein showed a clear preference for the de novo produced 18:1/16:0 MGDG in in-vitro assays. Furthermore, labeling kinetics of lipid classes after pulse-chase with [1-^{14}C]acetate suggested that galactolipids in *C. reinhardtii* are a major source of fatty acids for TAG production under nitrogen deprivation (Sect. 7) (Li et al. 2012a).

To conclude this section, we would like to note that a literature survey identified algal galactolipids of both micro- and macroalgae as potent therapeutic agents with anti-inflammatory and anti-viral properties (Banskota et al. 2013a, b; de Souza et al. 2012). Galactolipids from microalgae were reported to exhibit nitric oxide-inhibitory activity against lipopolysaccharide-induced nitric oxide production in a macrophage culture (Banskota et al. 2013a, b).

5.3 SQDG

An anionic sulfolipid SQDG is abundant in the thylakoids fractions of cyanobacteria, algae, and higher plants. A sulfonate group of quinovose (the sugar headgroup of SQDG) provides a negative charge of this polar lipid (Fig. 11). Sulfoquinovose is a derivative of glucose (Glu) in which the six-hydroxyl group is replaced by a sulfonic residue at C6. The biosynthesis of SQDG has been elucidated in photosynthetic bacteria, the higher plant *A. thaliana*, the green microalga *C. reinhardtii* by radiolabelling studies and utilizing mutants impaired in activities essential for SQDG biosynthesis (Benning et al. 1993; Benning 1998; Sato 2004). Beyond establishing the biosynthesis pathway, numerous studies with the mutants of *C. reinhardtii* underscored the role of this glycerolipid under conditions of sulfur (S) and phosphorous (P) deprivation. In the next section, we will mostly refer to the studies performed on *C. reinhardtii*.

The enzyme UDP-sulfoquinovose synthase, encoded by the *SQD1* gene, mediates the synthesis of UDP-sulfoquinovose from UDP-glucose (UDP-Glc) and sulfite SO_3^{2-} (Essigmann et al. 1998; Sanda et al. 2001; Sato 2004) (Fig. 11). The *SQD1* gene from *C. reinhardtii* was cloned and functionally characterized by two means: inactivation of the gene via insertional mutagenesis (Riekhof et al. 2003) and complementation of a SQDG-deficient phenotype in the SqdB mutant of *Synechococcus* sp. PCC7942 (Sato et al. 2003b). *SQD1* appeared to be highly expressed in ESTs of *C. reinhardtii*; high abundance of *SQD1* mRNA has been linked to necessity for increased abundance of the enzyme given a low kcat for the reaction catalyzed by SQD1 measured in vitro (Riekhof et al. 2003). SQDG synthase that is encoded by the *SQD2* gene transfers sulfoquinovose from uridine diphosphate (UDP)-sulfoquinovose to diacylglycerol (Yu et al. 2002; Yu and Benning 2003). Accordingly, the formation of SQDG in the chloroplast depends on the availability of both substrates: the activated sugar and DAG, and of course, is tightly connected to S metabolism. DAG for SQDG biosynthesis is produced in the above described prokaryotic pathway. The major fatty acid of SQDG is palmitic acid (16:0), often comprising over 60 % of the fatty acids; if present, C18 and C20 PUFA are localized to the *sn*-1 position of SQDG. For example, in the diatom *P. tricornutum*, the molecular species composition of SQDG is quite diverse, comprised of species 14:0/16:0, 16:0/16:0, 16:0/16:1, 16:1/20:4 and 20:5/16:0 (*sn*-1/*sn*-2) (Abida et al. 2015). In addition, the acylated derivatives of SQDG, 2′-O-acylsulfoquinovosyldiacylglycerols (ASQD), have been identified in *C. reinhardtii* (Riekhof et al. 2003) and *P. tricornutum* (Naumann et al. 2011; Abida et al. 2015). While SQDG of *C. reinhardtii* is composed mainly of forms 18:1/16:0, a triacylated form of ASQD harbors unsaturated $18:3^{\Delta 5,9,12}$ and $18:4^{\Delta 5,9,12,15}$ acyl groups (Riekhof et al. 2003). Given that these PUFA are components of extraplastidial glycerolipids in *C. reinhardii*, it seems likely that the biosynthesis of ASQD is associated with the outermost plastid envelope membrane, most proximal to the ER. In *P. tricornutum*, 2′-acylation of the sulfoquinovose moiety produces

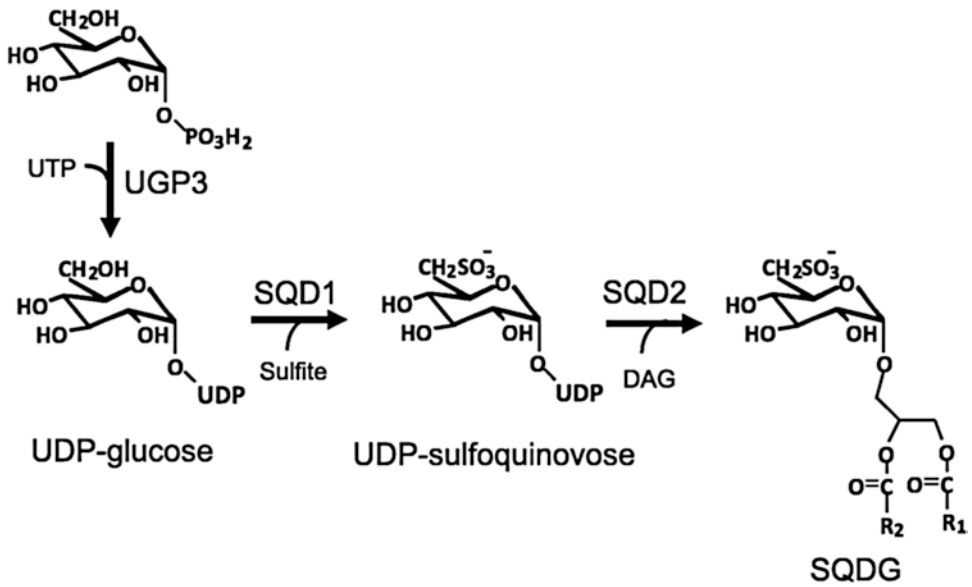

Fig. 11 Schematic representation of the reactions of the biosynthesis of SQDG. See text for details (Reproduced from Shimojima (2011); courtesy of Elsevier)

sn-1: C16:0/*sn*-2: C16:0/2′ C20:5 and *sn*-1: C20:5/*sn*-2: C16:0/2′ C20:5 ASQD (Naumann et al. 2011). Further investigations are needed to elucidate the origin of the unsaturated acyl groups and the cellular localization of the additional acylation reactions on sulfoquinovose.

Origin of the UDP- glucose in plastid required for SQDG biosynthesis has been addressed in recent studies. In plastid of higher plants and green algae cells, the most abundant is though adenosine diphosphate activated ADP-glucose, a substrate of ADP-glucose pyrophosphorylase, the key enzyme in starch biosynthesis (Dörmann 2005). It is known that the UDP-sugar pyrophosphorylase (USPase; EC 2.7.7.64) catalyzes a reversible transfer of the uridyl group from UTP to sugar-1-phosphate, producing UDP-sugar and pyrophosphate, the UDP-activated sugar that is utilized in versatile biochemical reactions in the cell (Kleczkowski et al. 2011). It was suggested that a plastid pool of Glu-1-P could be a branching point between starch and SQDG biosynthesis (Shimojima 2011). Quite recently it had been shown that synthesis of UDP-Glu in plastid is accomplished by the action of the plastid isoform of UDP-Glu pyrophosphorylase (UGP3) (Okazaki et al. 2009). The experiments with the recombinant UGP3 from *A. thaliana* demonstrated that it is able to catalyze the formation of UDP-glucose from Glu-1-P and UTP. Phylogenic analysis revealed that *Arabidopsis* UGP3 has close homologs in the green microalgae *O. tauri* and *C. reinhardtii* (Okazaki et al. 2009) but absent in cyanobacteria, raising the question on the origin of activated sugar group for SQDG synthesis in cyanobacteria. The similarity search on genome data on additional recently sequenced algal species reveals additional gene homologs within the green algae and the diatoms; for example, a putative plastid UDP-Glu pyrophosphorylase is annotated in the genome of the *P. tricornutum* (PHATRDRAFT_23639). The diatom *T. pseudonana* has clear homologs for SQDG biosynthesis proteins that are up-regulated under conditions of P starvation when synthesis on the non-phosphorous lipids is enhanced (Martin et al. 2011; Dyhrman et al. 2012).

A large body research has been conducted utilizing *C. reinhardtii* mutants impaired in SQDG biosynthesis to illuminate the function of SQDG in the photosynthetic microalgal cells. Mutants deficient in SQDG formation were also obtained in a photosynthetic bacterium *Rhodobactor sphaeroides* (Benning et al. 1993), and in cyanobacteria *Synechococcus* spp. PCC7942 and PCC6803 (Güler et al. 1996). Studies on the *C. reinhardtii* low-phosphate bleaching (lpb1) mutant shed some light on the function of UDP-Glu pyrophosphorylase in the green microalgae. This mutant of *C. reinhardtii* appeared to be more sensitive than the wild-type to S and P deprivation but did not feature a phenotype in response to nitrogen starvation (Chang et al. 2005). A *LPB1* gene encodes a protein that is 45 % identical to *Arabidopsis* UPG3 and is predicted to possess an organelle targeting signal. Given the role of SQDG under P and S deprivation it seems very likely that the protein encoded by *LPB1* is a functional UDP-synthase required for SQDG biosynthesis (Chang et al. 2005).

Indeed, a role for SQDG was intensively investigated in *Chlamydomonas* in relation to P and S starvation (Riekhof et al. 2003; Chang et al. 2005; Sugimoto et al. 2007, 2008, 2010). Given that SQDG is one of the three non-phosphorous chloroplast glycerolipids under conditions of P starvation, SQDG synthesis as well as expression of the genes encoding SQDG biosynthesis increase to compensate for the decline of phospholipids due to their turnover under these conditions (Riekhof et al. 2003; Sugimoto et al. 2008). A *C. reinhardtii* mutant, *Δsqd*1, carrying a deletion of the CrSQD1 gene proposed to encode UDP-sulfoquinovose synthase, lacked both ASQD and SQDG, and demonstrated impaired photosynthesis and growth under phosphate starvation (Riekhof et al. 2003). Remarkably, under S starvation, the SQDG synthesis capacity was increased by 40 %, with a sixfold elevation in the mRNA level of the SQD1 gene (Sugimoto et al. 2010). In agreement with that an increase in all three transcripts coding enzymes of SQDG biosynthesis was determined in a RNA-seq study of S-deprived *C. reinhardtii* collected for RNA isolation following 1, 6, and 24 h after being transferred to TAP and TAP-S medium (González-Ballester et al. 2010). Apparently recycling and redistribution of S is an essential survival strategy of the alga under these conditions.

An essential role of SQDG in *C. reinhardtii* under S-starvation providing a source of S for protein synthesis at early stages following S removal was inferred from several studies. Such as, in the cells that were pre-incubated with the radiolabelled [^{35}S] sulfate and transferred to the same medium but lacking sulfate radioactivity in SQDG reduced by 85 % within first 6 h concomitantly with the increase in radioactivity of the protein fraction (up to 83 %) (Sugimoto et al. 2007). These results raised possibility that protein synthesis under S-starvation predominantly utilizes S released from SQDG and reinforced a primary role of SQDG in sulfur storage. Notably, SQDG was not degraded under P- or N-limited conditions within the same experimental period (Sugimoto et al. 2007). Enzymatic steps and genes encoding them that are involved in SQDG degradation under S-starvation are not well known, however by applying inhibitors of the de novo protein synthesis it was shown that the machinery required for SQDG degradation is produced de novo under these conditions. By means of RNA-seq of S-deprived *C. reinhardtii*, transcripts encoding potentially involved in SQDG degradation were revealed and transcript encoding a putative lipase that were increased during exposure of *Chlamydomonas* to − S conditions (please refer for further details to González-Ballester et al. 2010).

A SQDG-defective mutant (hf-2) of *C. reinhardtii* features no detectable amount of SQDG and demonstrates

reduced photosystem II (PSII) activity with little effect on photosystem I (PSI) activity (Sato et al. 1995a, b). Latter, photosynthetic characterization of this mutant allowed suggesting that SQDG is indispensable for PSII activity in *Chlamydomonas* by maintaining PSII complexes in their proper state (Minoda et al. 2002). Recovery of PSII from the heat-induced damage was suppressed in this mutant Sato et al. (2003a). When compared with the wild type under S-deprivation a hf-2 mutant demonstrated decreased protein accumulation, photosystem PS I stability and growth rate (Sugimoto et al. 2010). The sulfur acclimation (sac) genes mutants of *C. reinhardtii* are impaired in their response to S-limitation (Davies et al. 1994, 1996). Studies on these mutants revealed transcriptional and posttranscriptional control of biosynthesis of the S-containing amino acid cysteine (Ravina et al. 2002); cac3 mutant appeared to be deficient in the plant-specific *SN*F-1 related kinase *SN*RK2.2 which is responsible for repression of S-inducible genes under S-replete conditions (Gonzalez-Ballester et al. 2008). Induction of SQDG degradation was largely repressed in sac1 and sac3 mutants when S-deprived implying that a role of SQDG in S storage is under the control of both SAC1 and SAC3 genes (Sugimoto et al. 2010). To conclude this section, an interesting hypothesis on the novel role of SQDG in sustaining of cell cycle and DNA synthesis was drawn by Aoki et al. (2012), based on the fact that the SQDG-deficient mutant cells of *Synechocystis* sp. PCC 6803 attained elevated level of cell mass, but repressed DNA synthesis and were impaired in completion of the cell cycle.

5.4 Phosphatidylglycerol (PG)

An anionic phospholipid PG is the only phospholipid present in cyanobacteria and in the thylakoid membranes of the photosynthetic cells, but is also ubiquitous in extraplastidic membranes, such as the ER, plasma membrane and membranes of mitochondria (Xu et al. 2002; Dörmann 2005). It should be noted here that the unique fatty acid 16:1Δ3*trans* is exclusively found in this lipid in photosynthetic organisms and is specifically esterified at the *sn*-2 position of the chloroplast PG, having very important implications for sustaining photosynthesis, and particularly at decreased temperatures (McCourt et al. 1985; Siegenthaler and Trémolières 1998).

PA produced in the plastid in the prokaryotic pathway is used in a reaction catalyzed by CDP-DAG synthase (CDS) to form CDP-DAG, a precursor for the anionic chloroplast lipid PG according to the scheme:

$$\mathrm{CTP + PA \rightarrow CDP\text{-}DAG + Pi}$$

CDS enzymes are integral membrane proteins; two plastidial isoforms of CDS that are required for PG biosynthesis were identified in *Arabidopsis* with similar and likely redundant catalytic properties (Haselier et al. 2010). PG is formed in two consecutive reactions from CDP-DAG and G-3-P by the sequential action of phosphatidyl glycerophosphate synthase (PG-P synthase; PGP, EC 2.7.8.5) producing phosphatidylglycerol phosphate (PG-P) (Xu et al. 2002; Babiychuk et al. 2003), followed by phosphate group removal by PGP phosphatase (EC 3.1.3.27) according to the scheme:

$$\mathrm{CDP\text{-}DAG + G-3-P \rightarrow PGP + CDP \rightarrow PG-P + H_2O \rightarrow PG + Pi}$$

PG formed in mitochondria can be further utilized in the synthesis of diphosphatidylglycerol (cardiolipin). Two PGP isoforms are known in *Arabidopsis*; a mutation mapped to the chloroplast targeted PGP1 caused 30 % reduction in PG content, severe decrease in the content of photosynthetic pigments as well in the PGP synthase activity in isolated chloroplasts (Xu et al. 2002). Remarkably, *Arabidopsis* PGP1 was identified in the proteome of chloroplast envelope (Ferro et al. 2003) and carries the organelle targeting presequence that enables dual targeting to both plastids and mitochondria (Babiychuk et al. 2003). The analysis of a loss-of-function *pgp*1 mutant that was not able to grow photoautotrophically revealed the severely decreased content of plastidial PG and the aberrant chloroplast ultrastructure, however, PGP1 was not absolutely required for the biogenesis of thylakoid membranes. Remarkably, the null mutation did not impair accumulation of CL which is produced in mitochondria, implying that mitochondria could import PG from the ER (Babiychuk et al. 2003).

A role of PG photosynthesis is intensively studied employing the mutants of *Synechococcus* sp. PCC 7942 produced by inactivation of the genes coding for the enzymes of PG biosynthesis (see e.g. Sakurai et al. 2007; Bogos et al. 2010; among many others). Such as, purified PSII complexes from a PG-depleted mutant of *Synechococcus* sp. PCC 7942 exhibited approximately 50 % of the oxygen-evolving activity as compared to PSII complexes isolated from the wild-type cells (Sakurai et al. 2007). Mutants of the eukaryotic alga *C. reinhardtii* that are deficient or impaired in plastid PG synthesis displayed decreased capacity to stabilize the light-harvesting complex (LHC) in a functional trimeric state, instability of the core LHC of photosystem I (PSI), as well as impaired synthesis of PSII core complexes (Dubertret et al. 2002; Pineau et al. 2004). A hypothetical protein (encoded by CHLREDRAFT_194257 gene) was putatively annotated in the genome of *Chlamydomonas* as PGP due to the strong similarity to PGP of *Arabidopsis* albeit without predictable cellular localization; homologues to *At*PGP with

about 40 % similarity are identifiable in some other sequenced green algae and diatoms.

6 Extraplastidial Phospho- and Betaine Lipids

PC and PE are the major components of the extraplastidial cellular membranes in plants. In many classes of microalgae, the betaine lipids either partially or completely replace phospholipids in the extraplastidial membranes (Sect. 3). PE is a non-bilayer-forming lipid, whereas PC supports bilayer formation. Extraplastidial PG and cardiolipin (CL) are major phospholipids in mitochondrial membranes. Biosynthesis of these important membrane components has been intensively studied in various organisms; for a detailed overview of phospholipid biosynthesis please refer to Kinney (1993), Kent (1995), Dormann (2005), Carman and Han (2011), and Kelly and Jacobs (2011). In many instances, early biochemical data on activities of the respective enzymatic steps were later supported by molecular studies. Apart from their structural role, PC and, possibly, PE in microalgae play a pivotal role in fatty acid modifications, such as desaturation, and in providing the precursors for TAG biosynthesis. We will discuss these functions further on.

PA produced in the ER by sequential action of GPAT and LPAAT is a common precursor for the de novo biosynthesis of the membrane phospholipids. The biosynthesis of PI, similar to that of PG, proceeds via the CDP-DAG pathway and is initiated by the action of the microsomal (ER-localized) isoform of CDS on PA and G-3-P. PG is formed from G-3-P and PA in analogy to the above described plastidial route. PI is produced from CDP-DAG and free myo-inositol via the action of PI synthase (reviewed in Dorman 2005). As noted in Sect. 3, PI has been found in the plasma membrane of microalgae and serves as a precursor for the synthesis of various PIPs implicated in signaling (Thompson 1996; Munnik 2001 and references therein).

Biosynthesis of the major membrane phospholipids PC and PE de novo proceeds via the DAG pathway and requires formation of 1,2-DAG by dephosphorylation of PA mediated by PAP/ PAH (referred to as lipin) and involves the cytidine 5′-diphosphate (CDP)-containing aminoalcohols CDP-choline (CDP-Cho) and CDP-ethanolamine (CDP-Etn) (Fig. 12). The pathway is initiated in the cytosol with phosphorylation of choline and ethanolamine with ATP by choline kinase (CK; EC 2.7.1.32) and ethanolamine kinase (EK; EC 2.7.1.82), respectively, resulting in the formation of Cho-P and Etn-P and liberation of inorganic phosphate. Sequential activities of CTP-phosphocholine cytidylyltransferase (CCT) (EC 2.7.7.15) and CTP-phosphoethanolamine cytidylyltransferase (ECT) (EC 2.7.7.14) yield CDP-Cho and CDP-Etn, respectively. Finally, CDP-Cho and CDP-Etn react with 1,2-DAG to form PC and PE, respectively; this step is catalyzed by CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT) (EC 2.7.8.1) and CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (EPT) (EC 2.7.8.1) which transfer the aminoalcoholphosphate moiety to the *sn*-3 position of 1,2-DAG (Fig. 12).

Aside from its uptake from the environment, free choline that is required for de novo PC biosynthesis is produced by catabolism of PC and other choline-containing compounds (Kinney 1993). Ethanolamine for the biosynthesis of PE is produced by decarboxylation of L-serine mediated by serine decarboxylase; similarly, decarboxylation of PS by PS decarboxylase (EC 4.1.1.65) represents another route for PE formation. However, PS is a minor membrane lipid in plants and in microalgae, and this reaction is therefore not considered significant in PE formation. In yeast, PS is synthesized

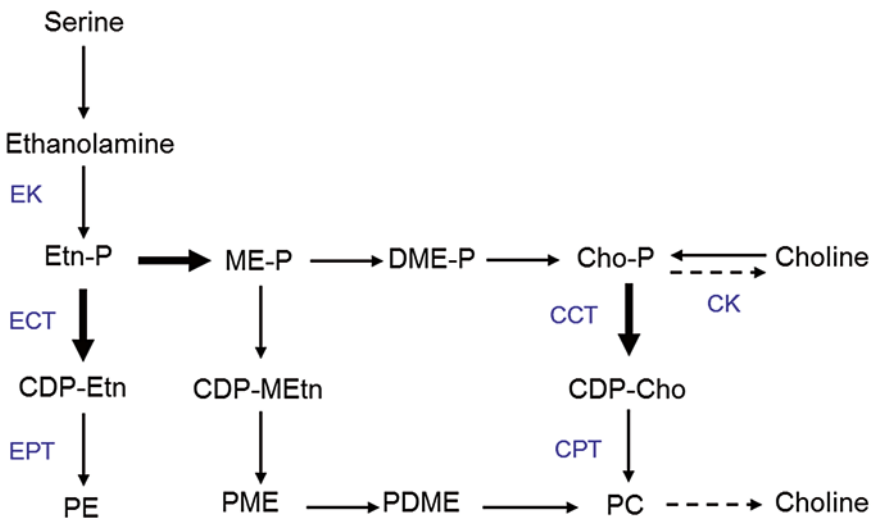

Fig. 12 Simplified schematic representation of phosphatidylcholine (*PC*) and phosphatidylethanolamine (*PE*) biosynthesis pathways (Please refer to the text for details)

from CDP-DAG and L-serine, with this reaction being indispensable for phospholipid biosynthesis and cell growth (Kinney 1993). *Arabidopsis* does not contain CDP-DAG-dependent PS synthase, which utilizes CDP-DAG and L-serine for PS biosynthesis (Li-Beisson et al. 2010), whereas three isoforms of PS decarboxylase have been identified, one localized to the mitochondria and two to the extraplastidial membranes (Nerlich et al. 2007; Li-Beisson et al. 2013). In *Arabidopsis*, PS is enriched in non-photosynthetic tissues and contains very-long-chain fatty acids (up to C24:0) (Yamaoka et al. 2011). Little data exist on the occurrence, biosynthesis and function of PS in microalgae but this lipid is detectable by lipidomics means (e.g. Martin et al. 2014).

Biosynthesis of PE and PC is closely linked via the PE *N*-methyltransferase (PEMT) pathway that is the second major pathway for PC biosynthesis. This pathway involves a three-step S-adenosyl methionine (AdoMet)-dependent methylation reaction (Kinney 1993; Carman and Han 2011) catalyzed by specific methyltransferases—two in yeast: PE methylase and *N*-methylphospholipid methyltransferase (PLMT), and a single methylase in animals. Furthermore, Etn-P produced by EK is an important intermediate for both PE and PC biosynthesis in plants, since it can also be sequentially methylated with three molecules of AdoMet, a versatile methylation agent in the cell (Kinney 1993).

A base-exchange pathway involves exchange of a head group with a preexisting phospholipid (Kinney 1993). This pathway, operating in animals cells, is involved in the biosynthesis of PS by PS synthase (PSS), exchanging either the Cho or Etn residue with serine in PC and PE, respectively, to produce PS. In plants, functional expression of a single *Arabidopsis* PS synthase in *E. coli* suggested that PE is the predominant substrate for this activity given the decreased share of PE and a concomitant rise in that of PS in cellular glycerolipids (Yamaoka et al. 2011). It should be noted that earlier biochemical evidence suggested the possibility of ethanolamine exchange for a pre-existing PC head group, suggesting another possible route for PE biosynthesis by head-group exchange in plants (Yang et al. 2004a and references therein) and possibly in microalgae (Bigogno et al. 2002a).

Since the phospholipids PC and PS are absent in *C. reinhardtii* (Giroud et al. 1988), this organism provides a unique opportunity to study PE biosynthesis as a single major phospholipid in the absence of PE-PC interconversion. The genome of *C. reinhardtii* does not encode the three enzymes in the CDP-Cho pathway of PC biosynthesis—choline kinase, CCT, and CPT, or PS decarboxylase, PS synthase or phospholipid base-exchange enzymes (Yang et al. 2004a; Riekhof et al. 2005b); on the other hand, two enzymes that are essential for PE biosynthesis—ECT and EPT—have been cloned and characterized from *C. reinhardtii* (Yang et al. 2004a, b).

ECT is considered the main regulatory enzyme in the de novo biosynthesis of PE in the CDP-Etn pathway. The ORF of CrECT encodes a 443-amino acid polypeptide featuring characteristic repetitive sequences in both its N and C halves comprising conserved HXGH motifs in analogy to human, rat and yeast ECT (Yang et al. 2004a). The putative protein also featured other signatures of a typical cytidylyltransferase but appeared to be longer than ECT of human, rat or yeast origins, due to the N-terminal extension, predicted to encode an organelle-targeting pre-sequence. Analysis of the distribution of ECT activity in different subcellular fractions of the cell-wall-less mutant of *C. reinhardtii* strain CC406 cw15, indicated mitochondrial localization of the ECT in this microalga given the tight correlation of the total and specific activities of ECT with those of the mitochondrial marker enzyme fumarase. Thus, the subcellular localization of ECT enzyme seemed to differ in the photosynthetic *C. reinhardtii* vs. mammalian cells, where ECT was shown to be a cytosolic enzyme. *A. thaliana* ECT, encoded by *PECT1*, was later shown to also be localized to the mitochondria employing tagging to the fluorescent protein (Mizoi et al. 2006). The function of *Cr*ECT was confirmed by heterologous expression in *E. coli* which demonstrated the production of CDP-Etn from [^{14}C]Etn-P and CTP in the presence of Mg^{2+} by the recombinant protein (Yang et al. 2004a). ECT activity seemed to be regulated during the cell cycle of *C. reinhardtii*, increasing during the dark period and reaching a maximum before the onset of light and initiation of cell division, which may require enhanced de novo PE synthesis; however, the mRNA level did not show any significant change under these same conditions. The authors thus suggested that ECT activity is regulated at the post-translational level; some evidence of transcriptional regulation of *ECT* was provided by studying the flagellation/deflagellation process in *C. reinhardtii*.

A cDNA encoding CrEPT was cloned by Yang et al. (2004b) and the function of the encoded protein was demonstrated by functional complementation in a *Saccharomyces cerevisiae* double mutant deficient in both CTP and ETP activities. Analysis of the deduced protein sequence revealed that CrEPT is a member of the CDP-alcohol phosphatidyltransferase family, featuring this family's conserved signature sequence D-G-x(2)-A-R-x(8)-G-x(3)-D-x(3)-D. CrEPT is a membrane-bound protein predicted to possess seven TMD and have a putative C-terminal ER membrane-retention signal, TPKR, resembling the canonical di-lysine ER-retention signal, the KKXX-like motif. Remarkably, the EPT of *C. reinhardtii* was capable of synthesizing both PC and PE in in vitro assays, even though PC is not present in *C. reinhardtii*. Moreover, CrEPT clearly preferred CDP-Cho to CDP-Etn in the competition assays. Taking into account the data on dual-substrate utilization by CrEPT as well as of the initial kinase step, the authors suggested that lack of CCT limits PC synthesis in *C. reinhardtii* in the CDP-DAG path-

way (Yang et al. 2004b). Furthermore, *C. reinhardtii* lacks the second pathway of PC biosynthesis, which is a PE *N*-methylation pathway, as implied by analysis of the genome data (Riekhof et al. 2005b).

Remarkably, CTP of *S. cerevisiae*, as well as human CTP, demonstrated a strict preference for CDP-Cho, whereas both human and yeast EPT were capable of utilizing both CDP-Cho and CDP-Etn (Hjelmstad and Bell 1987; Henneberry and McMaster 1999; Mancini et al. 1999). Higher plant amino alcohol phosphate transferase, so-called because of its dual activity toward both CDP-Cho and CDP-Etn (Goode and Dewey 1999), strongly prefers CDP-Cho over CDP-Etn. A recently cloned and characterized CPT from *Phytophthora infestans*, a LC-PUFA-producing oomycete, demonstrated CTP activity exclusively (Chen et al. 2013). Thus, high CTP activity of CrEPT is interesting from an evolutionary standpoint, particularly with respect to partial or complete substitution of PC by DGTS in many microalgal species (Yang et al. 2004b).

CPT of higher plants do not exhibit substrate preference toward DAG substrates with different fatty acid composition (Vogel and Browse 1996), but its activity in the reverse direction plays an important role in enriching the DAG pool with unsaturated fatty acids. Remarkably, functional expression of *P. infestans* CTP in a yeast double-mutant strain (ΔcptΔept) revealed that the recombinant protein favors LC-PUFA-containing DAG, such as 18:0/20:4 and 18:0/22:6, over di16:0, di16:1, di18:0, di18:1 and di18:2 DAG. The results of this study may be pertinent to the elucidation of mechanisms governing LC-PUFA production and accumulation in microalgae, providing a possible mechanism for channeling unsaturated DAG species to PC (Chen et al. 2013).

In *C. reinhardtii*, DGTS completely replaces PC in extraplastidial membranes, both structurally and functionally (Schlapfer and Eichenberger 1983; Moore et al. 2001). The biosynthesis of DGTS in microalgae was progressively disclosed by using biochemical and ultimately, molecular genetic approaches (Sato 1988; Vogel and Eichenberger 1992; Moore et al. 2001; Riekhof et al. 2005b). A few earlier studies examined the biosynthetic origin of a head group of DGTS in several organisms, including *C. reinhardtii*, by radiolabeling with [^{14}C]methionine as a potent precursor for both the homo-Ser moiety and the methyl units bonded to the amine group of the homo-Ser (Sato 1988; Vogel and Eichenberger 1992). By using both [^{14}C]carboxyl- and [^{3}H] methyl-labeled AdoMet [alternatively S-adenosyl-L-Met (SAM)], Moore et al. (2001) were able to prove that AdoMet is absolutely required for DGTS biosynthesis and serves as a precursor for the homo-Ser moiety, as well as methyl-group donor. The stepwise methylation pathway was presumed to occur in analogy to the methylation pathway of PC biosynthesis, and short-lived intermediates of the biosynthetic pathway were detected. The highest activity for DGTS formation was determined in a high-speed microsomal fraction that was rich in ER-membrane fragments, obtained by cellular fractionation of the cell-wall-deficient *C. reinhardtii* strain CC400 cw-15.

A few years later, a bioinformatics approach identified a putative protein in the genome of *C. reinhardtii*, encoded by *BTA1Cr* (GenBank Acc. No. AY656806), which appeared to be sufficient for the biosynthesis of DGTS. It was deduced according to its sequence similarity to two proteins, $BtaA_{Rs}$ and $BtaB_{Rs}$, of the bacterium *Rhodobacter sphaeroides*, required for the biosynthesis of DGTS in two steps (Riekhof et al. 2005a, b; Moellering et al. 2009). *BTA1*Cr appeared to encode a bifunctional protein with two functional domains of the AdoMet:DAG 3-amino-3-carboxypropyltransferase and the AdoMet-dependent diacylglycerolhomoserine (DGHS) methylase, respectively. Heterologous expression of *BTA1*Cr alone led to DGTS accumulation in *E. coli* with molecular species 32:1 and 34:2 characteristic of *E. coli* membrane lipids. Site-directed mutagenesis of the AdoMet-binding sites along with analysis of the pathway intermediates in in vitro assays inferred that the sequential reactions catalyzed by the two domains of BTA1Cr do not involve substrate channeling; rather, a BtaB-like DGHS-methylase domain was able to take out DGHS from the membrane and use it as a substrate (Riekhof et al. 2005b). Notably, BTA1Cr was present as the second major protein in the purified LD fraction of *C. reinhardtii* (Moellering and Benning 2010). It was therefore suggested that DGTS may have a role in LD formation similar to that of PC in other organisms. This assumption seems likely since DGTS, which possesses properties analogous to PC, replaces the latter in the extraplastidial membranes of *C. reinhardtii*.

A central role for PC in plant lipid metabolism, encompassing both glycerolipid and TAG biosynthesis, is now widely recognized (Bates and Browse 2012; Bates et al. 2013). PC is a major lipid substrate for acyl-group modifications, such as desaturation in plants and in microalgae, as well as hydroxylation in plants. Furthermore, PC is recognized as the major phospholipid substrate implicated in the acyl-editing machinery (Fig. 13). Remodeling or retailoring of the principal extraplastidial phospholipids by acyl-group editing is mediated by the Lands cycle (Lands 1958). This pathway increases the diversity of the acyl-group composition of glycerolipids, as well as the acyl-group signatures acquired due to acyl-CoA selectivity of GPAT and LPAAT in the Kennedy pathway (Wang et al. 2012b). Rapid turnover and dynamic exchange of acyl groups, predominantly between the *sn*-2 position of PC and acyl-CoA pools in higher plant cells, suggested to be governed by the action of the CoA-dependent lysophosphatidylcholine acyltransferase (LPCAT; EC 2.3.1.23) and the CoA-independent phospholipase A2 (PLA_2; EC 3.1.1.4) releasing free fatty acids. PLA_1, acting on the *sn*-1 position of phospholipids, is also known.

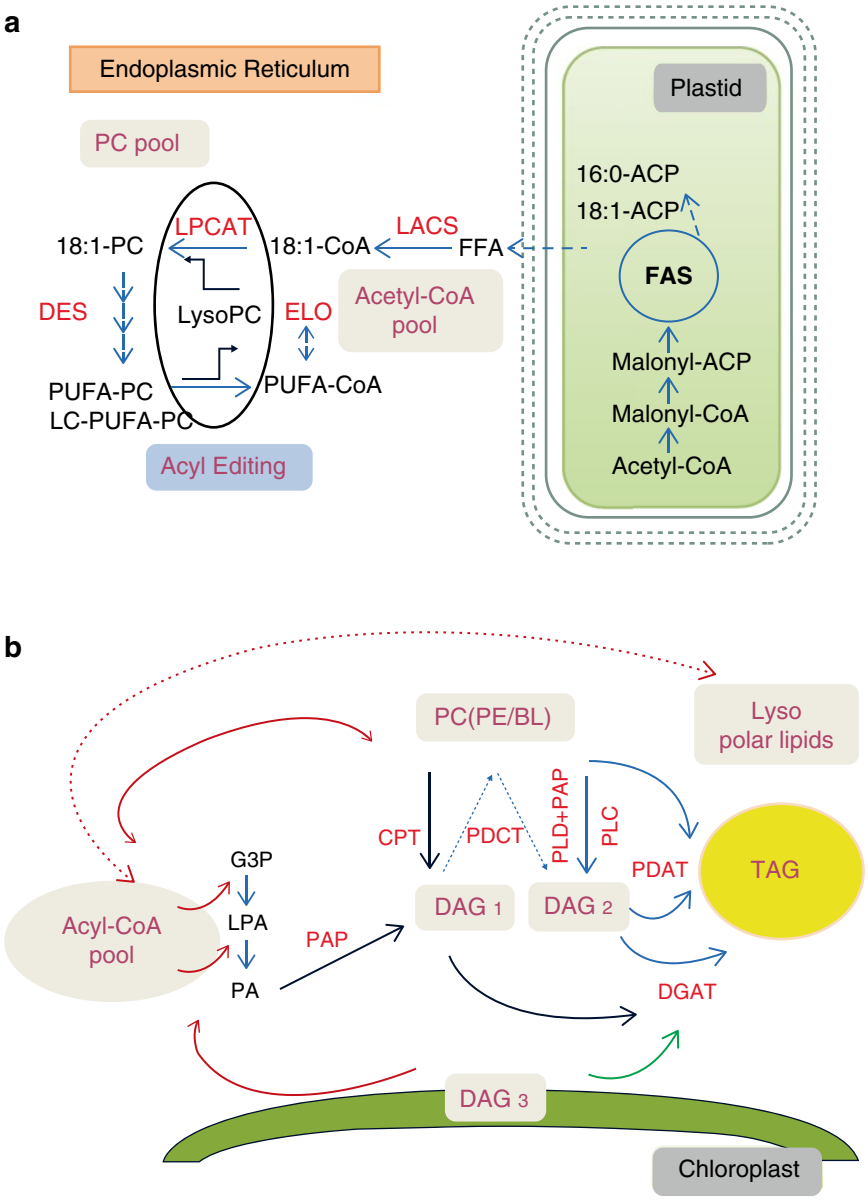

Fig. 13 Central role of DAG: possible acyl-editing reactions (**a**) and different routes contributing to DAG production (**b**) in the extraplastidial compartment. DAG_1 and DAG_2 are de novo synthesized and unsaturated PC-derived DAG pools, respectively. Extraplastidial lipids (PE and betaine lipids, BL) may also be involed in unsaturated DAG formation and acyl editing in microalgae. Chloroplast-derived DAG_3 may contribute to TAG formation in microalgae. Note, that in some microalgae de novo synthesized 16:0 and 16:1 are incorporated into the extraplastidial lipids. Please refer to text for details (Adopted and modified from Bates et al. (2013); courtesy of Elsevier)

One recent study identified, for example, a patatin-related phospholipase pPLAIIIδ in *Arabidopsis* with a role in fatty acid remodeling and glycerolipid production (Li et al. 2013). The Lands cycle in the versatile eukaryotic organisms effectively bypasses the de novo biosynthesis of phospholipids and is essential for phospholipid turnover, as well as the release and uptake of acyl groups (Kazachkov et al. 2008; Riekhof et al. 2007; Wang et al. 2012b; Robichaud et al. 2013 among others). Active polar lipid remodeling is implicated in the nutrient stress response in microalgae, and nutrient depletion activates expression of the genes involved in membrane glycerolipid turnover (Miller et al. 2010; Urzica et al. 2013; Li et al. 2014).

In plants, rapid deacylation and reacylation of the *sn*-2 position of PC, which is presumably governed by bidirectional action of the *sn*-2-specific LPCAT, enables incorporation of the de novo-synthesized fatty acids as well as the release of unsaturated acyl-CoA to the cytoplasmic pool (Bates et al. 2009, 2013) (Fig. 13). At least one candidate gene showing similarity to the *Arabidopsis* LPCAT and putatively encoding a hypothetical protein of the membrane-bound O-acyltransferase family (MBOAT) family is identifiable in the genomes of sequenced algae, such as the chlorophytes (*Coccomyxa subellipsoidea* C-169l, *Chlorella variabilis*, *Micromonas* sp. RCC299, *M. pusilla* CCMP1545, *Ostreococcus tauri*, *O. lucimarinus* CCE9901, *Volvox carteri*, *Bathycoccus prasinos*). Additional candidates exist in the genomes of the diatoms *P. tricornutum* and *T. pseudonana* CCMP1335, and of the rhodophytes, such as *Galdieria sulphuraria*, but with a lower degree of similarity.

There are several possible options for DAG production from phospholipids and presumably from the betaine lipids in microalgae in the extraplastidial compartment, apart from the aforedescribed PAP activity on PA. Notably, in the well-studied *Arabidopsis*, at least 11 candidate genes tentatively encoding PAP are known, with only two of them—*PAH1* and *PAH2*—having actually been characterized (Nakamura et al. 2009; Eastmond et al. 2010). PC-derived DAG may participate in glycerolipid and TAG biosynthesis via several routes, as affirmed in studies with plants (Bates et al. 2013) (Fig. 13). First, the significance of the reverse activity of CTP for enriching the DAG pool with unsaturated DAG species has been documented (Slack et al. 1983, 1985; Vogel and Browse 1996). Second, the activity governed by the recently cloned phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT), which is encoded by the Reduced Oleate Desaturation1 (*ROD1*) gene in *Arabidopsis*, catalyzes the direct symmetrical transfer of the phosphocholine head group from PC to DAG (Lu et al. 2009); rod1 mutants demonstrated drastically decreased flux of unsaturated 18:2 and 18:3 toward TAG biosynthesis, implying that PDCT mediates one of the major routes by which, from one side, 18:1 enters PC for further desaturation to 18:2 and 18:3, and from the other, unsaturated DAG becomes available for TAG biosynthesis. There is evidence that PDCT acts in planta to enhance the fluxes of modified fatty acids through PC to TAG (Hu et al. 2012). Furthermore, analysis of the triple *rod1*/*lpcat1*/*lpcat2* mutant of *Arabidopsis* with drastic reduction in PUFA (18:2 and 18:3) content in the seed TAG (up to 30 % of the wild-type level) clearly indicated that control of most acyl fluxes for unsaturated TAG synthesis resides in both the PC acyl editing (LPCAT) and PC head group-exchange (PDCT) mechanisms (Bates et al. 2013). The third route for DAG formation is mediated by activities of phospholipase C and phospholipase D, the latter acting in conjunction with PAP (Bates et al. 2013) (Fig. 13).

In this context, it should be noted that a tentative survey of microalgal genomes by similarity search to *Arabidopsis* PDCT did not reveal a candidate microalgal PDCT, except for the putative PAP2-like hydrolase of the diatom *T. pseudonana*, showing about 30 % identity to *Arabidopsis* PDCT. Hence, the existence of this route in microalgae is uncertain. In addition, metabolism of PE and the betaine lipids may generate DAG with unsaturated acyl groups in microalgae as was suggested by radiolabelling in several aforementioned studies with microalgae (e.g. Bigogno et al. 2002a; Khozin-Goldberg et al. 2002b) (Fig. 13). Again, lipid metabolism in these organisms has not yet been sufficiently studied and many candidate enzymes' functions are awaiting experimental confirmation.

The microalgal enzymes that have been studied most extensively are those that are involved in fatty acid modifications, such as desaturases and elongases, and these will be covered in the next section. Much effort has been invested in cloning and characterizing the desaturases and elongases involved in LC-PUFA biosynthesis in microalgae, as well as in using microalgal genes in plant metabolic engineering to reconstitute LC-PUFA biosynthesis in oil-seed plants. The main practical focus of these investigations has been to provide a sustainable feedstock of LC-PUFA for human consumption as an alternative to fish oils, whose supply is decreasing and quality deteriorating. At the same time, such research has enabled an elucidation of the constraints and bottlenecks in LC-PUFA accumulation within TAG in heterologous organisms, that is required affective acyl-editing mechanisms for acyl-group exchange between phospholipids and the acyl-CoA pool (for further and more recent information refer to Haslam et al. 2013; Ruiz-López et al. 2013).

7 Fatty Acids Modifications and LC-PUFA Biosynthesis

Desaturases are non-heme monooxygenases featuring three conserved histidine-box motifs [HX_3-4H, $HX_{2-3}HH$, and $(H/Q)X_{2-3}HH$] with generally eight essential histidines, that are important to formation and co-ordinate function of the di-iron center essential for fatty acid desaturase (FAD) activity (Shanklin et al. 1994, 2009; Shanklin and Cahoon 1998). Double-bond formation by aerobic fatty acid desaturation involves an enzymatic reaction in which a double bond is introduced into an acyl chain and a molecule of oxygen is completely reduced to water, while four H atoms are abstracted. Desaturase activity requires molecular oxygen, reducing equivalents and electron donors; the chloroplast desaturases use a soluble flavoprotein ferredoxin as the electron donor, whereas cytochrome b5 is used as a primary electron donor in fatty acid desaturation in the ER. Some desaturases in the ER rely on free cytochrome b5, but cytochrome b5 may appear fused with the desaturase domain in a single protein at either the N- or C- terminus. Many excellent reviews and book chapters have been written on fatty acid desaturases and their role in modifying membrane unsaturation level in response to environmental cues, particularly low temperature (Heinz 1993; Murata and Wada 1995; Somerville and Browse 1996; Shanklin and Cahoon 1998; Napier et al. 1999; Hildebrand et al. 2005; Napier 2007; Shanklin et al. 2009).

The desaturation reactions in the plastid and in the ER are catalyzed by the membrane-bound desaturases; they generally prefer glycerolipid substrates to acyl-CoA and are therefore referred to as acyl lipid desaturases. Remarkably, Acyl-CoA-dependent desaturases that are common in non-plant eukaryotic organisms have been identified and characterized in microalgae.

Fatty acids that remain in the plastid (16:0 and 18:1) are incorporated into the chloroplast glycerolipids and undergo

sequential lipid-linked desaturation. All desaturation steps in the chloroplast but one, which is catalyzed by a soluble acyl-ACP desaturase (Sect. 4.3), occur when acyl groups are attached to glycerolipids. SAD introduces a double bond at the Δ9 position of 18:0-ACP; however, ACP-desaturases capable of placing a double bond in other positions, such as Δ4, Δ6 and Δ9 in saturated fatty acids of different chain lengths, have been identified in higher plants (reviewed by Hildebrand et al. 2005 and references therein) and possibly in microalgae, given the variability of monounsaturated fatty acids in these organisms.

The red microalga *Cyanidioschyzon merolae* lacks the plastidial Δ9 acyl-ACP desaturase and the entire plastidial desaturation pathway but possesses the ER-localized stearoyl-CoA desaturase with the C-terminal cytochrome b5 domain (Itoh et al. 1998; Sato and Moriyama 2007). Delta-9 (Δ9) desaturases acting on acyl-CoA substrates, such as the stearoyl Δ9 desaturase of *S. cerevisiae* commonly have the cytochrome b5 domain fused to the carboxyl terminus (Mitchell and Martin 1995). Four candidate isoforms of the Δ9 desaturase containing a C-terminal cytochrome b5 domain were recently identified in the oleaginous diatom *Fistulifera sp*. along with two candidate plastidial Δ9 acyl-ACP desaturases (Muto et al. 2013). An unusual desaturase, containing a cytochrome b5-like domain at its N terminus, was cloned from the marine diatom *T. pseudonana* (Tonon et al. 2004). The deduced amino acid sequence of TpDESN displayed typical features of the ER-localized desaturases involved in the biosynthesis of LC-PUFA, but this desaturase was specifically active on saturated 16:0, desaturating it to $16{:}1^{\Delta 11}$ when expressed in yeast, but was not active on supplemented PUFA.

Palmitic acid (16:0) and oleic acid (18:1) attached to the *sn*-2 and *sn*-1 positions of the glycerol backbone, respectively, are sequentially desaturated by plastidial desaturases, most commonly yielding trienoic acids, the *n*-3 hexadecatrienoic acid ($16{:}3^{\Delta 6,9,13}$) and LNA ($18{:}3^{\Delta 9,12,15}$), in plants and many species of green microalgae. All desaturases acting in the plastid were cloned and characterized in the higher plants (Somerville and Browse 1996; Heilmann et al. 2004; Hildebrand et al. 2005; Li-Beisson et al. 2010, 2013), including the one that appeared to be responsible for the production of $16{:}1^{\Delta 3t}$ in chloroplast PG (FAD4), as affirmed by Gao et al. (2009). Remarkably, this desaturase features only two histidine boxes and was shown to fall into a distinct class of FAD. We will further cover the most recent data on the characterization of microalgal desaturases, starting with plastidial desaturases.

MGDG of *C. reinhardtii* comprises the major molecular species $18{:}3^{\Delta 9,12,15}$/$16{:}4^{\Delta 4,7,10,13}$ (*sn*-1/*sn*-2) (Giroud et al. 1988; Riekhof et al. 2005b; Zäuner et al. 2012). The non-typical for higher plants fatty acid $16{:}4^{\Delta 4,7,10,13}$ is particularly enriched in this lipid in *Chlamydomonas*, whereas it is present in DGDG in only trace amounts. While many genes encoding various desaturases from *C. reinhardtii* have been cloned, the desaturase that introduces the Δ4 bond remained elusive. A candidate gene encoding the MGDG-specific Δ4-desaturase from *Chlamydomonas* (CrΔ4FAD) was identified based on the deduced protein's similarity to the previously characterized and ER-localized Δ4-desaturases from *Euglena gracilis* (Meyer et al. 2003) and *Thalassiosira pseudonana* (Tonon et al. 2005b). Remarkably, the deduced amino acid sequence contained both desaturase and N-terminal cytochrome b5 domains, as is typical for the ER-located desaturases involved in the LC-PUFA biosynthesis, along with a chloroplast-targeting signal. Chloroplast localization in the alga was confirmed by expressing a green fluorescent protein (GFP)-tagged *Cr*Δ4FAD and analyzing the transformants by confocal microscopy. The GFP signal was evident in the chloroplasts of *Chlamydomonas* cells, supporting the predicted plastidial localization. The same construct was overexpressed in the *C. reinhardtii* strain UVM-4 shown to effectively express foreign and homologous genes (Neupert et al. 2009), to elucidate the impact of overproduction of *Cr*Δ4FAD on fatty acid and acyl lipid composition. Interestingly, *Cr*Δ4FAD overexpression led to an increase in $16{:}4^{\Delta 4,7,10,13}$ out of total lipids, while the fatty acid composition of MGDG did not change, it appeared that the increase in total MGDG levels was responsible for those alterations. Reciprocally, when expression of *CrΔ4FAD* was reduced using an amiRNAi construct, the proportion of MGDG declined. The authors concluded that a compensatory system is operating in *Chlamydomonas* that is capable of adjusting the abundance of MGDG and its molecular species composition in response to decrease in desaturation (Zäuner et al. 2012). The functional role of the fused cytochrome b5 domain in the plastidial desaturase requires further investigation; nevertheless, the results on in vitro expression of the cytochrome b5 domain were consistent with the major role for a plastidial ferredoxin in providing electrons to *Cr*Δ4Cytb5.

A single putative ω-3 desaturase (*Cr*FAD7) is encoded by the *Chlamydomonas* genome as was reaffirmed in a recent study published by Nguyen et al. (2013). They isolated a mutant showing a significant decrease in the content of LNA ($18{:}3^{\Delta 9,12,15}$) as compared to the wild-type strain; the mutation was mapped to the insertion of a TOC1 transposon 113 bp upstream of the ATG start codon of a putative ω-3 desaturase (*Cr*FAD7). Localization studies with CrFAD7 by immunofluorescence in situ hybridization revealed its localization in the chloroplast. Furthermore, the presence of a single plastid-located ω-3 desaturase seems not to be restricted to *C. reinhardtii*. BlastP search identified a single homolog in some other sequenced green algae (*Chlorella variabilis* NC64A, *Coccomyxa subellipsoidea* C-169 and *Volvox carteri*). Green algae homologs differ in the length of the pre-

dicted N-terminal pre-sequence. Functional complementation of the crfad7 mutant with chloroplast biased version of *CrFAD7* restored the content of ω-3 fatty acids. Furthermore, a comparative lipidomic analysis of the crfad7 mutant and wild type revealed reductions in all ω-3 fatty acid-containing plastidial and extraplastidial glycerolipid molecular species, implying that a mutation in *CrFAD7* might have impacted glycerolipids in both compartments. The authors proposed a plausible explanation in which *Cr*FAD7 has access to both plastidial and ER-located acyl lipid substrates through the ER-plastid contact sites; alternatively, the trafficking of precursors might be considered (Nguyen et al. 2013).

The chloroplastic lipids of the diatom *P. tricornutum* contain an unusual isomer of *n*-6 hexadecatrienoic acid ($16:3^{\Delta6,9,12}$), rather than the *n*-3 hexadecatrienoic acid ($16:3^{\Delta7,10,13}$) and LNA ($18:3^{\Delta9,12,15}$) which, as noted above, are common in chloroplast membrane lipids of higher plants and green algae (Domergue et al. 2002). The gene encoding PtFAD6 was cloned and expressed in a strain of the cyanobacterium *Synechococcus* which is characterized by the absence of di- and trienoic fatty acids. The heterologous expression of *PtFAD6* in the photosynthetic cells resulted in the production of $16:2^{\Delta6,9}$, in support of the suggestion that it encodes a plastidial enzyme. The N terminus of PtFAD6 bore a bipartite sequence typical of the nuclear-encoded and chloroplast-targeted diatom proteins required for transport into the complex diatom plastids, surrounded by four membranes. The plastid localization of PtFAD6 was confirmed when the N-terminal 113 amino acids were fused with EGFP (enhanced green fluorescent protein) and expressed in *P. tricornutum* cells. In contrast, the microsomal (ER-localized) desaturase PtFAD2 was shown to be active when expressed in yeast and specific for oleic $18:1^{\Delta9}$ acid, indicating that PtFAD2 requires cytochrome b5/cytochrome b5 oxidoreductase and is likely involved in the biosynthesis of EPA in the ER.

Lipid-linked desaturation is considered the one of the major routes operating in the ER of microalgae, analogous to the C18 PUFA biosynthesis pathway in higher plants when acyl groups are sequentially desaturated being attached to PC; nevertheless, acyl-CoA dependent desaturases are known. Microalgae are primary producers of LC-PUFA, and many algal species therefore possess the machinery for sequential alternating desaturation/elongation steps. In the lipid-linked route, acyl groups are desaturated, being attached to phospholipids, such as PC and PE, or to the betaine lipids, such as DGTS, that is abundant in many classes of microalgae. Acyl-CoA dependent desaturation and elongation of C18 and C20 PUFA utilizes CoA-activated acyl moieties in the cytoplasmic acyl-CoA pool.

The biosynthesis of LC-PUFA is initiated by Δ12 desaturation of oleic acid (OA, $18:1^{\Delta9}$), generating LA ($18:2^{\Delta9,12}$), which in turn might be further desaturated by an ω-end-specific Δ15 desaturase giving rise to ALA ($18:3^{\Delta9,12,15}$) (Fig. 14). LA as well as ALA, are further processed stepwise via the so-called Δ6 desaturase/Δ6 elongase pathway, giving rise to ARA (20:4 *n*-6) and EPA (20:5*n*-3), respectively. This route begins with the Δ6 desaturase and includes alternating desaturation and elongation steps mediated by Δ6 PUFA elongase and Δ5 desaturase (Cohen et al. 1992; Abbadi et al. 2004; Meyer et al. 2004; Wallis et al. 2002). The respective pathways initiating with either LA or ALA (the so-called ω-6 and ω-3 pathways) comprise the *n*-6 or *n*-3 PUFA intermediates, respectively. The ω-3 pathway leads to the synthesis of EPA and DHA, whereas the ω-6 pathway generates ARA. The alternative Δ9 pathway initiates in turn with Δ9-specific elongation of LA or ALA to EDA ($20:2^{\Delta11,14}$) or eicosatrienoic acid (ETA, $20:3^{\Delta11,14,17}$), respectively, followed by sequential Δ8 and Δ5 desaturations. This alternative pathway, bypassing Δ6 desaturation, is found to occur in many microalgae, such as the haptophytes *I. galbana* (Qi et al. 2002) and *Pavlova salina* (=*Rebecca salina*) (Zhou et al. 2007; Robert et al. 2009), the coccolithophore *E. huxleyi* (Sayanova et al. 2011) and the euglenophyte *Euglena gracilis* (Wallis and Browse 1999).

Furthermore, an ω-3 desaturation can link two routes by conversion of *n*-6 to *n*-3 C18 PUFA and LC-PUFA. For example, ARA, which is produced via the *n*-6 pathway, is assumed to be converted to EPA by the ω-3 desaturase in the eustigmatophytes *Nannochloropsis* sp. (Schneider and Roessler 1994) and *Monodus subterraneus* (= *Monodopsis subterranea*) (Khozin-Goldberg et al. 2002b), and the red microalga *P. cruentum* (Khozin et al. 1997). In the freshwater trebouxiophyte *Lobosphaera incisa*, the major LC-PUFA ARA is produced via the *n*-6 pathway, as was elucidated by analysis of fatty acid composition of individual lipid classes (Bigogno et al. 2002b), radiolabeling studies (Bigogno et al. 2002a) and gene cloning (Iskandarov et al. 2009, 2010) whereas conversion of ARA to EPA by a putative ω-3 desaturase is scant. It seems likely that analogous to *Chlamydomonas* CrFAD7 (Nguyen et al. 2013) *L. incisa* has a single ω-3 desaturase with preference to C18 fatty acid. As shown in earlier works, both the ω-3 and ω-6 pathways are suggested to operate in the diatom *P. tricornutum*, and their intermediates may contribute to the biosynthesis of EPA (Arao et al. 1994; Domergue et al. 2002).

LC-PUFA biosynthesis in microalgae involves cooperation between different cellular compartments—the chloroplast and the ER and different extraplastidial lipids not limited to PC—as such the final step in EPA biosynthesis may be localized to different cellular compartments. In the ARA-producing *L. incisa*, it was suggested that three extraplastidial lipids are involved in various steps of ARA (20:4 *n*-6) biosynthesis: PC and DGTS are involved in the Δ 12

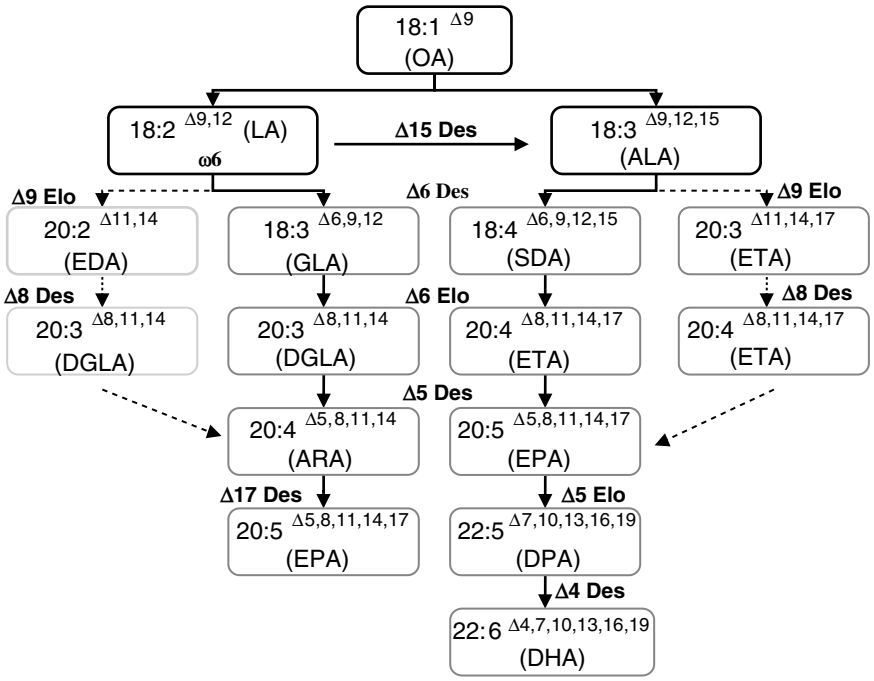

Fig. 14 Pathways for the biosynthesis of LC-PUFA in microalgae

and subsequently Δ6 desaturations, whereas primarily PE as well at some extent PC are the suggested as major substrates for the Δ 5 desaturation of 20:3 *n*-6 to 20:4 *n*-6 (Bigogno et al. 2002a), indicative to active metabolic interactions between different lipids involved in the biosynthesis of ARA in this alga. The *sn*-2 position of PC is involved in production of ARA from the C18 precursors while conversion of ARA to EPA that is mediated by a putative ω-3 desaturase (acting as the Δ17 desaturase) is suggested to occur in the *P. cruentum* chloroplast, involving chloroplast-lipid-linked desaturation (Khozin et al. 1997). The existence of the plastidial ω-3 desaturase was supported by the recently released genome data of *P. purpureum* (a taxonomic synonym of *P. cruentum*) where a gene sequence putatively encoding for the plastidial ω-3 desaturase most similar to the hypothetical one of the bryophyte *Physcomitrella patens* was identified (Bhattacharya et al. 2013). To date, the best functionally characterized ω-3 desaturases with preference to ARA are of the fungal origin (e.g. Pereira et al. 2004a), whereas such microalgal ω-3 desaturases have not been cloned or characterized; however, their occurrence has been further indicated at the genome level (e.g. Vieler et al. 2012b). LC-PUFA produced in the ER can be exported to the plastid (Khozin et al. 1997; Sukenik 1999), probably by the described above mechanisms analogous to trafficking of the ER-produced C_{18} PUFA in higher plant cells.

Production of the highly unsaturated C22 LC-PUFA such as DHA (22:6 *n*-3) from EPA requires two additional steps, including action of the Δ5-specific C_{20} PUFA elongase, catalyzing the C_{20}-specific 2-C chain elongation of EPA to docosapentaenoic acid (DPA, 22:5*n*-3) and Δ4 desaturase. The consecutive activity of Δ4 desaturase acting on DPA is necessary to accomplish DHA biosynthesis in microalgae. These metabolic steps have been shown to occur in the C_{22} LC-PUFA-producing microalgae (Meyer et al. 2004) and are different from mammals and fish, where biosynthesis of DHA from EPA involves the formation of polyunsaturated C_{24} intermediates by sequential elongations and desaturation, followed by chain shortening via β-oxidation of 24:6 *n*-3 to 22:6 *n*-3 in the peroxisomes (Sprecher 2000). In the latter pathway, DPA is further elongated to 24:5 *n*-3, followed by Δ6 desaturation to 24:6 *n*-3 and chain shortening via β-oxidation to DHA in peroxisomes. Several genes encoding for Δ4 desaturases were cloned from DHA-producing microalgal species (Meyer et al. 2003; Pereira et al. 2004b; Zhou et al. 2007). The PsΔ4Des of *Rebecca salina* was able to desaturate both $22{:}4^{\Delta7,10,13,16}$ and $22{:}5^{\Delta7,10,13,16,19}$ at the Δ4 position when expressed in the yeast *S. cerevisiae* (Pereira et al. 2004b). The Δ4 desaturase of *Euglena gracilis* showed strict Δ4-regioselectivity and required the presence of a Δ7-double bond in the substrate acyl group; the enzyme featured a broad substrate specificity and activity was not limited to C22 PUFA and also efficiently desaturated C16 PUFA such as $16{:}3\ 3^{\Delta7,10,13}$ (Meyer et al. 2003). Some those candidate genes were used in plant metabolic engineering to mediate a terminal activity to reconstitute DHA biosynthesis in higher plants.

The desaturase enzymes (Δ4, Δ5, Δ6, and Δ8 DES) engaged in the above described LC-PUFA biosynthesis pathways belong to the "front-end" desaturases which introduce a new double bond between the preexisting double bond and the carboxyl end of the fatty acid (Sayanova and Napier

2004; Napier 2007; Haslam et al. 2013). Various desaturases involved in LC-PUFA biosynthesis share similar structural features of front-end desaturases: an N-terminal cytochrome b5-fused domain, which serves as an electron donor for desaturase, and the presence of three conserved histidine-rich motifs. Functional expression and characterization of the putative cloned desaturase is essential for its proper designation (Sayanova et al. 2011), that is commonly performed by expression of the algal protein of interest in a heterologous eukaryotic system, such as the yeast *S. cerevisiae*. This yeast species has a very simple FA profile and lacks desaturases other than Δ9 desaturase; it can therefore be fed various FA substrates and the respective product analyzed for rate of substrate conversion. Numerous front-end desaturases have been cloned and characterized in this way from various LC-PUFA-producing algae mentioned in this section. Furthermore, numerous microalgal desaturases and elongases have been successfully utilized in studies on reconstruction of the LC-PUFA pathway in oil-seed plants (Qi et al. 2004; Napier and Graham 2010; Sayanova and Napier 2011; Ruiz-Lopez et al. 2013) and most recently in microalgae (Hamilton et al. 2014). Noteworthy, *C. reinhardtii* appeared to contain the unusual desaturase harboring the cytochrome b5 domain displaying, however, *n*-13 desaturase activity with preference for Δ9 unsaturated C18/C20 fatty acids (Kajikawa et al. 2006). This desaturase seems to be involved in the biosynthesis of the non-methylene-interrupted C18 PUFA $18{:}3^{\Delta5,9,12}$ and $18{:}4^{\Delta5,9,12,15}$ in *C. reinhardtii* almost exclusively found in DGTS of this alga.

Recently, it has been shown that some algal Δ6 and Δ5desaturases may act on CoA-activated PUFA, similar to mammalian front-end desaturases (Domergue et al. 2005; Hoffmann et al. 2008; Petrie et al. 2010b). This biochemical feature, allowing for more efficient substrate exchange between desaturases and elongases in the cytoplasmic acyl-CoA pool, offers great promise in plant biotechnology for the metabolic engineering of oilseed plants to produce LC-PUFA (for further information please refer to Ruiz-Lopez et al. 2013; Haslam et al. 2013), as well as in the metabolic engineering of microalgae for enhanced production of LC-PUFA. A significant increase in DHA content was observed in the diatom *P. tricornutum* through the expression of C20 Δ5-elongase in conjunction with the endogenous Δ4-desaturase, accompanied by a concurrent decrease in EPA (Hamilton et al. 2014). The results implied a sufficient shift of the LC-PUFA biosynthesis pathway toward the production of DHA, which is not accumulated at high levels in this diatom. Notably, some of the recently cloned microalgal acyl-CoA-dependent desaturases have been shown to be highly specific for the ω-3 substrates and thus provide a useful gene resource for the metabolic engineering of ω-3 LC-PUFA production in higher plants (Sayanova et al. 2011).

Fatty acid elongation is an essential step in the LC-PUFA biosynthesis and involves four sequential enzymatic reactions: condensation with malonyl-CoA (KCS, β-ketoacyl-CoA synthase), ketoreduction (β-ketoacyl-CoA reductase), dehydration (hydroxyacyl-CoA dehydratase) and enoyl reduction (enoyl-CoA reductase). Microalgal PUFA elongases are structurally similar to the ELO family of enzymes that catalyze the condensation step of fatty acid elongation in animals and fungi (Meyer et al. 2004; Paul et al. 2006). These elongases are different from the condensing enzymes that participate in microsomal elongation of saturated and monounsaturated fatty acids, noteworthy to mention that these elongases were also characterized in some microalgae (reviewed by Khozin-Goldberg et al. 2011). Three major types of PUFA elongases, regarding substrate specificity, were characterized from LC-PUFA-producing microalgae: Δ6 C18-PUFA-specific elongases were shown to be involved in the elongation of C18 PUFA (18:3 *n*-6 and 18:4 *n*-3) and the production of C20 LC-PUFA, ARA and EPA, while Δ5 C20-PUFA-specific elongases are engaged in the elongation of 20:5 *n*-3 (EPA) in the pathway of DHA biosynthesis, in some marine species. In an alternative route, Δ9 -specific elongation of 18:2 *n*-6 and 18:3 *n*-3 to the respective C20 intermediates precedes sequential Δ8 and Δ5 desaturations to form ARA and EPA, respectively. For functional characterization, these enzymes are expressed in yeast where the recombinant proteins are suggested to accomplish a condensing step in conjunction with host activities of ketoreduction, dehydration and enoyl reduction. For example, elongases cloned from the marine haptophytes of the *Pavlova* genus, being expressed in yeast, catalyzed the conversion of EPA into DPA (22:5 *n*-3) (Pereira et al. 2004b; Robert et al. 2009). This enzyme appeared to be specific towards both ω6 and ω3 C20-PUFA substrates and did not show activity towards C18- or C22-PUFA substrates.

It should be mentioned that in some primitive marine eukaryotes of the Thraustochytriaceae family, the alternative polyketide synthase pathway (PKS) catalyses the production of LC-PUFA (Metz et al. 2001). The PKS pathway does not require aerobic desaturation while the double bonds are introduced during the process of fatty acid synthesis. The PKS pathway is predominant in *Schizochytrium*, whereas a desaturation/elongation pathway acts in *Thraustochytrium* of the same family. The incomplete and possibly remnant desaturase-elongase pathway in Schizochytrium sp. lacks a crtical Δ12 desaturase and not capable of synthesizing PUFAs de novo (Lippmeier et al. 2009). The homology between the prokaryotic *Shewanell*a and eukaryotic *Schizochytrium PKS* genes suggested that the PUFA PKS pathway has undergone lateral gene transfer (Metz et al. 2001). The PKS-like encoding genes are

annotated in the microalgal genomes, future research perhaps will unveil their functions.

8 TAG Assembly and Metabolism

The mechanisms implicated in TAG biosynthesis and metabolism have been best studied in developing oil seeds, yeast and mammalian fat-storing and lipogenic cells. Recent intensive research has targeted the biochemical and molecular mechanisms underlying microalgal accumulation and mobilization of TAG reserves, as well as the regulatory mechanisms involved in the expression of lipid-biosynthesis genes and proteins. Recent studies on unicellular microalgae induced to accumulate TAG by nutritional stresses have led to some interesting discoveries and have shown the existence of distinct mechanisms in some species leading to increased TAG deposition in the cell under nutrient depletion (Liu and Benning 2013). For instance, biochemical mechanisms by which the cells of *C. reinhardtii* deposit TAG within chloroplastic LD and involvement of the chloroplast in supplying the fatty acids and DAG skeletons for TAG biosynthesis have attracted substantial attention (Goodson et al. 2011; Fan et al. 2011, 2013; Liu and Benning 2013). Apart from their importance to the field of microalgal lipid research and microalgal biotechnology for the production of biodiesel precursors and value-added lipids, novel insights into TAG production in unicellular photosynthetic organisms hold great promise in the prospect of enhancing TAG deposition in photosynthetic cells of higher-plant vegetative tissues (Sanjaya et al. 2013). So far, TAG-biosynthesis pathways have been annotated in numerous microalgae, for example, in *C. reinhardtii* (Riekhof et al. 2005b; Boyle et al. 2012), *N. oceanica*, *N. gaditiana* (Vieler et al. 2012b; Radakovits et al. 2011; Li et al. 2014), *Neochloris oleoabundans* (=*Ettlia oleoabundans*) (Rismani-Yazdi et al. 2012), *Phaeodactylum tricornutum* (Valenzuela et al. 2012; Guerra et al. 2013) and *Monoraphidium neglectum* (Bogen et al. 2013). The latter microalga has been recently identified as a promising candidate for biofuel applications, demonstrating robust growth characteristics combined with efficient neutral lipid accumulation. The green alga *C. reinhardtii* and the diatom *P. tricornutum* are currently among the leading microalgae in terms of number of cloned and functionally characterized genes coding for enzymes involved in TAG biosynthesis.

8.1 Biosynthesis of TAG

TAG biosynthesis is a complex process involving diverse enzymatic activities and intermediates produced in different cellular compartments; it shares many common metabolites and enzymatic steps with the pathways of phospholipid biosynthesis (Bates and Browse 2012; Bates et al. 2013; Chapman and Ohlrogge 2012; Merchant et al. 2012). Enzymes engaged in TAG assembly may play an important role in establishing its fatty acid composition; moreover, the reaction mediated by the terminal acyltransferases involved in TAG biosynthesis is regarded as the rate-limiting step (Weselake et al. 2008, 2009), and therefore the genes that encode them are considered potential candidates for metabolic engineering of TAG-production capacity in various organisms, including microalgae.

The canonical de novo pathway of TAG biosynthesis in the ER, known as the Kennedy pathway, proceeds via four sequential steps and is initiated by the action of GPAT on G-3-P and acyl-CoA. Three types of ER-resident acyltransferases, GPAT, LPAAT and acyl-CoA:diacylglycerol acyltransferase (DGAT), operating in the Kennedy pathway catalyze sequential *sn*-specific esterification of G-3-P (Fig. 15). Current opinion suggests that these enzymes function cooperatively with acyltransferases engaged in acyl editing and enzymes involved in phospholipid-DAG interconversion, and thus play a key role in determining TAG's acyl composition and final content (Chapman and Ohlrogge 2012; Bates and Browse 2012; Bates et al. 2013). Candidate genes which putatively encode acyltransferases of the Kennedy pathway have been identified by analysis of genomic and transcriptomic data from approximately ten microalgal species. A number of genes encoding acyltransferases from microalgae have been characterized by cloning and overexpression, as well as by functional expression in heterologous organisms (e.g. Wagner et al. 2010; Guihéneuf et al. 2011; Deng et al. 2012; Boyle et al. 2013; Gong et al. 2013).

GPAT, LPAAT, and PAP functions in the membrane glycerolipid biosynthesis are covered in the previous sections. The significance of the PA hydrolysis catalyzed by lipins in neural lipid metabolism and LD biogenesis in eukaryotic organisms (yeast, mammals and plants) is recently emerged and has been reviewed in the several recent publications (Csaki and Reune 2010; Mietkiewska et al. 2011; Bates et al. 2013; Pascual and Carman 2013) and should be emphasized in this section. As summarized by Pascual and Carman (2013) PAP activity influence numerous cellular physiological and biochemical functions including phospholipid synthesis gene expression, nuclear/ER membrane growth, LD formation, and vacuole homeostasis and fusion. Disruption of PAH1 in yeast impaired LD formation concomitant with an accumulation of neutral lipids in the ER (Adeyo et al. 2011). Mutation in the lipin-coding gene in mice appeared to be responsible for a lipodystrophy phenotype indicating that lipin-mediated activity is required for normal adipose tissue development (Péterfy et al. 2001), as opposed by the obesity-promoting effects of the enhanced lipin expression (Phan and Reue 2005). We already noted that PAP-encoding genes

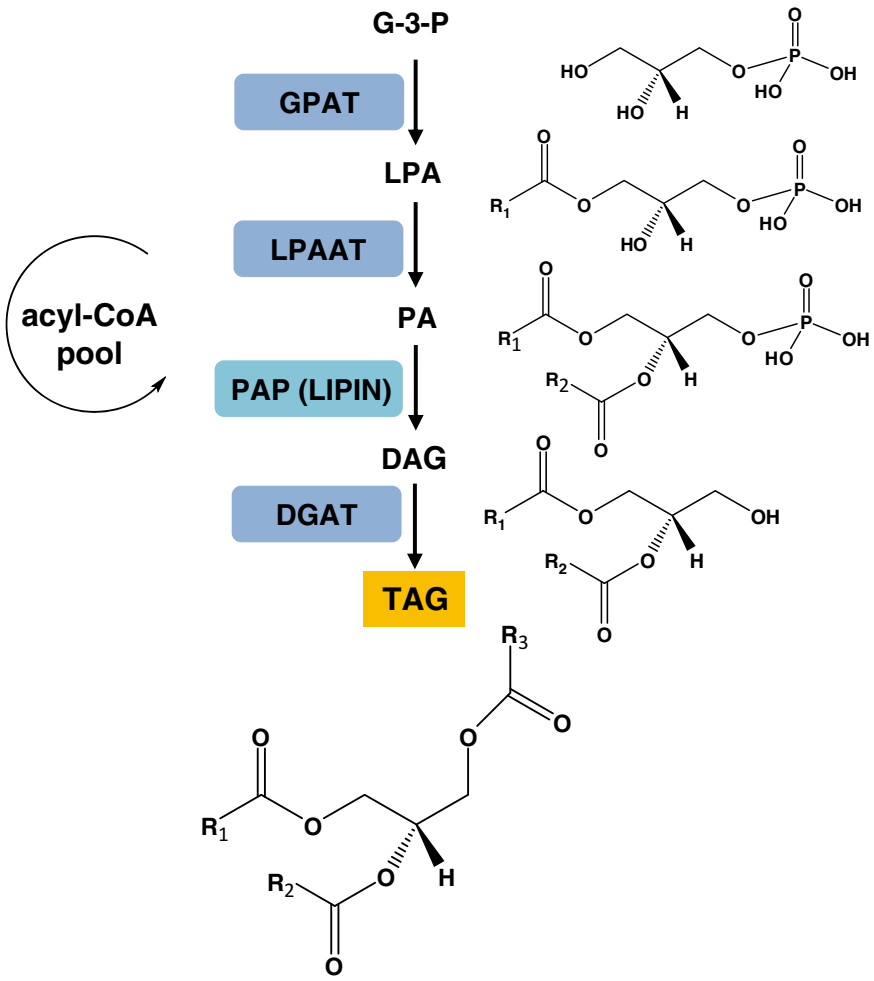

Fig. 15 The Kennedy pathway of triacylglycerol (*TAG*) biosynthesis

with the conserved in lipins N- and C-terminal domains were identified in microalgae which display differential expression under conditions triggering TAG accumulation. Given importance of lipins in storage lipid metabolism, lipins may represent potential targets for genetic manipulation of oil storage in microalgae but further research efforts are required to elucidate their function in microalgae.

DGAT (EC 2.3.1.20) catalyzes the committed step of TAG biosynthesis: the terminal esterification by acyl-CoA of the *sn*-3 position of *sn*-1,2-DAG. Two major types of unrelated DGAT proteins, DGAT1 and DGAT2, are known; they are dissimilar in primary structure and distinguished topologically. DGAT1 and DGAT2 are assumed to play distinct and non-redundant roles in TAG production in oil seeds (Lung and Weselake 2006; Snyder et al. 2009), and hence their properties have been investigated in many oil-seed plant with a focus on their different roles in those plants that have TAG with unusual fatty acid compositions (e.g. Li et al. 2010b, c; Pan et al. 2013).

DGAT1 (type 1) are members of the membrane-bound O-acyltransferase (MBOAT) family; the first member of this type was cloned from a mouse cDNA library based on sequence similarity shared with mammalian sterol:acyl-CoA acyltransferase (Cases et al. 1998). DGAT1 are integral membrane proteins typically predicted to have up to ten transmembrane domains (TMD); however, experimental verification has only revealed three TMD in the murine DGAT1 protein with the N terminus oriented toward the cytosol (McFie et al. 2010). Furthermore, the N-terminal domain of DGAT1 is assumed to not be required for catalytic activity but rather it involves in dimer/tetramer formation (McFie et al. 2010). The mammalian DGAT1 is a multifunctional acyltransferase which is also capable of synthesizing DAG, retinyl, and wax esters (McFie et al. 2010). A previously uncharacterized DGAT of the MBOAT family with *sn*-3 acetyltransferase activity was identified with the help of deep transcriptional profiling of the developing seeds of *Euonymus alatus* which produce unusually high levels of 3-acetyl-1,2-diacylglycerols (Durrett et al. 2010).

The first member of the DGAT2 (type 2) protein family was isolated from the oleaginous fungus *Umbelopsis ramanniana* (formerly *Morteriella*) (Lardizabal et al. 2001) and later, the genes encoding DGAT2 were cloned and characterized from numerous organisms, including microalgae (Cases

et al. 2001; Oelkers et al. 2002; Shockey et al. 2006; Burgal et al. 2008; Stone et al. 2009; Li et al. 2010b, c; Wagner et al. 2010; Zhang et al. 2012a; Gong et al. 2013; Sanjaya et al. 2013; Zhou et al. 2013). DGAT2 are generally predicted to have two or three TMD and are similar in their protein sequence to the members of the monoacylgycerol acyltransferase (MGAT) family. MGAT are well characterized in mammals where these enzymes play an important role in the absorption of dietary fat in the small intestine by catalyzing the re-synthesis of TAG from MAG and acyl-CoA (Cao et al. 2004; Yen et al. 2002; Yen and Farese 2003). The lack of similarity between type 1 and type 2 DGAT proteins led to the proposition that these proteins represent a case of functional convergent evolution (Shockey et al. 2006). Furthermore, DGAT1 and DGAT2 were shown to have different fatty acid specificities and were localized to non-overlapping subdomains in the ER (Shockey et al. 2006). Most organisms, albeit with some exceptions, have both types of DGAT (Chen and Smith 2012). A DGAT1 homolog is missing in the genome of the yeast *Saccharomyces cerevisiae*, while DGAT1 with experimentally confirmed function in TAG production was identified in the genome of the oleaginous yeast *Yarrowia lipolytica* (Zhang et al. 2012a). Multifunctional DGAT2-related acyltransferases esterifying a wide range of acyl acceptors with acyl-CoA were recently identified in the protozoan ciliate *Tetrahymena thermophila* (Biester et al. 2012).

A bifunctional DGAT capable of mediating both wax synthase and DGAT function (WS-DGAT) was isolated and characterized in the gram-negative bacterium *Acinetobacter calcoaceticus* (Kalscheuer and Steinbüchel 2003). The prokaryotic DGAT are reviewed in Wältermann et al. (2007), and a few bifunctional WS-DGAT have been characterized in higher plants; however, these are likely involved in surface-lipid biosynthesis rather than storage-lipid biosynthesis (King et al. 2007). A deduced protein featuring WS and DGAT domains was identified in the diatom *P. tricornutum* (Cui et al. 2013) showing the highest similarity to a putative WS-DGAT of the alveolate *Perkinsus marinus*, a member of a recently established phylum that is close to the ancestors of ciliates, dinoflagellates, and apicomplexans (Joseph et al. 2010). Further research is obviously needed to elucidate the putative WS-DGAT's function in diatom cells.

A novel class of soluble DGAT3 (type 3) has been relatively recently discovered (Saha et al. 2006) and its function in plants is currently being investigated. A soluble *Arabidopsis* DGAT3 homolog appeared to contribute to TAG biosynthesis in *Arabidopsis* seed oil-catabolism mutants. The soluble DGAT3 appeared to function in recycling 18:2 and 18:3 fatty acids into TAG when seed oil breakdown is blocked (Hernández et al. 2012). Topology, primary structure and functional properties of various DGAT types from various organisms are detailed in several comprehensive reviews (Yen et al. 2008; Liu et al. 2012a)

The acyl-CoA-independent pathway may also contribute significantly to the final step in TAG production. The activities of phospholipid:diacylglycerol acyltransferase (PDAT) (EC 2.3.1.158) (Dahlqvist et al. 2000; Banaś et al. 2000) and the putative DAG:DAG transacylase (DGTA) (Stobart et al. 1997) utilize the central lipid intermediate *sn*-1,2-DAG as an acceptor of the acyl group transferred directly from the *sn*-2 position of a phospholipid or a second DAG molecule to produce TAG, releasing lyso-PC or monoacylglycerol (MAG), respectively. PDAT enzyme is distantly related to the mammalian enzyme lecithin:cholesterol acyltransferase and is encoded by the *LRO1* gene in yeast (Oelkers et al. 2000). PDAT1 of *S. cerevisiae* contributes to TAG accumulation mainly in the exponential growth phase (Oelkers et al. 2000), whereas PDAT of the oleaginous yeast *Y. lipolytica* is responsible for 30 % of total lipid production, as was demonstrated by analysis of a *PDAT* gene-disruption mutant (Zhang et al. 2012a). Multiple *PDAT* genes are commonly found in plants (Ståhl et al. 2004; van Erp et al. 2011; Pan et al. 2013). Two candidates PDAT were identified in the genome of *N. oceanica* (Vieler et al. 2012b). Transcriptome de novo sequencing of *Dunaliella tertiolecta* identified of a transcript putatively encoding PDAT, while transcriptome analysis did not enable identification of PDAT *in Ettlia* (*Neochloris*) *oleoabundans* (Rismani-Yazdi et al. 2011, 2012).

8.2 Identification and Characterization of Genes Encoding TAG-Metabolism-Related Enzymatic Steps in Microalgae

Recent sequencing of algal genomes has paved the way to the identification and functional characterization of acyltransferases involved in the production of TAG and specifically of TAG with specialized acyl composition. A genome-wide survey for DGAT-like genes in the representative sequenced and publically available algal genomes was recently performed by Chen and Smith (2012). The searched genomes included those of six green algae (*C. reinhardtii*, *Coccomyxa* C-169, *Volvox carteri*, *Micromonas pusilla*, *Ostreococcus lucimarinus* and *O. tauri*), the red alga *C. merolae*, three diatoms (*P. tricornutum*, *T. pseudonana* and *Fragilariopsis cylindrus*), the recently sequenced haptophyte *Emiliania huxleyi*, and the filamentous multicellular brown alga *Ectocarpus siliculosus*. In general, only one candidate gene for DGAT1 was present in many of the algal classes, whereas DGAT2 were generally present in multiple isoforms (Chen and Smith 2012). Such as, five putative DGAT2 were annotated in the genome of *C. reinhardtii* (Moellering and Benning 2010). Remarkably, *DGAT1* was not annotated in the first version of the *C. reinhardtii* genome, but its full

length has been only recently assembled based on an improved genome version (Boyle et al. 2012). Expression patterns of *CrDGAT1* were studied under conditions of nutrient depletion, and the functionality of the encoded protein was confirmed in a yeast expression system. Expression of *CrDGAT1*, but of only some *DGAT2* isoforms (e.g. DGTT1), was consistently upregulated, accumulating their mRNA under conditions of nitrogen deprivation (Boyle et al. 2012; Schmollinger et al. 2014) and stressing the importance of the terminal step in TAG molecule assembly. A partial gene sequence encoding a hypothetical protein was assembled into the functional DGAT1 protein with the help of the available EST database in the diatom *P. tricornutum* (Guihéneuf et al. 2011); *PtDGAT1* was shown to be expressed at higher levels under nitrogen-starvation conditions and an intron-retention mechanism seems to regulate the amount of transcript encoding the active DGAT1 under these conditions. *DGAT1* genes are identifiable in the genome of the green algal classes Volvocales and Trebouxiophyceae, whereas the genomes of the picoplanktonic microalgae *M pusilla*, *O. lucimarinus* and *O. tauri* (Prasinophyceae) did not reveal any DGAT1 candidate. The absence of DGAT1 in *O. tauri* had been previously noted by Wagner et al. (2010). Remarkably, the prasinophytes also displayed peculiar features as compared to other representatives of the green algal lineage, lacking a heteromeric ACCase form in the plastid (Sect. 4.2). One candidate gene for DGAT1 was found in the genome of the eustigmatophyte *Nannochloropsis oceanica* CCMP1779 with the deduced protein featuring an MBOAT domain typical of DGAT1s; however, there is no EST support for this gene model. On the other hand, 11 putative DGAT2 were identified that were supported by EST (Vieler et al. 2012b). More recent studies have identified two DGAT1 isoforms in the genome of *Nannochloropsis* IMET 1 and confirmed the presence of 11 DGAT2-encoding genes (Li et al. 2014). A temporal transcriptomics analysis revealed differential expression of numerous DGAT genes during TAG accumulation and illuminated the potent role for cellular compartmentalization in accommodating certain reactions of the TAG assembly (Li et al. 2014). The individual DGAT-encoding genes demonstrated distinct gene-expression patterns upon transfer to nitrogen starvation; out of 12 DGAT genes, 7 were upregulated and 6 were downregulated under nitrogen-starvation conditions. A chloroplast affiliation was predicted by bioinformatics means for at least one DGAT1 and DGAT2 isoform along with one GPAT, two LPAAT and one PAP acting upstream of DGAT. Based on these data and evidence obtained from the lipidomics analysis on the abundance of TAG molecular species with C16 fatty acids bound to the *sn*-2 position (amounting to more than 80 % of total TAG), the authors proposed that the chloroplast-localized pathway significantly contributes to TAG production in *Nannochloropsis* under nitrogen starvation. An abundance of DGAT2-encoding candidates in *Nannochloropsis* showing similarity to MGAT might suggest that the MGAT pathway of TAG biosynthesis functions in *Nannochloropsis* (Vieler et al. 2012b). There are indications that the MGAT-mediated pathway contributes to TAG formation in plants (Tumaney et al. 2001), and perhaps in algae as well. This pathway seems to be amenable to reconstruction in plants by genetic engineering (Petrie et al. 2012c) and thus hold promises in engineering of lipid biosynthesis routes in microalgae.

Two putative PDAT-encoding genes were tentatively identified in *N. oceanica* CCMP1779 (Vieler et al. 2012b). The candidate genes were putatively assigned as *PDAT* in assembled microalgal genomes by similarity to plant and yeast PDAT; as will be described further on, a novel, recently discovered microalgal PDAT-like gene cloned from *C. reinhardtii* demonstrates multiple activities in vitro (Yoon et al. 2012). Obviously, the candidate genes should be cloned and the functionality of the putative proteins confirmed before proposing their role in overall TAG production or their contribution of a particular activity to the acyl composition of the produced TAG.

Functional complementation assay using a neutral lipid-deficient mutant of *S. cerevisiae* is a widely used tool in in vivo experiments designed to characterize the functionality of putative DGAT and PDAT; it is also seems instrumental in examining substrate preferences of candidate acyltransferases. The quadruple *S. cerevisiae* mutant (strain H1246 carries four disrupted genes: *DGA1*, *LRO1*, *ARE1*, and*ARE2*, encoding DGAT2, PDAT1, and duplicate acyl-CoA:sterol acyltransferases ASAT1 and ASAT2, respectively; this strain is unable to produce storage lipids—TAG and steryl esters—in the stationary phase (Sandager et al. 2002). Sterol acyltransferases possess some DGAT activity, and they must also be disrupted to investigate the significance of neutral lipid formation. The strain appeared viable under standard nutrient-replete growth conditions, indicating that neutral lipids are not essential for *S. cerevisiae* under those conditions; however, the mutant yeast was unable to grow in the presence of exogenous fatty acids, due to their lipotoxicity, caused by the yeast's inability to sequester acyl groups in the form of storage lipids within LD (Siloto et al. 2009). It should be noted that not every foreign acyltransferase was successfully expressed in the neutral lipid-deficient mutant of *S. cerevisiae*; for example, *Arabidopsis* DGAT1 was able to restore TAG synthesis, whereas DGAT2, PDAT2, and PDAT1 failed to complement TAG synthesis (Zhang et al. 2009). Therefore, a clue as to the overlapping essential roles of PDAT and DGAT1 in *Arabidopsis* was derived from RNAi silencing of *PDAT1* in a *dgat1-1* background and of *DGAT1* in a *pdat1-1* background: dramatic decreases in seed oil content, and disruptions in embryo development were thus obtained (Zhang et al. 2009). Remarkably, functional expression of

Arabidopsis DGAT2 was recently achieved in a different expression system, by transient overexpression in the *Nicotiana benthamiana* leaf (Zhou et al. 2013).

Many DGAT1 have been cloned from different oil-seed plants, and their mutants and knockdowns have been used to study the roles of these enzymes in the production of TAG in oil seeds. For example, mutagenesis or downregulation of DGAT1 expression was shown to significantly decrease TAG accumulation in the seeds (Routaboul et al. 1999; Zou et al. 1999; Zhang et al. 2009), whereas seed-specific overexpression resulted in increased oil content in the seed (e.g. Jako et al. 2001). A recent study on the functional characterization of DGAT1 from flax (*Linum usitatissimum* L.) accumulating about 70 % of $18{:}3^{\Delta 9,12,15}$ (ALA) in its seed oil reinforced a role for DGAT1 in the nonselective transfer of acyl moieties to TAG during the final stages of seed development (Pan et al. 2013). While the acyl substrate-selective PDAT displayed a preference for the major fatty acyl component of TAG (Pan et al. 2013). *PDAT* and *DGAT2* transcripts seemed to occur at much higher levels in the developing seeds of plants accumulating unusual fatty acids in their oils compared to *Arabidopsis* and soybean (Li et al. 2010b). Furthermore, expression of both DGAT1 and DGAT2 from *Vernonia galamensis* resulted in epoxy fatty acid accumulation in soybean seeds, which are typically rich in C18 PUFA. Expression of DGAT2 of castor bean (*R. communis*) along with fatty acid hydroxylase in *Arabidopsis* gave significant yields of hydroxy fatty acid content in the seed oil and production of ricinoleic acid-containing TAG molecular species (Burgal et al. 2008). Expression of castor bean PDAT1A and PDAT1-2 in *Arabidopsis* was reported to favor the production of ricinoleic acid-containing TAG (van Erp et al. 2011; Kim et al. 2011).

Enzymatic activities responsible for channeling LC-PUFA into TAG in those microalgal species that are capable of accumulating LC-PUFA in their storage lipids are also of special interest for pathway-engineering approaches and a general understanding of the mechanisms responsible for channeling LC-PUFA into TAG in some species (Bigogno et al. 2002a, b; Tonon et al. 2002a; Wagner et al. 2010; Guihéneuf et al. 2011). Metabolic engineering approaches will be applicable to various microalgae when adequate genetic tools, such as stress-inducible promotors, become available for more species. The microalga *Ostreococcus tauri* produces TAG rich in the *n*-3 LC-PUFA DHA, which may account for more than 25 mol% of the TAG and is significantly less abundant within phospholipids and glycolipids (less than 5 mol%). This indicates the existence of mechanisms directing DHA to TAG (Wagner et al. 2010). Three putative DGAT candidates were identified in the genome of *O. tauri*, all of which appeared to belong to the DGAT2 family, while no homologs of type 1 or type 3 DGAT were found (Wagner et al. 2010). Only *DGAT2B* seemed to encode the functional protein as shown by expression of the codon-optimized form in the neutral lipid-deficient mutant strain of *S. cerevisiae*, restoring LD and TAG production. TAG formation was also observed in in vitro enzymatic DGAT assays with homogenates of transformed mutant yeast cells. Similarly, among four DGAT2-like isoforms identified in the genome of *P. tricornutum*, showing a different number of predicted TMD and limited sequence similarity, only the isoform PtDGAT2B (GenBank acc. no. JQ837823) showed efficient functional complementation of the neutral lipid-deficient phenotype of the *S. cerevisiae* mutant (Gong et al. 2013). By feeding the recombinant yeast with various fatty acids, it was shown that OtDGAT2B accepts a wide array of acyl substrates, ranging from saturated fatty acids to PUFA and LC-PUFA, indiscriminately incorporating both *n*-3 and *n*-6 substrates into TAG. Given the substrate promiscuity of OtDGAT2B, further elucidation of the activity responsible for the incorporation of DHA into TAG is of importance. Studies on PDAT may provide further insight into the potential role of an acyl-CoA-independent pathway for LC-PUFA-rich TAG in this alga.

In fact, broad substrate specificity was demonstrated by DGAT of both major types cloned from microalgae and expressed in the mutant *S. cerevisiae* strain H1246. For example, DGAT2 of *C. reinhardtii* did not show any preferential incorporation of exogenous oleic acid or EPA (Zhang et al. 2013). Similarly, the recombinant DGAT1 of the diatom *P. tricornutum* (PtDGAT1) was shown to incorporate various C18 PUFA and C20 LC-PUFA into TAG (Guihéneuf et al. 2011). Interestingly, PtDGAT1 favored endogenous saturated fatty acids, as manifested by increased proportions of 16:0 and 18:0 over monounsaturated fatty acids in TAG of the PtDGAT1-complemented yeast. Recent studies in our laboratory examined acyl preference of the cloned DGAT1 of the ARA-producing *Lobosphaera incisa* also demonstrated a promiscuous nature of the enzyme being expressed in *S. cerevisiae* (S. Sitnik, I. Khozin-Goldberg, personal communication). Earlier research in our laboratory has shown that both acyl-CoA-dependent and independent pathways seemed to be involved in TAG formation in *L. incisa* (Shrestra et al. 2004). Microsomes and isolated oil bodies of *L. incisa* were able to mediate TAG formation when 1,2-[^{14}C] oleoyl-DAG or 1-stearoyl-2-[^{14}C]arachidonoyl-DAG were supplied as the sole acyl donors, indicating a possible transacylase activity. DGAT activity with lower specific activity was also observed in both cellular fractions.

As already noted, DGAT enzymes represent potential targets for altering TAG-accumulating capacity by molecular engineering because overexpression of DGAT may provide a larger sink for allocation of the photosynthates to storage oil. Overexpression of DGAT of both major types was effective at increasing TAG content not only in oil seeds, but also in green vegetative leaf tissues (e.g. Bouvier-Navé et al. 2000;

Lardizabal et al. 2008; Andrianov et al. 2010; Oakes et al. 2011; Zhou et al. 2013). Thus this group of enzymes has captured the interest of researchers engaged in metabolic engineering of photosynthetic microalgae for increased TAG content. Furthermore, deep transcriptional profiling data generated under nitrogen and iron deprivation further revealed transcriptional upregulation of three acyltransferases—DGAT1, DGTT2 (DGAT2) and PDAT involved in the final steps of TAG assembly as well as polar lipid remodeling for TAG formation in *Chlamydomonas* (Miller et al. 2010; Boyle et al. 2012; Urzica et al. 2013).

Several DGAT2 candidate genes were overexpressed or downregulated in *C. reinhardtii* in an attempt to decipher their role in, and manipulate, TAG production (Deng et al. 2012; La Russa et al. 2012). Candidate *DGAT2* were selected for overexpression in a study by La Russa et al. (2012) based on their expression pattern under nitrogen depletion (Miller et al. 2010). *C. reinhardtii* DGAT2 transformants, overexpressing either of the three selected full-length genes driven by the powerful photosynthetic *PsaD* promoter, indeed displayed increased mRNA levels of the corresponding genes, especially of *CrDGAT2a* (XP 001702848); however, no significant impact was observed on growth parameters on TAP+N or TAP-N media, and there were no significant alterations in either the fatty acid profile of TAG or TAG production under nutrient-replete conditions or under nitrogen or sulfur deprivation (La Russa et al. 2012). Nevertheless, the authors could not exclude the possibility that post-transcriptional or post-translational inhibition mechanisms hinder production of the functional DGAT protein (La Russa et al. 2012). Another study reported slight effects of amiRNAi silencing of two *DGAT2* genes on lipid content in *C. reinhardtii* grown under photoautotrophic conditions (Deng et al. 2012), this technique however, does not allow obtaining null (knockout) mutants, and, perhaps, more investigations are thus required to decipher the role of multiple DGAT2 and DGAT1 in *Chlamydomonas*.

A detailed functional analysis of *DGTT* genes (encoding DGAT2 isoforms) of *Chlamydomonas* in the heterologous system was performed by expressing them in yeast as well as *Arabidopsis*. The coding sequences of *DGTT2–5* were isolated and expressed in *S. cerevisiae* strain H1266, a triple-knockout mutant with low background DGAT activity (Sanjaya et al. 2013). Among the cloned and examined genes, only *DGTT2* and *DGTT3* expression in yeast resulted in significant accumulation of TAG as determined by ESI-MS (Hung et al. 2013; Sanjaya et al. 2013). Microsomal activity assays with radiolabeled acyl-CoA and dioleoyl DAG demonstrated that DGTT2 and DGTT3 of *Chlamydomonas* prefer 16:0-CoA; moreover, DGTT2 showed activity toward long-chain monounsaturated 22:1-CoA in competition assays (Sanjaya et al. 2013). *DGTT2* was expressed in *Arabidopsis* under the control of a constitutive promoter to examine its impact on the acyl-CoA pool and TAG production in the vegetative tissues. Two transformant lines with increased levels of *DGTT2* transcript contained TAG at about 1.0 % of dry weight in leaves, compared with 0.04 % in the wild type. Remarkably, oil droplets were observed in the leaf cells of one of the transformant lines localized in the proximity to chloroplasts. Furthermore, the molecular species containing long-chain fatty acids (C20 and longer) were detected in the TAG. Overall, expression of the microalgal DGAT2 enzyme had multiple effects on *Arabidopsis* leaf lipid metabolism as manifested by increased TAG formation in the leaves, redirection of very-long-chain fatty acids from sphingolipids into TAG, and increased caloric value of the vegetative tissues. This interesting research paves the way for future exploitation of acyltransferases from single-cell photosynthetic microalgae for the engineering of TAG biosynthesis in higher plants as well as microalgae. The challenge of selecting an inducible promoter to drive sufficient expression of the recombinant target gene was circumvented in a recent report on the successful overexpression of DGAT2 (DGTT4) in *C. reinhardtii* under phosphate starvation by choosing the promoter of a sulphoquinovosyldiacylglycerol 2 (*SQD2*) gene, induced under phosphate shortage (Iwai et al. 2014).

Further insights into the mechanisms underlying TAG formation under conditions of nitrogen starvation in *C. reinhardtii* were obtained by exploring mutants impaired in starch biosynthesis generated in earlier studies (Li et al. 2010c; Work et al. 2010; Goodson et al. 2011) or recently identified mutants with altered TAG contents (Li et al. 2012a, b). Microscopic examination of the cell-wall less strain *C. reinhardtii* CC-4349 cw15 and its starch-less mutant *cw15 sta6* strain CC-4348 revealed that *Chlamydomonas* may accumulate two types of LD in response to nitrogen starvation (Goodson et al. 2011). A mutant strain bafJ5 that carries a deletion of the *STA6* gene which encodes the small subunit of ADP-glucose pyrophosphorylase essential for starch biosynthesis (Zabawinski et al. 2001) was unable to accumulate starch but produced higher TAG contents under stressful conditions as expressed on a dry weight basis, namely a tenfold increase in TAG production from 2 to 20.5 % of dry weight as compared to the wild type (Li et al. 2010c). Another study indicated the importance of examining TAG content per cell when comparing the starch-less mutants to their progenitors (Siaut et al. 2011).

Nitrogen starvation caused massive formation of cytoplasmic LD in the cells of the parent strain, whereas the mutant starch-less strain cells also produced chloroplastic LD that were amply accumulated in the stroma (Goodson et al. 2011). Close association of cytoplasmic LD with both the ER membrane and the outer membrane of the chloroplast envelope was revealed by deep-etch electron microscopy, suggesting that both compartments participate in LD biogen-

esis (Goodson et al. 2011). The capacity to divert carbon flux from starch to storage TAG formation was related to the ability to accumulate LD in the chloroplast (Goodson et al. 2011). However, this ability did not completely compensate for the lack of starch; the starch-less mutants demonstrated accelerated loss of chlorophyll and more severely attenuated O_2 evolution affecting cell viability under nitrogen starvation (Work et al. 2010). In the wild-type cells of *Chlamydomonas*, TAG accumulation under nitrogen starvation lagged behind that of starch, and rapid oil synthesis occurred only when the carbon supply exceeded the capacity for starch synthesis whereas in the mutant acetate is directly utilized for TAG biosynthesis (Fan et al. 2012). Examining the effect of cerulenin indicated the importance of de novo fatty acid synthesis for TAG production under nitrogen starvation; however, TAG formation was only partially inhibited (Fan et al. 2011, 2012). The inhibition was reversed by adding oleic acid, indicating that acyl-CoA groups can be readily incorporated directly into TAG. Notably, *C. reinhardtii* induced autophagy-related events under nitrogen starvation (Wang et al. 2009 and references therein) that were triggered by the acetate boost with a dependence on initial inoculum conditions, growth stage and cell concentration (Work et al. 2010; Goodson et al. 2011).

Chlamydomonas reinhardtii lacks PC the major player in the eukaryotic pathway of glycerolipid synthesis, leading to the hypothesis that chloroplast glycerolipids participate in the biogenesis of LD (Fan et al. 2011). Positional analysis of acyl group distribution in the TAG glycerol backbone demonstrated preferential localization of the chloroplast-derived C16 PUFA at the *sn*-2 position, indicating that the DAG moiety for TAG assembly in *C. reinhardtii* is likely derived from the chloroplast lipids. The current model of TAG biosynthesis in *C. reinhardtii* under nitrogen starvation suggests TAG formation at least via two pathways: a large fraction of TAG is assembled near the site of fatty acid synthesis in the envelope membrane of the chloroplast, and the other part is assembled by the conventional ER-localized Kennedy pathway (Liu and Benning 2013) (Fig. 16). It was postulated that consistent with the absence of PC, which has been shown to be engaged in the export of fatty acids via an acyl-editing mechanism, TAG assembly in the chloroplast envelope membrane may substantially contribute to the efficiency of fatty acid export (Liu and Benning 2013).

Further and novel insights into TAG biosynthesis in *C. reinhardtii* were recently gained by the discovery of a novel galactolipid-linked pathway contributing de novo-synthesized fatty acids to TAG formation. A mutant displaying the chlorotic phenotype and a twofold decrease in TAG content under nitrogen deprivation was identified by screening mutants generated by random insertional gene disruption of the cell-wall-less *C. reinhardtii* strain dw15-1 (Li et al. 2012b). In contrast to the parent strain in which pigment content declines gradually, the mutant cells showed accelerated loss of photosynthetic pigments upon transfer to nitrogen-depleted medium and were completely bleached after prolonged nitrogen starvation. A mutation responsible for the phenotype was identified in the gene referred as to *plastid galactoglycerolipid degradation* 1 (*pgd1*); the deduced PGD1 protein appeared to feature a conserved catalytic triad of Ser-Asp-His, a typical motif identified in lipases (acyl hydrolases). Fatty acid composition of extraplastidial lipids

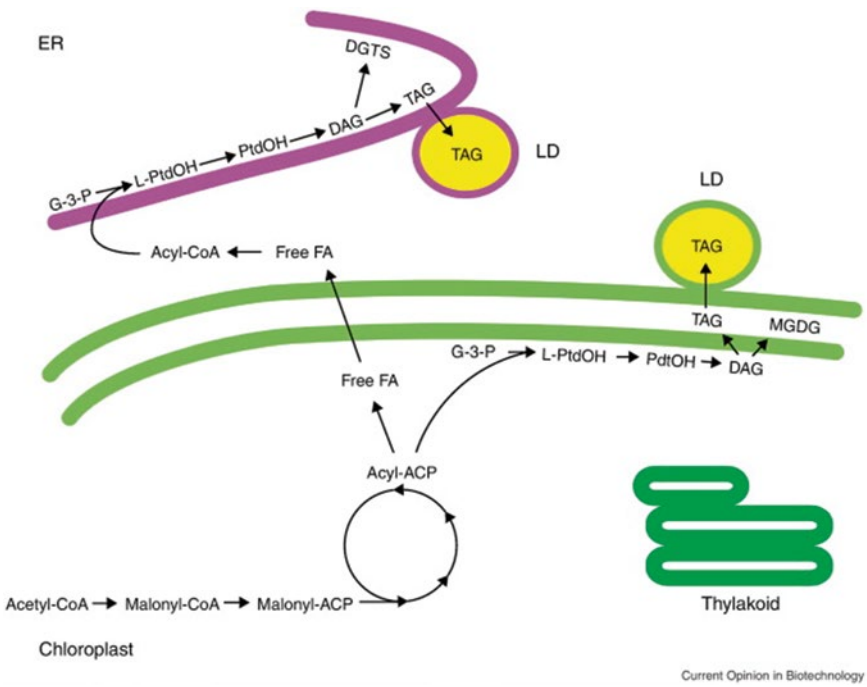

Fig. 16 Hypothetical routes for TAG biosynthesis in *Chlamydomonas reinhardtii* (Reproduced from Liu and Benning (2013); courtesy of Elsevier)

was altered in the *pgd1* mutant, with the extraplastidial lipids DGTS, PE and TAG displaying the reduced 18:1 proportions. When expressed in *E. coli*, the recombinant PGD1 displayed low activity toward the predominant 18:3/16:4 (*sn*-1/*sn*-2) MGDG species of *C. reinhardtii*, but favored the less unsaturated MGDG species produced by *E. coli* via the activity of a recombinant plant MGDG synthase. Furthermore, it appeared that PGD1 does not act on DGDG in vitro and prefers acyl groups at the *sn*-1 position over the *sn*-2 position of MGDG. A comparison of the patterns of galactolipid and TAG labeling with [^{14}C]acetate in the parent strain and in the mutant re-affirmed that PGD1 mediated the release of acyl groups from the de novo-produced 18:1$^{\Delta 9}$/16:0 MGDG for TAG formation under nitrogen starvation. Remarkably, acetate-labeling data and lipid analysis did not provide evidence for a substantial role of fatty acid flux through DGTS in TAG formation, at least via the route affected by the *pgd1* mutation (Li et al. 2012b).

Based on these data, a novel mechanism responsible for the release of the de novo-produced fatty acids from MGDG via PGD1 was postulated. According to this model, turnover of de novo-synthesized MGDG releases 18:1 to the cytosolic acyl-CoA pool, which contributes acyl groups for acylation of the *sn*-3 position in TAG. Turnover of mature MGDG and other thylakoid lipids generates another dominant DAG pool for TAG assembly at the plastid envelopes under conditions of nitrogen starvation in *Chlamydomonas*. Indeed, genes encoding numerous lipases and lipase-like enzymes are induced upon transfer of *C. reinhardtii* to nitrogen starvation (Miller et al. 2010; Boyle et al. 2012). Polar lipid modification and turnover at the ER comprise another source of acyl-CoA for DGAT-mediated TAG assembly.

Yoon et al. (2012) hypothesized that an acyl-CoA-independent enzymatic route can contribute to TAG formation via utilization of the DAG pool generated as a result of chloroplast-membrane turnover in *Chlamydomonas* and could be mediated by a PDAT-like protein functioning as a galactolipid:DAG acyltransferase. A gene encoding the single PDAT-like protein of *C. reinhardtii* displayed an increasing expression pattern in response to short-term nitrogen depletion (Boyle et al. 2012). The cloned *CrPDAT* gene was shown to encode a functionally active protein that was heterologously expressed in yeast, whereas downregulation of its expression by RNAi silencing in *Chlamydomonas* led to attenuated TAG production under nitrogen deprivation (a 25 % decrease) (Boyle et al. 2012). Cloning of the full-length gene and sequence analysis of the deduced protein revealed the presence of several domains typical to members of the lecithin:cholesterol acyltransferase (LCAT) family and found in functionally characterized PDAT of yeast and *Arabidopsis*, namely, the α/β hydrolase domain comprising the catalytic triad Ser-Asp-His (Dahlqvist et al. 2000; Ståhl et al. 2004). Furthermore, the deduced protein bore a predicted 92-amino acid N-terminal chloroplast transit peptide, suggesting its possible chloroplast localization and consistent with identification of this protein in isolated chloroplasts of *Chlamydomonas* in a proteomic study (Terashima et al. 2011). The open-reading frame (ORF) of *CrPDAT* featured a PDAT-characteristic TMD but contained additional amino acid stretches that are absent in most characterized and putative PDAT. Phylogenetic analysis that included putative PDAT from microalgae showed that microalgal PDAT-like proteins are more closely related to the fungal PDAT than to those of higher plants. Both protein and mRNA expression data showed transient upregulation of CrPDAT several hours following nitrogen depletion.

Detailed biochemical analysis of *Cr*PDAT in the yeast *Pichia pastoris* revealed that *Cr*PDAT is a multifunctional enzyme with broad substrate specificity, displaying both acyltransferase and lipase activities. Both the full-length and truncated (lacking the N-terminal TMD) recombinant forms demonstrated PDAT transacylase activity when provided with different phospholipids, displaying some preference for PA, PS, PI and PG over PC and PE; remarkably, both forms also displayed, albeit at a lower level, DAG:DAG transacylase activity (DGTA). Next, an activity assay using MGDG, DGDG and SQDG as acyl donors was implemented, given the presence of the chloroplast-targeting pre-sequence and the suggested function in chloroplast membrane lipid turnover. The full-length CrPDAT was indeed able to use MGDG for TAG formation demonstrating previously unreported galactolipid:DAG acyltransferase activity.

Formation of free fatty acids in the enzymatic assays led to the assumption that CrPDAT can exhibit lipase activity; this was reinforced by computerized structural analysis of the secondary protein structure, and was confirmed in assays utilizing a wide range of different lipid classes. The recombinant enzyme could release fatty acids from neutral lipids such as TAG, DAG and MAG, and steryl esters, preferred MGDG over DGDG and could hydrolyze a variety of phospholipids, including PC, which is absent in *Chlamydomonas*.

To investigate the role of CrPDAT in vivo, amiRNAi mutants were employed. Quantification of TAG production by ESI-MS in *CrPDAT*-knockdowns revealed a substantial decrease in TAG formation only after 24 h following transfer to nitrogen-depleted conditions as compared to the control lines, indicating that activity encoded by *Cr*PDAT does not contribute significantly to TAG production under prolonged nitrogen deprivation. Profiling of TAG molecular species demonstrated that the C50 molecular species (18/16/16) show a more drastic decline in content than the C52 (18/16/18) and C54 (18/18/18) molecular species in knockdown lines as compared with the control, leading to the assumption that CrPDAT prefers the prokaryotic-type DAG.

Overall, the results of this work produced further novelty in terms of discovery of enzymes with multiple functions in

the lipid metabolism that may enable rapid membrane turnover and recycling of biosynthetic precursors. A candidate lipase that is potentially involved in rapid TAG turnover upon nitrogen replenishment following nitrogen deprivation in *Chlamydomonas* was identified by Li et al. (2012b) among other candidate lipase genes showing fluctuating expression levels in a transcriptomic study (Miller et al. 2010). The candidate genes were expressed in a *S. cerevisiae* tgl3Δ tgl4Δ double-knockout mutant deficient in the major TAG lipases (encoded by *TGL3* and *TGL4*) and it displayed the increased TAG phenotype. Remarkably, these TAG lipases play multiple roles in yeast lipid metabolism, reciprocally contributing to both anabolic and catabolic processes (Rajakumari and Daum 2010a, b) and thus allowing for the active contribution of storage lipids to cell metabolism. Expression of *CrLIP1* (GenBank acc. no. XP_001694945) resulted in a reduction in TAG content in mutant yeast cells, hence this gene was therefore characterized in more detail. *CrLIP1* seemed to be expressed at high levels during vegetative growth and downregulated under nitrogen deprivation in *Chlamydomonas*. The deduced CrLIP1 protein also featured considerable sequence similarity to the human *sn*-1-specific DAG lipases DAGLα and DAGLβ. The recombinant purified protein appeared capable of hydrolyzing glycerophospholipids in vitro (PE of *E. coli* and all major glycerolipids of *Chlamydomonas* with a preference for PE and PG), but not of TAG under the experimental conditions tested. When expressed in yeast CrLIP1 seemed to release either fatty acid from the *sn*-1 position of PC and at longer incubation was also able to hydrolyze *sn*-2 lyso-PC.

A major catabolic role was inferred for CrLIP1 given the inverse relationships between gene expression and amount of TAG formed under conditions of both nitrogen depletion and nitrogen replenishment. Repression of gene expression by amiRNAi silencing resulted in a significant delay in TAG mobilization following nitrogen replenishment and a transient increase in DAG, implying an indirect role in TAG turnover. The authors concluded that CrLIP1 participates in TAG turnover in *Chlamydomonas* by facilitating DAG removal during TAG mobilization upon nitrogen resupply. Further research into microalgal TAG lipases will provide more knowledge on the process of TAG reserve mobilization in microalgae. In this respect, earlier works carried out with the red microalga *Porphyridium cruentum* (= *P. purpurum*) and the green microalga *L. incisa* should be noted that pointed out the importance of TAG mobilization in microalgae accumulating LC-PUFA in their TAG following growth recovery from unfavorable conditions, and for nutrient-replete growth of microalgae exposed to swift changes in ambient temperature (Cohen et al. 2000; Bigogno et al. 2002c; Khozin-Goldberg et al. 2005). A mutant of *P. cruentum* that featured impaired growth at low temperatures and decreased content of the major LC-PUFA EPA showed a delayed and lesser decline in the label incorporated into TAG following a pulse with radioactive fatty acids. This was accompanied by reduced relocation of the label from TAG to chloroplast galactolipids, particularly to the eukaryotic-like molecular species of MGDG (Cohen et al. 2000; Khozin-Goldberg et al. 2000). TAG mobilization was shown to occur in the green microalga *P. incisa* upon nitrogen replenishment and growth recovery from conditions of nitrogen starvation (Khozin-Goldberg et al. 2005), and beyond consumption for energy purposes to have buffering capacity for LC-PUFA relocation to chloroplast galactolipids.

Prevention of TAG degradation by downregulation of major TAG lipases synergistically with intensification of fatty acid synthesis and TAG assembly by the expression of relevant genes hold promise in the genetic engineering of microalgae for increased TAG production as considered being required for biodiesel production. Recent efforts in manipulating TAG production in vegetative tissues may hallmark the progress in this engineering approach (Kelly et al. 2013; Vanhercke et al. 2013). The *A. thaliana* mutant in sugar-dependent1 (sdp1) TAG lipase featured increased TAG contents in non-storage organs, whereas more significant TAG accumulation in leaves (up to 5–8 % of dry weight) was achieved when the sdp1 mutation was combined with overexpression of DGAT1 and the transcription factor WRINKLED1 (WRI1), a master transcriptional regulator orchestrating the expression of genes involved in fatty acid synthesis and plastidial glycolysis (Kelly et al. 2013). Combining upregulation of fatty acid biosynthesis with increasing TAG assembly, referred to as a "push-pull" approach was utilized to induce TAG accumulation in tobacco leaves by coexpression of the transcription factor WRI1 and DGAT1 with a synergistic effect of the two genes (Vanhercke et al. 2013). Simultaneous overexpression of *DGAT2* and *ACCase* genes driven by a strong endogenous promoter exerted a synergistic effect on TAG accumulation in the oleaginous yeast *Y. lipolytica* (Tai and Stephanopoulos 2013). Lessening TAG breakdown may further increase TAG in the so-called "accumulation" approach (Vanhercke et al. 2013) and the strategy of downregulation of TAG lipolytic activities holds great promise in the metabolic engineering of oleaginous microalgae. The promise of this approach was recently documented in the diatom *Thalassiosira pseudonana* when antisense expression of a multifunctional lipase/phospholipase/acyltransferase (Thaps3_264297) resulted in elevated lipid yields under nutrient-sufficient conditions (Trentacoste et al. 2013). The target lipase gene was identified based on gene-expression analysis of the alga under silicon-limited conditions. Deficiency in this nutrient, which is indispensable for this diatom, leads to cell-cycle arrest and among other metabolic responses, causes accumulation of TAG. One of the numerous lipase-encoding genes displayed strong downregulation upon transfer to silicon-limited con-

ditions, prompting the authors to assume that this lipase is implicated in TAG degradation during nutrient-sufficient growth. Remarkably, Thaps3_264297 is a homolog of CGI-58 (29 % identity), which is an α/β hydrolase-type lipase essential for cellular TAG homeostasis, and its deficiency causes LD formation, specifically in the human neutral lipid storage disease Chanarin-Dorfman syndrome. Disruption of this gene in *Arabidopsis* resulted in elevated TAG formation in mature *A. thaliana* leaves (James et al. 2010). Indeed, knockdown of this gene's expression led to increased lipid production and, importantly, without the compromising growth characteristics of the recombinant lines of *T. pseudonana* as compared to the wild type.

Several strategic steps for the metabolic engineering of microalgae aimed at increasing TAG production have been proposed (Rosenberg et al. 2008; Beer et al. 2009; Radakovits et al. 2010). Among these are overexpression of ACCase and FAS enzymes, increasing the availability of acetyl-CoA precursor by overexpression of the plastidial enzymes involved in its production or downregulation of PEP conversion to oxaloacetate, and interference of competing pathways leading to TAG degradation, e.g. inhibition of β-oxidation and lipolysis. So far, metabolic engineering approaches involving the individual overexpression of a "push" gene such as that encoding ACCase (Roessler et al. 1994; Sheehan et al. 1998) and a "pull" gene such as that encoding DGAT (La Russa et al. 2012) have not been effective at increasing TAG content in microalgae, suggesting that this strategy paves the way to enhancement of TAG accumulation by metabolic engineering. Combination of push and pull approaches with suppression of fatty acid β-oxidation in peroxisomes and/or TAG lipolytic activities seems to be a potent efficient mean to modulate TAG storage capacity in the single-cell photosynthetic microalgae (Fig. 17) as well as in vegetative tissues of higher plants (Slocombe et al. 2009; Chapman et al. 2013). Such genetic modifications, however, should not impose a severe growth effect, and thus reduce the productivity of microalgal cultures. These approaches will be supplemented by the development and application of novel tools in genetic engineering, enabling targeted gene modifications and genome editing in microalgae (Daboussi et al. 2014).

8.3 The Significance of TAG Accumulation in Microalgae

The significance of TAG accumulation in microalgal cells under stress conditions, particularly nitrogen starvation and increased photosynthetically active radiation (PAR), is elaborated upon in several review publications (e.g. Hu et al. 2008; Solovchenko 2012 and references therein). TAG overproduction under conditions of nitrogen starvation is seen as an efficient means of consuming excess photoassimilates, thereby preventing photooxidative injury under stressful conditions (Solovchenko 2012; Solovchenko et al. 2013b). Under nitrogen deprivation, the de novo production of fatty acids continues and circumvents over-reduction of the electron transport chain by diverting excess electrons from the

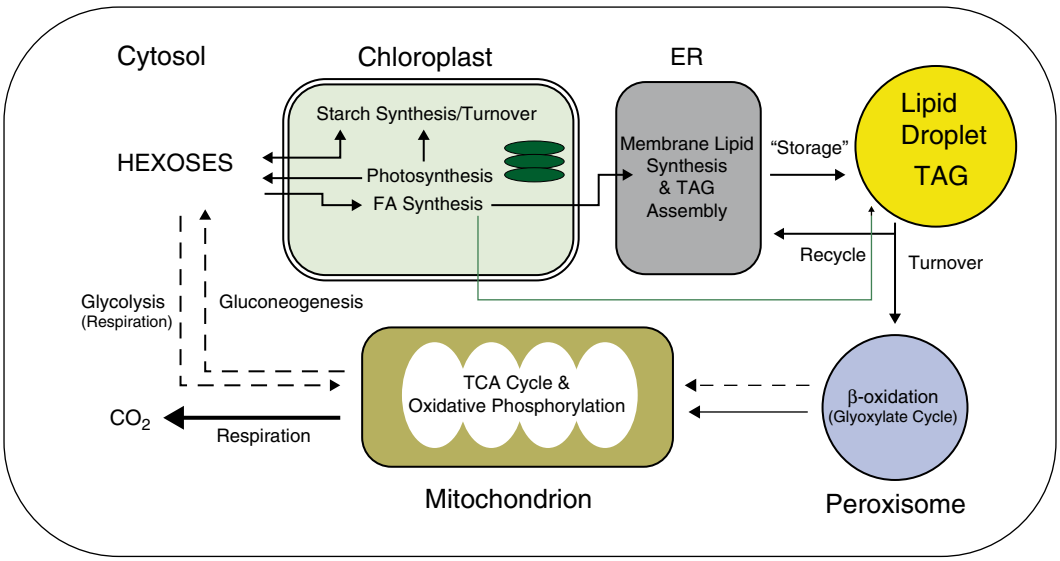

Fig. 17 Simplified scheme of the pathways of carbon flow toward TAG accumulation in a photoautotrophic oleaginous green microalga, amendable for genetic engineering (Modified from Chapman et al. (2013) with permission from the authors and the publisher (Elsevier))

production of reactive oxygen species (ROS) to that of NADPH, which is then utilized in reactions with FAS (Hu et al. 2008). Enhanced formation of ROS was indeed measured in the nitrogen-depleted cultures of the aforementioned *pgd1* mutant of *C. reinhardtii* which is impaired in TAG accumulation, as compared to the parental strain under the same conditions (Li et al. 2012b).

In the cells of microalgae that are able to deposit LC-PUFA in TAG, these TAG reserves are utilized to some extent for membrane lipid buildup when growth conditions require it, such as upon nitrogen replenishment or transfer to low temperature (Cohen et al. 2000; Bigogno et al. 2002c; Khozin-Goldberg et al. 2005). This strategy is particularly important for species isolated from environments in which they are subjected to swift changes in environmental conditions.

Optimization of the biotechnology of, and light utilization by, photosynthetic microalgal cultivation for cost-effective production of energy-rich TAG storage lipids is the subject of a plethora of investigations, given the biotechnological prospects for biodiesel production (Scott et al. 2010; Wijffels and Barbosa 2010; Georgianna and Mayfield 2012; Perrine et al. 2012; Gimpel et al. 2013). Biotechnological prospectives and implications of lipid production by microalgae are widely debated and different opinions exist (e.g. Ratledge and Cohen 2008; Lundquist et al. 2010; Tredici 2010; Stephens et al. 2010; Wijffels and Barbosa 2010; Hall and Benemann 2011). Microalgae accumulate TAG under stressful conditions, mainly caused by nutrient deprivation or limitation and exposure to increased light intensities (Roessler 1990; Hu et al. 2008). Among nutritional factors, nitrogen limitation and starvation trigger TAG accumulation most markedly, and silica deficiency induces TAG accumulation in diatoms with a siliceous cell wall. Sulfur deficiency also induces TAG production in *Chlamydomonas* (Boyle et al. 2012; Sato et al. 2014). In correlation with the amount of TAG produced, nitrogen starvation elicited more profound increases in DGTT1 and DGAT1 mRNA as compared to sulfur deprivation; however, the expression of PDAT remained unaltered, implicating stress-specific mechanisms in the regulation of acyl-CoA-dependent and independent pathways of TAG biosynthesis, possibly at the post-transcriptional level (Boyle et al. 2012). As might be expected under stressful conditions, cell cycle and consequently growth of microalgal cultures are hampered, and therefore obtaining sufficient and sustainable biomass and lipid productivities is not trivial, especially under outdoor conditions. Optimized productivity can be achieved by cultivation of the most robust species under conditions that allow at least partially circumventing the negative consequences of nutrient or light stress, such as optimized cell densities, incident light, light path and geometry of the cultivation facility, growth stage of the inoculum, etc. However, a discussion of the prospects of microalgal biotechnology for cost-effective and sustainable production of biofuel, and the biotechnological aspects of microalgal cultivation for neutral lipid production are beyond the scope of this chapter.

Strain selection for the most robust microalgae in terms of biomass and lipid productivities is exemplified by Rodolfi et al. (2009) who examined 39 strains under laboratory conditions and selected the four most promising strains for examination outdoors (among them *Nannochloropsis* spp.). Indeed, species of the genus *Nannochloropsis* emerged among the leading organisms for the microalgal biotechnology field and are cultivated on a large scale by a number of commercial ventures, as a source of both EPA and TAG (Khozin-Goldberg et al. 2011). Conditions detrimental to total fatty acid productivity in laboratory cultures were dissected by Pal et al. (2011), and a combined effect of increased PAR and salinity level had a severe negative impact on biomass productivity of the cultures and decreased cellular lipid content. A strong emphasis is placed on industrial CO_2 mitigation by microalgae for the purpose of biomass and oil production. Recent developments in microalgal biotechnology for CO_2 mitigation by microalgal cultures, as well as mechanisms of high CO_2 tolerance and impacts on lipid metabolism are discussed in Solovchenko and Khozin-Goldberg (2013).

8.4 TAG Molecular Species Analysis

Intensive studies on TAG metabolism in microalgae have prompted significant advances in state-of-the-art analytical methods for microalgal TAG (Yu et al. 2009; Danielewicz et al. 2011; MacDougall et al. 2011; Řezanka et al. 2012; Liu et al. 2013; Li et al. 2014). TAG profiling by ESI-MS was performed in two diatoms—*P. tricornutum* and *T. pseudonana*—over the time course of nitrogen and silica starvation (Yu et al. 2009). Whereas *P. tricornutum* had little requirement for silica, exposing *T. pseudonana* to silica deprivation led to a 25 % increase in TAG production as compared to nitrogen starvation. The major molecular species of TAG in the starved T. *pseudonana* (almost 60 %) contained 16:0 in combination with 16:1 and 14:0; the LC-PUFA EPA and DHA were shown to be major components of the higher molecular weight molecular species of TAG detected in *T. pseudonana* cells that had been nitrogen- or silica-starved. Partitioning of LC-PUFA into TAG in the stationary phase of growth had been previously demonstrated in this alga (Tonon et al. 2002b). TAG molecular species 48:1 (16:0/16:0/16:1) and 48:2 (16:0/16:1/16:1) constituted the main species at different growth stages, whereas molecular species comprising of LC-PUFA became more diverse, such

as TAG 52:6 (16:0/16:1/20:5), 56:10 (14:0/20:5/20:5), 58:11 (16:0/20:5/22:6), and 58:12 (16:1/20:5/22:6).

Danielewicz et al. (2011) analyzed and compared TAG composition in hexane extracts of four marine microalgal species: the diatom *P. tricornutum*, the two eustigmatophytes *N. salina* and *N. oculata*, and the prasinophyte *Tetraselmis suecica*, all producing LC-PUFA, using MALDI-TOF MS, ESI linear ion trap-orbitrap (LTQ Orbitrap) MS, and ^{1}H NMR spectroscopy preceded by pigment and polar lipid elution by an additional column-chromatographic step. Remarkably, TAG molecular species with EPA occupying from one to three positions on the glycerol backbone were detected in all species examined. Řezanka et al. (2012) identified more than 100 molecular species of TAG in *P. tricornutum* and studied the composition of positional isomers of the highly unsaturated species that were isolated by reverse-phase (RP)-HPLC followed by chiral HPLC as influenced by nitrogen, phosphate, sulfur and silica starvation. These authors also detected highly unsaturated 60:15 TAG with all *sn* positions occupied by EPA (20:5/20:5/20:5), as well as, for example, 62:16 TAG (20:5/20:5/22:6), composed of EPA and DHA. Chiral separation of molecular species containing EPA in combination with one or two palmitoyl groups (56:10 and 52:5 TAG) allowed for quantifying the relative proportions of the positional isomers; it seemed that starvation conditions favor the formation of symmetrical molecular species, such as 52:5 (20:5/16:0/20:5 -) with a C16 acyl group located at the *sn*-2 position and two palmitoyl groups at the *sn*-1,3 positions, and 56:10 TAG with EPA attached to the *sn*-2 position (16:0/20:5/16:0). The green microalga T. *suecica* featured higher abundances of C18 PUFA-containing TAG molecular species as compared to the diatom and the eustigmatophytes, where different combinations of C16 and C14 were very abundant (Danielewicz et al. 2011). Investigation of total lipid extracts with a focus on TAG profiling in six microalgal species: *Botryococcus braunii*, *N. gaditana*, *Ettlia* (*Neochloris*) *oleoabundans*, *P. tricornutum*, *Porphyridium aerugineum*, *and Scenedesmus obliquus*, was performed by direct analysis on an UPLC/ESI/MS (MacDougall et al. 2011). TAG profiling appeared to be possible from the total lipid extracts and as expected, TAG molecular species composition in the stationary cultures differed across species. Two green algae—*B. braunii* and *S. obliquus*—were more enriched in TAG with C18 fatty acids and featured a broader range of molecular species; remarkably TAG species with very long chain mono- and dienoic fatty acids, such as 28:1, 28:2 and 30:1, 30:2, and 36:2, were detected in *B. braunii*. Quantification of TAG molecular species in two microalgae—*C. reinhardtii* and *N. oceanica*—cultivated in nitrogen replete and nitrogen-depleted media was achieved by UPLC-MS using endogenous compounds present in algal extracts as internal standards (Liu et al. 2013). Research exploring powerful lipidomics techniques in studies of lipid metabolism in microalgae in general, TAG in particular, is accumulating. The network approaches now allow integrative system-level analysis of microalgal cell responses to different conditions (Goodenough et al. 2014; Li et al. 2014; Schmollinger et al. 2014).

9 Lipid Droplet Formation and Proteins

Microalgae deposit neutral storage lipids, most commonly TAG, in a special type of subcellular organelle referred to as a lipid or oil globule (LG or OG, respectively), and very commonly as LD. It should be noted that some other designations exist in the literature, such as lipid particles, fat globules, oleosomes, and spherosomes. We will use the term LD in most cases for this organelle in microalgal cells, except instances where the major LD protein carries a different original OG designation.

Ambient conditions are implicated in TAG accumulation; for example, nitrogen depletion and/or exposure to elevated levels of PAR induce dramatic ultrastructural alterations in the cells of oleaginous microalgae, generally associated with an overall reduction in chloroplasts, and decreased stacking of thylakoid membranes concomitant with massive formation of LD (Merzlyak et al. 2007; Wang et al. 2009; Moellering and Benning 2010; Přibyl et al. 2012; Simionato et al. 2013, among others) (Fig. 18). However, the remaining chloroplasts may retain some photosynthetic activity even under prolonged nitrogen starvation contributing to the cells' viability (Simionato et al. 2013; Solovchenko et al. 2013b). The intensity of the ultrastructural changes, translated into a physiological cell response is, of course, dependent on the duration of the stressful conditions and severity of the stress applied. Some microalgal species endure long-term stresses by forming resting cells with abundant oil-storing cytoplasmic inclusions (Boussiba 2000; Zhekisheva et al. 2002; Merzlyak et al. 2007). Microalgae can mobilize TAG reserves stored in LD in response to growth recovery from unfavorable conditions; for example, nitrogen replenishment triggers LD degradation (Khozin-Goldberg et al. 2005; Dong et al. 2013; Přibyl et al. 2012). In the oleaginous *Chlorella vulgaris*, capable of accumulating more than 50 % of total lipids, nitrogen starvation promotes formation of initially small LD in both the cytoplasm and chloroplast, while a huge LD fills up virtually the entire volume of the cell as nitrogen starvation progresses. The LD is progressively broken up into smaller ones upon nutrient replenishment in association with recovery of growth and chloroplast ultrastructure (Přibyl et al. 2012). Remarkably, during TAG remobilization, numerous electron-dense structures with random granular structure were observed at the interface between the LD and the cytosol, resembling the

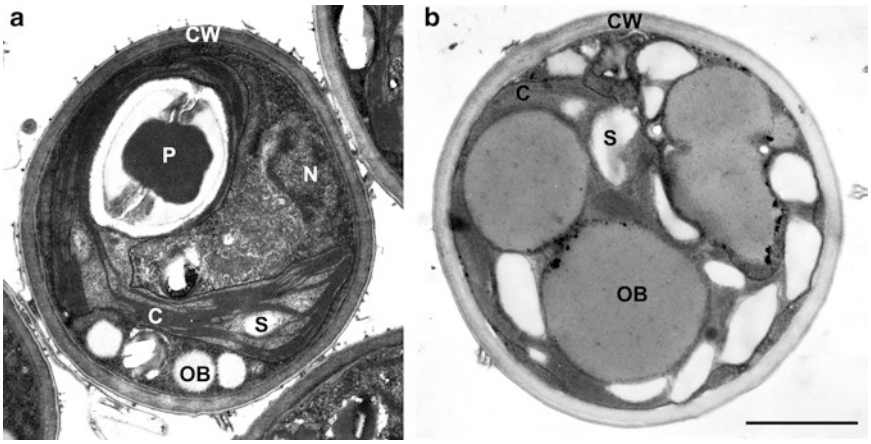

Fig. 18 The ultrastructure (TEM micrograph) of the microalga *Desmodesmus* sp. (Solovchenko et al. 2013a) grown in (**a**) N-replete and (**b**) N-depleted meda. *C* chloroplast, *CW* cell wall, *N* nucleus, *OB* oil body, *P* pyrenoid, *S* starch grain. Scale bar: 1 μm (Courtesy of O. Gorelova and O. Baulina, Moscow State University)

association of microperoxisomes in the vicinity of LD in adipocytes (Blanchette-Mackie et al. 1995).

In the cells of the microalga *L. incisa*, small LD initially appear in the cytoplasm following transfer to nitrogen-depleted medium, and then they increase in number and size and only partially coalesce as nitrogen starvation continues (Merzlyak et al. 2007). In certain species, such as *H. pluvialis* and *L. incisa*, the cytoplasmic inclusions sequester the secondary carotenoid pigments along with TAG; the physiological role of this response has been recently reviewed (Solovchenko 2012).

LD as major cellular depots of energy-rich reserve lipids are widely present in eukaryotes, as well as in the cells of some prokaryotes, such as the gram-positive bacterium *Rhodococcus* sp. (Huang 1992; Ding et al. 2012; Murphy 2012). In mammals, various types of cells harbor LD, such as the specialized fat-storing adipocytes, macrophages and anti-inflammatory mast cells (Dichlberger et al. 2013). The reader is referred to earlier and more recent excellent reviews on the biogenesis and function of these ubiquitous organelles in different organisms and cell types (e.g. Huang 1992; Frandsen et al. 2001; Daum et al. 2007; Farese and Walter 2009; Chapman et al. 2012; Murphy 2012; Yang et al. 2012a; Dichlberger et al. 2013). As might be expected, the field of LD metabolism is central to studies of obesity-related disorders and metabolic diseases, and a strong emphasis is placed on studies of LD formation in yeast cells and oil-seed plants, and more recently in the cells of oleaginous algae driven by a growing interest in algal oil production.

Studies in various organisms infer that LD are metabolically active, dynamic organelles that actively participate in cellular lipid homeostasis and cellular lipid trafficking, rather than being simply inert fat reservoirs playing a role in ensuring energy supply upon changes in cellular needs (Wagner and Daum 2005; Daum et al. 2007; Farese and Walter 2009; Zehmer et al. 2009; Grillitsch et al. 2011; Murphy 2001; 2012). TAG in microalgal cells may serve as a source of membrane-building components when growth or nutritional conditions require membrane expansion or buildup (Cohen et al. 2000; Khozin-Godberg et al. 2005). In fact, the dynamic nature and complex role of LD have been affirmed by the numerous proteomic studies performed on isolated LD revealing proteins associated with lipid trafficking, lipid biosynthesis and catabolism, cell signaling and interactions with different organelles (reviewed in Beller et al. 2010). Robust protocols have been developed for the isolation of LD from various cell types and organisms, to characterize their proteomes (Ding et al. 2012). Proteomic studies in microalgae support the dynamic role of LD in these organisms (Moellering and Benning 2010; Nguyen et al. 2011; Davidi et al. 2015).

LD biogenesis is still not completely understood, and it is being actively elucidated through the use of molecular tools that allow manipulating LD proteins' expression and tracing their cellular localization during oil deposition and mobilization in yeast, *Drosophila* and mammalian cells. Different modes of LD biogenesis have been suggested, with the prevailing hypothesis being that it is initiated by the accumulation of TAG between the two leaflets of the ER bilayer, followed by enlargement of a nascent LD and eventually, its budding off into the cytoplasm (Chapman et al. 2012 and references therein). This mechanism explains the origin of the phospholipid monolayer surrounding the mature LD, but it does not explain how the mature LD grows in size (Prinz 2013); such as, a single LD is often present in adipose cells, occupying essentially most of the cell volume. Recent investigations in yeast and adipose cells have provided many novel insights into the biogenesis of LD. LD appear to be functionally connected to the ER membrane and this allows for the efficient partitioning of membrane proteins engaged in TAG biosynthesis between the two compartments (Jacquier et al. 2011; Wilfling et al. 2013; Prinz 2013). Wilfling et al. (2013) were able to follow the fate of fluorescently tagged GPAT4 in adipocytes actively forming LD upon provision of free oleic acid; Fig. 19 illustrates a proposed route for GPAT4 relocalization from the ER to a subset of enlarging LD, where it becomes stably associated and is required for LD growth (Wilfling et al. 2013).

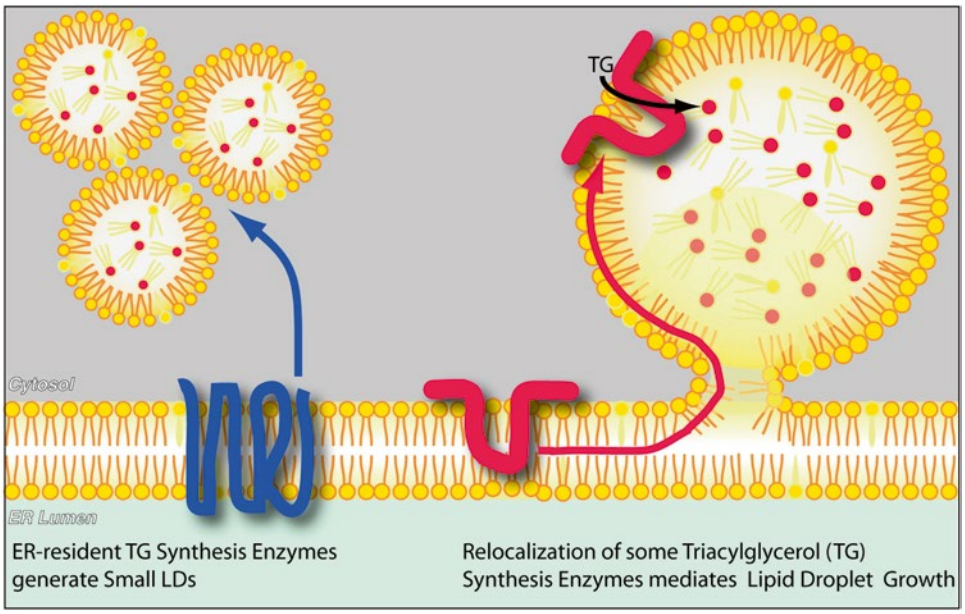

Fig. 19 Lipid droplet biogenesis and relocalization of triacylglycerol biosynthesis enzyme from the ER to lipid droplets (Reproduced from Wilfling et al. (2013); courtesy of Elsevier)

LD surface proteins play an important structural role and are implicated in the regulation of TAG storage and lipolysis via the recruitment of TAG lipases. Oleosins are abundant structural proteins on the surface of seed OGs that prevent them from coalescencing and thus are responsible for OG stability during desiccation, and for lipase binding during imbibition and germination (Huang 1992). These negatively charged proteins possess a characteristic and unique hydrophobic stretch comprising the so-called proline knot motif (Pro-knot; PX5SPX3P), which plays a pivotal role in immersion of the protein in the hydrophobic matrix of the OG. Remarkably, a recent study identified oleosin-3 in immature peanut seeds as a functional protein with dual MGAT and phospholipase A2 activity (Parthibane et al. 2012). Amphiphilic proteins of the PAT (perilipin, adipophilin and TIP47) protein family are the main structural proteins of mammalian LD, and they also play an important role in LD dynamics (Bickel et al. 2009). Perilipin was localized to the surface of LD in adipocytes (Blanchette-Mackie et al. 1995).

The hydrophobic core of microalgal LD consists of TAG (accumulation of steryl esters in LD is not common in algae or plants) enveloped by a monolayer of polar lipids. The phospholipids PC and PE are the most common constituents of the LD half-membrane in various cell types. The major extraplastidial betaine lipid DGTS was detected in LD-enriched fractions of the green microalga *C. reinhardtii* (Wang et al. 2009; Moellering and Benning 2010). In *H. pluvialis*, which can accumulate astaxanthin esters along with TAG in the LD, the phospholipid PC and the betaine DGTS were observed in the lipid extracts of isolated LD (Peled et al. 2011). In *C. reinhardtii* and *H. pluvialis*, TAG comprised the major lipid class of the LD-enriched fractions. However, some chloroplast galactolipids, MGDG and DGDG, were also detected (Moellering and Benning 2010; Peled et al. 2011; Davidi et al. 2014), suggesting a close genuine association between LD formation and the chloroplast envelope as discussed above. Though, contamination during the homogenization and floatation steps of the isolation process cannot be ruled out co-precipitaion assay indicated that MLDP in *C. reinhardtii* recruits chloroplast membrane lipids and proteins to LD (Tsai et al. 2015).

Proteomic studies of LD in microalgae have revealed that putative structural proteins and those with various other functions comprise the repertoire of LD proteins in nitrogen-depleted cells (Wang et al. 2009; Moellering and Benning 2010; Nguyen et al. 2011; Davidi et al. 2015), implying a dynamic fate of LD in microalgal cells. For example, Nguen et al. (2011) identified more than 30 proteins in the LD proteome of *C. reinhardtii* that are putatively involved in the metabolism of acyl lipids, including several acyltransferases of the Kennedy pathway Kennedy (1961), and Rab-type GTPases, implicated in vesicle trafficking, supporting the notion that LD are active dynamic structures that are likely to be involved in processes such as TAG biosynthesis, catabolism and lipid homeostasis. The carotenogenic microalga *Dunaliella bardawil* (=*D. salina*) features two types of LD, cytoplasmic LD and β-carotene-enriched plastoglobuli formed in response to nitrogen starvation (Davidi et al. 2014, 2015). The two populations of LD have a very similar TAG composition yet very different proteomes, indicating their distinct functions in the cell as corroborated by the functional assignment of identified proteins. The less variable cytoplasmic LD proteome (consisting of 42 core proteins) contained a broad range of proteins involved in different steps of lipid biosynthesis and degradation of microsomal membrane lipids. Among 124 core proteins, the plastoglob-

uli were endowed with enzymes implicated in the catabolism of chloroplast lipids and vesicle trafficking, and contained all of the necessary machinery for β-carotene synthesis.

A major LD protein (termed MLDP) was initially identified by LD proteomics of nitrogen-limited cells of *C. reinhardtii* (Moellering and Benning 2010); this protein did not show similarity in either amino acid sequence or hydrophobicity profile to plant oleosins, the main structural protein of seed OG, or the PAT family proteins that are abundant on the surface of the mammalian LD (Moellering and Benning 2010). Expression of an MLDP-GFP fusion in *C. reinhardtii* was not successful; hence, its exact cellular location remained elusive. Abundance of MLDP mRNA and protein is correlated with TAG accumulation under nitrogen deprivation and TAG remobilization upon nutrient resupply (Li et al. 2012b; Tsai et al. 2014). RNAi silencing of MLDP in *C. reinhardtii* did not alter TAG accumulation under nitrogen deprivation, but it substantially affected the average size of the LD. LD increased in size in the transformant cells, implying a possible structural role for MLDP. Notably, oleosin content was shown to affect the size of OG in *Arabidopsis* in a similar fashion (Siloto et al. 2006). Besides the impact on LD size and number, reducing MLDP abundance by RNAimediated silencing impaired TAG breakdown and remobilization in the nitrogen-starved cells of *C. reinhardtii* upon nitrogen replenishment (Tsai et al. 2015).

Homologs of *C. reinhardtii* MLDP seem to be restricted to the green algal lineage (Chlorophyta) (Moellering and Benning 2010). Major LD proteins were identified and the genes encoding them were cloned in some other representatives of the Chlorophyta (Peled et al. 2011; Davidi et al. 2012), and more putative MLDP could be deduced in the genomes of the additional sequenced green microalgae. Phylogenetic analysis performed by Davidi et al. (2012) on the cloned and putative MLDP of the sequenced green algae of the phylum Chlorophyta revealed that the more closely related *C. reinhardtii*, *H. pluvialis* and *Volvox carteri* (Chlorophyceae) compose a subgroup that is separate from trebouxiophytes *Chlorella variabilis*, a *Coccomyxa* sp., *Prototheca wickerhamii* and the prasinophyte *Micromonas pusilla*. A major protein in the astaxanthin-enriched OG—Haematococcus OG protein (HOGP)—was isolated from nitrogen-depleted cells of *H. pluvialis* cultured under high light and was identified by 2D gel electrophoresis followed by MS/MS analysis (Peled et al. 2011). A similar approach was employed to isolate and clone the major protein of the cytoplasmic LD of the halophyte microalga *Dunaliella salina* cultivated under nitrogen starvation (Davidi et al. 2012). Since *D. salina* is a cell-wall-less alga, gentle cell breakage by osmotic shock preserves the intactness of the chloroplast and thus minimizes contamination with other organelles' debris during LD purification. Based on the amino acid sequence to that of *D. salina*, MLDP were cloned from two other *Dunaliella* species: *D. bardawil* (=*D. salina*) and *D. parva*. *D. bardawil* (=*D.salina*) produces two pools of LD (Davidi et al. 2014); a salient feature of the chloroplast-localized LD (plastoglobuli) is abundance of the carotenoid pigment β-carotene along with TAG (Katz et al. 1995). Previous research identified a major protein in the chloroplast lipid globules of *D. bardawil* (=*D. salina*) (Katz et al. 1995) which is dissimilar to MLDP in its amino acid sequence. The origin of the acyl groups for both pools of TAG is currently under investigation; electron microscopic analyses have revealed that in the early stages of nitrogen deprivation, cytoplasmic LD adhere to the chloroplast envelope membranes concomitant with the appearance of small plastoglobuli within the chloroplast, implying structural organizations within the chloroplast envelope, the complex cooperation of compartments in chloroplast TAG generation and the formation of plastoglobuli (Davidi et al. 2014).

The gene encoding HOGP in *H. pluvialis* displayed very low expression in the vegetative green cells. An increase in its expression occurred in correlation with astaxanthin and TAG accumulation in the cytoplasmic OG (Peled et al. 2011). Similarly, the expression level and protein abundance of MLDP in *D. salina* increased with duration of nitrogen starvation in strong correlation with TAG accumulation (Davidi et al. 2012). Heterologous expression studies in our laboratory allowed visualization of the GFP-tagged HOGP on the periphery of LD in the cells of a model transformable diatom *P. tricornutum*, confirming its LD localization (Z. Shemesh et al. in preparation).

Huang et al. (2013) investigated the localization of MLDP in *C. reinhardtii* LD; using immunofluorescence confocal laser-scanning microscopy (immuno-CLSM) with CrMLDP-specific antibodies, they observed that MLDP is not directly associated with LD but rather with the subdomains of the ER adjacent to the LD; partial co-localization with an ER-specific marker was also observed. On the other hand, MLDP was localized to the LD surface immunofluorescence microscopy in another study (Tsai et al. 2015) and by in situ immunogold-TEM in the cells of the microalga *D. salina* (Davidi et al. 2012). It is difficult to discuss this discrepancy, especially given that Huang et al. (2013) conducted a detailed time-course survey of MLDP localization under nitrogen starvation at different stages. It is conceivable that in single-cell organisms such as *C. reinhardtii*, tight wrapping of MLDP is not required because of the dynamic nature of cytoplasmic LD formation in microalgal cells as compared to OG in seeds destined for prolonged periods of desiccation. More research is obviously required to study the function and the fate of MLDP in green microalgae.

Furthermore, Huang et al. (2013) identified a previously nonannotated sequence in the genome of *C. reinhardtii* putatively encoding an oleosin-like protein referred to as "oleo-like". This is, in fact, the first evidence on the presence of

oleosin-like proteins in green microalgae, while the putative caleosin homologs have been annotated in the genome of *C. reinhardtii*. The deduced protein sequence of oleolike comprised 77 amino acid residues, with 18 Leu and 9 Pro, and possessed the characteristic 12-residue Pro-knot sequence of an oleosin but with shorter nonpolar arms than in higher plant oleosins (Huang et al. 2013). The transcript of oleolike was only expressed in zygotes and tetrads of *C. reinhardtii*, while MLDP transcript was expressed in the gametes, which are produced under conditions of nitrogen starvation. A lower plant—*Physcomytrella patens*—was then used for the expression of an oleolike-GFP fusion, and remarkably, oleolike-GFP was associated almost exclusively with LD in the transformed cells.

Recently, a major LD protein was identified in a representative of an evolutionary distinct species—in the oleaginous eustigmatophyte *Nannochloropsis* (Vieler et al. 2012a). The ca. 17-kD protein was enriched in nitrogen-depleted cells and appeared as the most prominent band following SDS-PAGE of the LD fraction. The LD fraction contained ample TAG and was enriched in carotenoids, as has been shown to occur in the LD fractions of some microalgae (Moellering and Benning 2010; Solovchenko et al. 2008, 2010; Peled et al. 2011). The protein, identified by MS, was designated as a LD surface protein (LDSP), and it's per cell content was quantified by immunodetection with specific antibodies, demonstrating a strong correlation with TAG deposition and mobilization during nitrogen depletion and replenishment, respectively. In addition, LDSP were detected in other members of the genus *Nannochloropsis*: *N. oceanica*, *N. gaditana*, *N. granulata*, and *N. salina* (Vieler et al. 2012a).

LDSP displayed several features resembling the higher plant oleosins, such as a sequence of 62 nonpolar amino acids forming a hydrophobic region, although it was shorter than in the oleosins. The relative content of prolines was high in this region, but the typical oleosin Pro-knot motif was not identified. The hydrophobicity profile was also found to be similar to that of the *Arabidopsis* oleosin OLE-1, whereas the overall calculated hydrophobicity was even higher in the LDSP. Predicting that these features were sufficient to allow the formation of a hairpin-like or similar structure on the surface of the LD in oilseed plants, the authors implemented expression of a cDNA of LDSP (tagged with either 6xHis or GFP at its C terminus) under the control of the *Arabidopsis* OLEO1 promoter in the T-DNA-insertion oleo1 mutant of *Arabidopsis*. Several lines of evidence confirmed LDSP's successful expression in oleo1-mutant plants, among them a visible GFP signal at the LD periphery in embryos and increased germination rate of seeds upon vernalization, the latter impaired in the mutant due to LD fusion in the absence of the structurally important OLEO1 in mature embryos. Microalgal LDSP expression led to a decrease in LD size in one of the transformants as compared to the nontransformed control, implying that the microalgal LD protein can at least partially rescue the impaired mutant phenotype. However, LDSP was incapable of rescuing the deficiency in TAG hydrolysis during germination; this would probably require additional factors and protein-protein interactions. Nevertheless, a study of LDSP provides evidence of LD proteins' functional expression in the heterologous higher plant hosts, and raises a question as to the evolutionary origin of LDSP in general. Furthermore, the promoter region of the LDSP gene appeared to be very effective at driving antibiotic resistance in the *Nannochloropsis* sp. genetic transformation system (Vieler et al. 2012a).

10 Concluding Remarks

To summarize, it is clear that complex lipid metabolism in microalgae still remains to be fully understood. Microalgae are capable of synthesizing a diverse assortment of fatty acids and lipid classes which are not just limited to those that are described in this chapter. Indeed this chapter does not deal with some additional and important aspects of lipid metabolism, such as biosynthesis of sphingolipids and their modifications, biosynthesis of sterols as well as enzymatic pathways of oxylipin formation from PUFA and LC-PUFA.

Many fundamental questions remain unanswered. A more in depth elucidation of the function of candidate genes in the microalgae's lipid biosynthesis, including TAG-biosynthesis machinery, is required, using genetic engineering tools. The development of genetic transformation systems for more species, including oleaginous and robust non-model microalgae with high biotechnological potential, will facilitate this type of research. Cellular-localization studies are also essential in order to advance our knowledge of the intracellular location of microalgal enzymes mediating the critical steps in carbon supply to fatty acid synthesis and glycerolipid biosynthesis. Metabolic engineering of lipid biosynthesis in microalgae is feasible and should be supported by the detailed physiological characterization of both wild type and engineered strains. These rewarding goals seem to be attainable with the further development of sophisticated molecular and analytical tools such as lipidomics and metabolomics.

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Sterols in Microalgae

John K. Volkman

1 Introduction

Sterols are tetracyclic triterpenoids biosynthesized by all eukaryotic organisms (Desmond and Gribaldo 2009). A generalized structure with carbon atoms numbered is shown in Fig. 1. Sterol distributions in microalgae display a wide range of structures reflecting subtle differences in the sterol biosynthetic pathway used by different organisms (e.g. Goad et al. 1974; Volkman 1986, 2003, 2005; Volkman et al. 1998; Nes 2011). In some cases the presence of specific sterols follows taxonomic classifications quite closely. In other classes, a wide range of structures can be found (Table 1) either because the taxonomic grouping is actually polyphyletic or the alga accumulates sterols produced at different stages in the biosynthetic pathway perhaps due to a defect in later biosynthetic steps.

Sterol biosynthesis occurred very early in the history of life on Earth (Kodner et al. 2008), and the presumed last common ancestor of the eukaryotes likely had a large panel of enzymes for sterol biosynthesis (Desmond and Gribaldo 2009). These biosynthetic pathways have continued to evolve over geological time and thus one might expect to see certain "unusual" or rare sterols in particular algal classes due to specific changes in one or more of the many genes needed for sterol biosynthesis.

This review provides characteristic features of the sterol distributions in each of the major microalgal classes and builds on previous reviews by this author (Volkman 1986, 2003, 2005; Volkman et al. 1998). However, a difficulty immediately arises as to which taxonomic scheme to use. Ideally this should reflect the evolution of the different algal classes, but a consensus is not yet available. There are 15 traditionally recognized phyla in microalgae and most of the papers cited in this review have assigned species based on this classification. These 15 phyla are: Cyanophyta (cyanobacteria, also referred to as blue-green algae in the older literature), Rhodophyta (red algae), Euglenophyta (euglenoids), Cryptophyta (cryptomonads), Pyrrophyta (dinoflagellates), Raphidophyta (raphidophytes), Chrysophyta (chrysophytes, golden-brown algae), Xanthophyta (=Tribophyta, yellow-green algae), Chlorophyta (green algae), Eustigmatophyta (eustigmatophytes), Phaeophyta (brown algae), Prasinophyta (prasinophytes), Bacillariophyta (diatoms), Glaucophyta (glaucophytes).

Undoubtedly these classification schemes will continue to be refined as new gene sequence data become available. While it would have been more satisfying to structure the paper around an agreed taxonomic scheme that reflects the evolution of microalgal classes, this has not been possible. The taxonomy of many microalgal groups is in a state of flux and it is not uncommon to see some species transferred to completely different classes and species names being changed several times. Accordingly the data are structured according to the major phyla and where possible assigned to different algal classes within each phylum. Revised taxonomic assignments have been used based on those presented in AlgaeBase (Guiry and Guiry 2015), with a reference back to the original classification used in the paper cited where there has been a revision. The chapter "Systematics, Taxonomy and Species Names: Do They Matter?" of this book (Borowitzka 2016) also provides the names and current taxonomically accepted affiliation of all taxa mentioned in the present chapter.

2 Sterol Biosynthesis

Sterols are an end-product of the cyclization of the C_{30} isoprenoid 2,3-oxidosqualene.

Cyclisation either produces lanosterol as in animals, fungi, and some algae and bacteria or cycloartenol as in higher plants and most algae (Fig. 2). Many plants also

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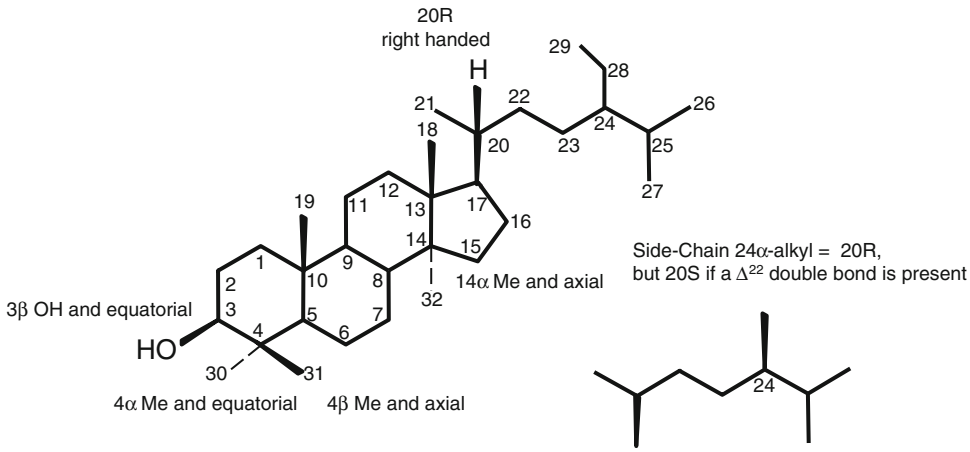


Fig. 1 A generalized structure of a sterol showing numbering of carbon atoms and positions of alkyl substituents and double bonds

Table 1 Systematic and trivial names for sterols found in microalgae

Sterol C. no.	Structure	Systematic name	Trivial name
27:0	IIb	5α-cholestan-3β-ol	5α-cholestanol
27:1	Ib	Cholest-5-en-3β-ol	Cholesterol
27:1	Ia	27-Nor-(24S)-cholesta-5,22E-dien-3β-ol	Occelasterol
27:2	Ic	Cholesta-5,22E-dien-3β-ol	22-dehydrocholesterol
27:2	Id	Cholesta-5,24-dien-3β-ol	Desmosterol
28:0	IIe	24α-methyl-5α-cholestan-3β-ol	Campestanol
28:1	Ie	24α-methylcholest-5-en-3β-ol	Campesterol
28:1	Ie	24β-methylcholest-5-en-3β-ol	5-ergostenol
28:1	IIIe	24β-methyl-5α-cholest-7-en-3β-ol	7-ergostenol
28:2	If	24α-methylcholesta-5,22E-dien-3β-ol	*epi*Brassicasterol or diatomsterol
28:2	If	24β-methylcholesta-5,22E-dien-3β-ol	Brassicasterol
28:2	Ih	23-methylcholesta-5,22E-dien-3β-ol	None
28:2	Ig	24-methylcholesta-5,24(28)-dien-3β-ol	24-methylenecholesterol
28:2	IVe	24β-methyl-cholesta-5,7-dien-3β-ol	5,7-ergostadienol
28:2	IIIf	24β-methyl-5α-cholesta-7,22E-dien-3β-ol	7,22-ergostadienol
28:3	IVf	24β-methyl-cholesta-5,7,22-trien-3β-ol	Ergosterol
29:0	IIi	24α-ethyl-5α-cholestan-3β-ol	Sitostanol
29:1	Ii	24α-ethylcholest-5-en-3β-ol	Sitosterol
29:1	Ii	24β-ethylcholest-5-en-3β-ol	Clionasterol
29:2	Ij	24α-ethylcholesta-5,22E-dien-3β-ol	Stigmasterol
29:2	Ij	24β-ethylcholesta-5,22E-dien-3β-ol	Poriferasterol
29:2	Ik	24α-ethylcholesta-5,24(28)E-dien-3β-ol	Fucosterol
29:2	Il	24α-ethylcholesta-5,24(28)Z-dien-3β-ol	Isofucosterol
29:2	IIIj	24β-ethyl-5α-cholesta-7,22E-dien-3β-ol	Chondrillasterol
29:3	IVj	24β-ethylcholesta-5,7,22-trien-3β-ol	7-dehydroporiferol
30:1	Im	22,23-methylene-23,24-dimethylcholest-5-en-3β -ol	Gorgosterol
30:0	Vn	4α,23,24-trimethyl-5α-cholestan-3β-ol	Dinostanol
30:1	Vo	4α,23,24-trimethylcholest-22E-en-3β-ol	Dinosterol
30:1	Vj	4α-methyl,24-ethyl-5α-cholest-22E-en-3β-ol	–
31:1	Fig. 2	4,4,14α-trimethyl-9,19-cyclo-5α-cholest-24-en-3β-ol	Cycloartenol
30:2	Fig. 2	4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol	Lanosterol

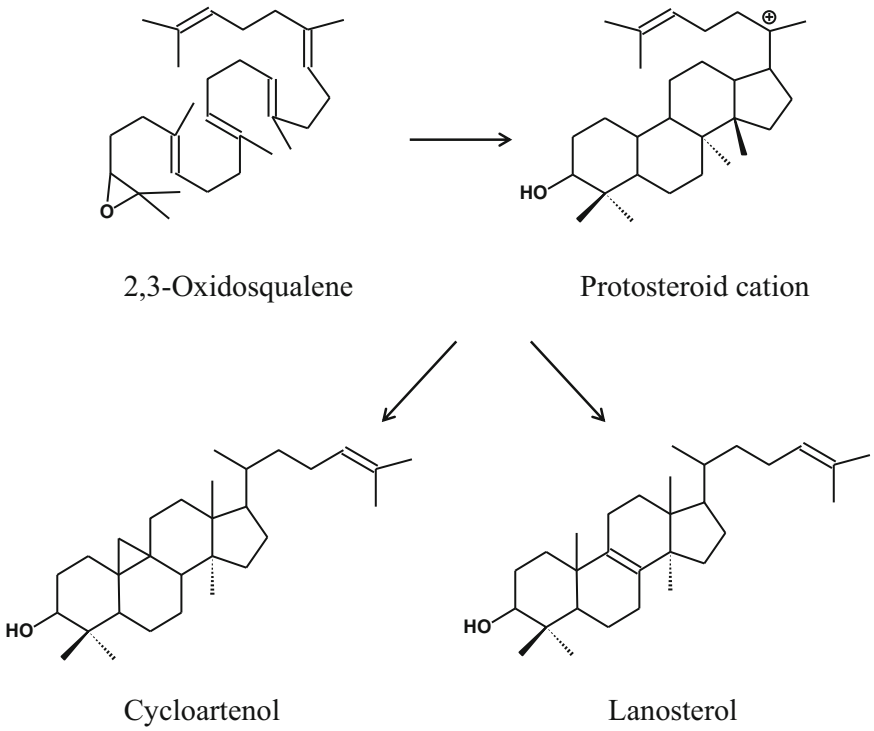


Fig. 2 The first products in sterol biosynthesis are either cycloartenol as found in higher plants and many microalgae or lanosterol as produced mostly by animals, fungi and some bacteria

convert 2,3-oxidosqualene to pentacyclic triterpenes such as α- and β-amyrin while bacteria generally synthesize cyclic triterpenes such as hopenes by cyclization of squalene rather than oxidosqualene (Buntel and Griffin 1994; Volkman 2005). For a comprehensive review of the biosynthesis of cholesterol and other sterols, primarily in higher plants and fungi, and the enzymes involved the reader is referred to the review by Nes (2011).

In microalgae, sterol biosynthesis can give rise to sterols having C_{26} to C_{31} carbon atoms and structural variations including 4,4-dimethyl substituents, a 4-methyl substituent or no methyl group at C-4 (so-called 4-desmethylsterols that comprise the majority of sterols in microalgae) (reviewed by Volkman 2003, 2005). Addition of one to four methyl substituents to the side-chain provides a wide variety of structures including 24-methyl, 24-methylene, 24-ethyl, 24-ethylidene, 24-*n*-propyl, 24-isopropyl, 23,24-dimethyl, 22,23-methylene (cyclopropyl) and 23-methyl sterols (Fig. 3).

Further variety is provided by the different locations for the double bonds including Δ^5, Δ^7, $\Delta^{8(9)}$, $\Delta^{8(14)}$, Δ^{22}, $\Delta^{5,7}$, $\Delta^{5,22}$, $\Delta^{5,24}$, $\Delta^{7,22}$, $\Delta^{5,24(28)}$, $\Delta^{8(14),24(28)}$ and $\Delta^{5,7,9(11)}$ amongst others. Structures of the main variants are shown in Fig. 3. Double bonds are generated by hydrogen transfers to produce different isomers. Early in the sterol biosynthesis pathway the double bond position is at Δ^8, which is subsequently isomerized to Δ^7. Introduction of a second double bond at C-5 leads to $\Delta^{5,7}$ sterols and reduction of the Δ^7 double bond gives rise to the Δ^5 sterols (Dempsey 1965; Doyle et al. 1972) (Fig. 4), which dominate the sterol distributions in most microalgae. Side-chain alkylation can occur at any of these stages giving rise to C-24 alkylated sterols with unusual double bond positions in the ring system.

The presence of particular sterols in an alga can often be understood by considering them as products of the many different steps involved in sterol biosynthesis. The biosynthesis of sterols will not be covered in detail here and the reader is referred to other papers outlining the key steps (e.g. Goad et al. 1974; Chappell 2002; Nes 2011; Miller et al. 2012; Xue et al. 2012). While sterol synthesis is undoubtedly very ancient, some steps may have evolved more recently.

2.1 Side-Chain Biosynthesis

The sterol distributions of most microalgae are characterized by a high proportion of sterols that are alkylated in the side-chain with either a methyl group, ethyl group (two methyl transfers), or rarely a propyl group at C-24 (e.g. Goad et al. 1974; Raederstorff and Rohmer 1984; Patterson 1994). Other variants include the 23,24-dimethyl side-chains found in the 4-methylsterols of dinoflagellates (e.g. Withers 1987) and in the 4-desmethylsterols of some diatoms and haptophytes (e.g. Volkman et al. 1993; Rampen et al. 2009b). These alkylations are brought about by S-adenosyl-L-methionine (AdoMet = SAM) sterol methyltransferases (SMTs: e.g. Nes 2003; Nes et al. 2003). SMTs do not occur in animal systems (Nes 2000). Goad et al. (1974) describe

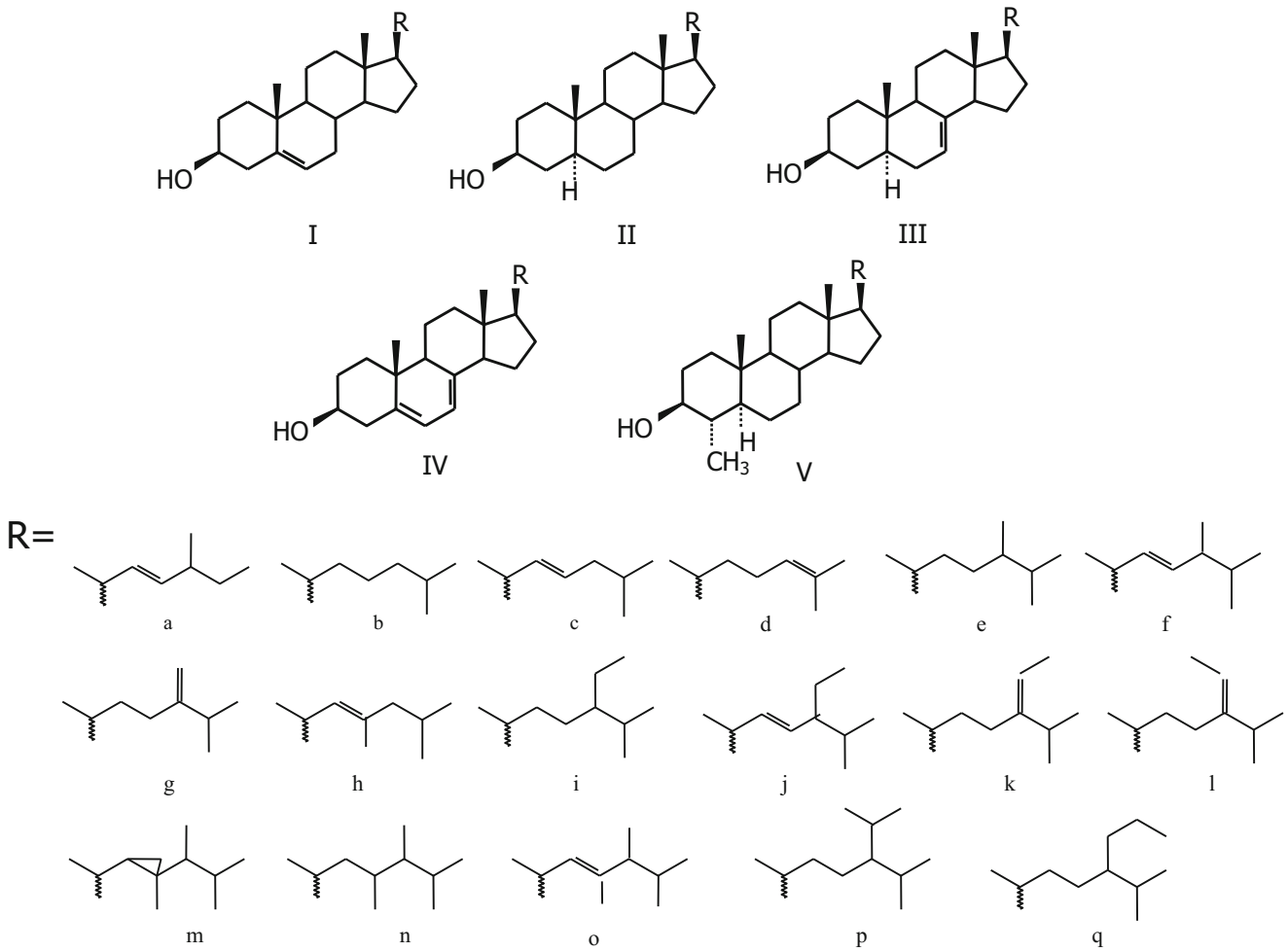
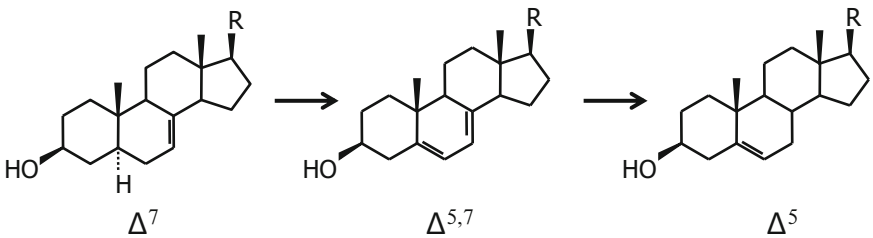


Fig. 3 Structural variation found in the main sterols of microalgae illustrating the main ring structures, double bond positions and alkylation (highly unusual sterols are not shown). R = sterol side-chain

Fig. 4 Stages in the formation of Δ^5 sterols from Δ^7 sterols in microalgae



six different mechanisms by which alkyl groups might arise in the side-chains of sterols. Nes and co-workers have proposed an active-site model, which they term the "steric-electric plug" model to describe C-24 alkylation (reviewed by Nes 2003; Nes et al. 2003). These pathways are complex and show phylogenetic groupings.

Work by Dennis and Nes (2002) showed that the SMT from *Glycine max* expressed in *Escherichia coli* cells catalyses the step-wise conversion of cycloartenol to 24(28)-methylenecycloartanol and then to a mixture of stereochemically related $\Delta^{24(28)Z}$-ethylidene-, $\Delta^{24(28)E}$-ethylidene- and $\Delta^{25(27)}$-24β-ethylcyclosterols. Campesterol (24α-methylcholesterol) and dihydrobrassicasterol (24β-methylcholesterol), typical C_{28} sterols in higher plants, are biosynthesized from desmosterol via 24-methylenecholesterol and 24-methyldesmosterol. The typical plant C_{29}-sterol, sitosterol (24α-ethylcholesterol), is produced from 24-methylenecholesterol via isofucosterol and 24-ethyldesmosterol.

2.2 C-24 Stereochemistry

The stereochemistry of the alkyl substituents in the side-chain can be α- or β- oriented depending on the biosynthetic pathways by which the side-chain is alkylated. Different algal classes usually produce sterols with either 24α or 24β stereochemistry, but only rarely do both occur (e.g. in some diatoms; Gladu et al. 1991b). For example, the C_{28} and C_{29} Δ^5 unsaturated sterols of vascular plants generally have the 24α configuration as do the C_{28} sterols of most diatoms (Maxwell et al. 1980; Nes and Nes 1980; Gladu et al. 1991b). In contrast, green algae and dinoflagellates biosynthesize sterols with the 24β configuration (Goad et al. 1974; Bohlin et al. 1981; Goad and Withers 1982; Miller et al. 2012).

Note that due to the IUPAC-IUB JCBN sequence rules, 24α corresponds to 24R when the side-chain is saturated, but it becomes 24S when a Δ^{22} double bond is present (Fig. 1). The side-chain α- and β- nomenclature is not related to that which is used to describe the orientation of methyl substituents on the ring system (see Goad and Akihisa 1997 for further explanation). 24β-Sterols (which are common in green algae) arise from $\Delta^{25(27)}$ sterols. Reduction of $\Delta^{24(28)}$ sterols can give rise to either 24α or 24β sterols (Goad et al. 1974). Fungi apparently use this pathway exclusively for the production of 24β epimers.

The majority of published data on algal sterols has been obtained by GC–MS analysis with reliance on relative retention data (e.g. Itoh et al. 1982) and reference mass spectra to identify specific isomers (e.g. de Leeuw et al. 1983; Jones et al. 1994; Gerst et al. 1997; Volkman et al. 1997). While GC–MS is a sensitive technique and very useful for identifying small amounts of sterols in complex mixtures it does not provide information on the configuration at C-24 (Gerst et al. 1997; Giner et al. 2008). The epimers can be separated on very long, polar glass capillary columns (Maxwell et al. 1980; Thompson et al. 1981; Ikekawa et al. 1989), but analysis times are very long and to date the method has not been widely exploited. HPLC techniques are also now available (Chitwood and Patterson 1991), but are also rarely utilized. Unambiguous assignment of C-24 stereochemistry can be obtained by NMR (e.g. Wright et al. 1978; Chui and Patterson 1981; Goad and Akihisa 1997; Miller et al. 2012), but this requires isolation and purification of each sterol within the complex mixtures found in most microalgae.

Identification of the stereochemistry at C-24 can indicate which of the many sterol pathways operates in that alga. Such data combined with information from incubation with [CD_3]-methionine and labeled mevalonate can be powerful tools for studying sterol biosynthesis in microalgae (Goad et al. 1974). Several algal species, including a diatom, two prymnesiophytes and two cryptophytes, have been shown to produce 24α-methylcholesta-5,22E-dien-3β-ol (*epi*brassicasterol; Rubinstein and Goad 1974; Maxwell et al. 1980; Goad et al. 1983). Green algae on the other hand mainly contain 24β sterols. In some cases both epimers can be found (Raederstorff and Rohmer 1984) as in an unidentified alga believed to be a chrysophyte (Kokke et al. 1984); 24α-ethylcholest-5-en-3β-ol comprised 10.2 % of 4-desmethyl sterols compared with 3.0 % for the 24β epimer.

C-24 stereochemistry is not always consistent within a single taxonomic grouping. For example, Gladu et al. (1990) examined nine strains of phytoplankton which contain 24-methylcholesta-5,22E-dien-3β-ol as their principal sterol. The two strains provisionally identified as *Isochrysis* (a haptophyte) contained brassicasterol (24β-methylcholesta-5,22E-dien-3β-ol); whereas all other species examined (two haptophytes, two rhodophytes and two diatoms) contained primarily *epi*brassicasterol 24α-methylcholesta-5,22E-dien-3β-ol).

2.3 Biochemical Forms of Sterols

The methods used in most studies of sterols in microalgae do not provide information about the chemical form in which the sterol exists since the extracts are usually saponified and any steryl esters are converted to the free sterols. In most microalgae, the sterols mainly exist in a non-esterified (i.e. free) form but this is not always the case. For example, Véron et al. (1996) studied the sterols of seven unicellular algae including representatives from the Prasinophyceae, Haptophyceae, Eustigmatophyceae, and Bacillariophyceae (as then defined). All synthesized free sterols and esterified forms (steryl esters, acyl steryl glycosides, and steryl glycosides), but free sterols predominated in five of the species. In contrast, the eustigmatophyte *Nannochloropsis oculata* and the diatom *Thalassiosira pseudonana*, contained mostly esterified sterols whereas in the diatom *Chaetoceros calcitrans* glycosylated forms represented over 60 % of total sterols. The pennate diatom *Haslea ostrearia* synthesized large amounts of steryl glycosides consisting mainly of the uncommon sterol 23,24-dimethylcholest-5-en-3β-ol.

2.4 Effects of Environmental Condition on Sterol Compositions

Minor differences in the relative proportions of sterols with culture age and growth state have been observed (Ballantine et al. 1979), but it comparatively rare for sterol distributions to change dramatically with changes in environmental conditions in contrast to fatty acid compositions. Sterol compositions thus are usually good chemotaxonomic tools for studying microalgal relationships and biochemistry. Sterol concentrations per cell or dry weight, however, can be affected. For example, Mercer et al. (1974) found no change

in sterol composition with culture age in three species of xanthophytes, but noted that aeration increased the cholesterol content. A detailed review of this topic is not possible here, but some leading references are provided to guide the reader.

Piepho et al. (2010) studied the sterol content in freshwater green algae *Desomodesmus communis* (*as S. quadricauda*) and *Chlamydomonas*, the cryptophyte *Cryptomonas ovata* and the diatom *Cyclotella meneghiniana*, and found that sterol contents increased significantly with increasing light in three out of four species and that sterol content decreased with increasing light at low phosphorus supply. Piepho et al. (2012) observed that sterol concentrations were higher at 25 °C than at 10 °C in *D. quadricauda* and *C. meneghiniana*, but were not affected by temperature in *C. ovata*. Temperature and phosphorus supply interacted to affect sterol concentrations in *C. meneghiniana* presumably due to the bioconversion of 24-methylene-cholesterol to 24-methylcholesta-5,22E-dien-3β-ol. Jo et al. (2004) found the same major sterols in autotrophically grown and heterotrophically grown *Tetraselmis suecica*, but the proportions of these and total amounts varied with culture age.

3 Sterols in Cyanobacteria

The suggestion that cyanobacteria (blue-green algae) contain sterols has been much debated. Some early studies suggested that their presence was due to contamination since on repeated purification the amount of sterols declined (Ourisson et al. 1987) and others have obtained similar data (Volkman, unpublished data; Summons et al. 2001). However, there are more than a dozen reports of sterols being found in cyanobacteria (summarized by Volkman 1986, 2003). These include simple mixtures in which cholesterol and 24-ethylcholest-5-en-3β-ol predominate, but Δ^7 and $\Delta^{5,7}$-sterols including ergosterol have also been reported (De Souza and Nes 1968). In almost all cases the amounts of sterols isolated are quite small (Hai et al. 1996). Summons et al. (2001) suggested that the sterols present in cyanobacterial cultures are derived from contaminating yeasts and fungi. The balance of evidence seems to indicate that cyanobacteria do not synthesize sterols although a few eubacteria can synthesize sterols that are not alkylated in the side-chain (reviewed by Volkman 2005).

4 Sterols in the Phylum Rhodophyta

The division or subphylum Rhodophyta contains both microalgae and macroalgae, generally termed red algae. Most members are marine macroalgae, but unicellular marine coccids are known including *Porphyridium*, *Rhodosorus* and *Rhodella*. Unicellular red algae that form mucilaginous colonies are considered primitive. The taxonomy has been through a number of changes. The Bangiophyceae, as defined traditionally, is paraphyletic and taxonomic identification of species has been difficult because of a lack of distinct morphological features, and the presumed morphological plasticity of the species. It has since been merged with the Floridophyceae to form the Rhodophyceae.

Most macrophyte red algae contain primarily C_{27} sterols with cholesterol predominant (e.g. Palmerno et al. 1984), although several species contain large amounts of cholesta-5,24-dien-3β-ol (desmosterol) (Gibbons et al. 1967). Only a few red macroalgae contain traces of C_{28} (e.g. 24-methylcholesta-5,22E-dien-3β-ol) and very rarely C_{29} sterols. This is in sharp contrast to the sterol distributions found in microscopic red algae which, while often dominated by cholesterol, can also show high contents of C_{28} 4-desmethyl sterols and occasionally biosynthetically "primitive" 4-methyl sterols.

4.1 Class Bangiophyceae

The sterols of the macroalgae *Pyropia*/*Porphyra* are dominated by cholesterol (60 %) whereas *Stylonema alsidii* (as *Goniotrichum elegans*) contains both 24-methylcholesta-5,22E-dien-3β-ol and cholesterol (Brothers and Dickson 1980). Sterol analyses of microalgae in this class appear not to have been reported.

4.2 Class Porphyridiophyceae

The microalga *Porphyridium cruentum* contains 24-methylcholesta-5,7,22-trien-3β-ol (ergosterol) and cholesta-5,22-dien-3β-ol (Beastall et al. 1971). In contrast, other species of *Porphyridinium* contain unusual 4-methyl Δ^8-unsaturated sterols including 4α-methyl-5α-cholesta-8,22-dien-3β-ol, 4α,24-dimethyl-5α-cholesta-8,22-dien-3β-ol, 4-methylcholest-8-en-3β-ol and 4,24-dimethylcholest-8-en-3β-ol (Beastall et al. 1974), perhaps due to the lack of a Δ^8 to Δ^7 isomerase. Duperon et al. (1983) showed that *Porphyridinium* sp. contains free sterols, steryl glycosides and acylated steryl glycosides.

4.3 Class Florideophyceae

Desmosterol was the major sterol found in three samples of the macroalga *Palmaria* (*Rhodymenia*) *palmata* (82 %, 97.2 % and 60.4 %) while cholesterol (92.3 %) predominated in a fourth (Idler et al. 1968). There was also significant variation in the principal sterol of two samples of the macroalga

Devaleraea ramentacea (as *Halosaccion ramentaceum*). One contained predominantly cholesterol (85.4 %), the other desmosterol (81.8 %).

4.4 Class Stylonematophyceae

The major sterol in the microalgae *Rhodosorus* sp. (CS-210) and *Rhodosorus lens* (= *R. salina*?) is the C_{28} sterol 24-methylcholesta-5,22E-dien-3β-ol (Gladu et al. 1990; Dunstan et al. 2005) as found in some cryptomonads, diatoms and haptophytes. *Rhodosorus* sp. (CS-210) contains in addition small amounts of cholesterol (2 %) and various Δ^7-sterols including the rare 4-methyl-5α-cholest-7-en-3β-ol (5.8 %) and 4-methyl-5α-cholesta-7,22-dien-3β-ol (12.3 %).

5 Sterols in the Phylum Chlorophyta

The Viridiplantae comprises the green algae and their descendants the land plants. One clade, the Chlorophyta, comprises the early diverging prasinophytes, which gave rise to the core chlorophytes. The other clade, the Streptophyta, includes the charophyte green algae from which land plants evolved. Many uncertainties about the evolution of this phylum persist, including the branching orders of the prasinophyte lineages, the relationships among core chlorophyte clades (Chlorodendrophyceae, Ulvophyceae, Trebouxiophyceae and Chlorophyceae), and the relationships among the streptophytes (Leliaert et al. 2012). As a consequence, it is difficult to classify some of the reported sterol distributions of various species due to changes in their taxonomic assignments.

The Chlorophyta contain chlorophylls *a* and *b* and store starch inside the chloroplast. There are thought to be between 9,000 and 12,000 species. Green algae became ecologically important about 600–800 Ma ago (Knoll et al. 2007; Kodner et al. 2008), possibly in response to increases in Fe content in the ocean (Canfield et al. 2008). Morphological analogues of the Ulvaphyceae have been found in Spitsbergen sediments 700–750 Ma (Butterfield et al. 1994). Green algae are thought to be responsible for the high abundance of C_{29} steranes seen in some Cambrian sediments and crude oils (e.g. Kodner et al. 2008). However, when one examines the sterols of modern groups of green alga the situation is much more complex. Many chlorophytes contain mixtures of Δ^7, $\Delta^{5,7}$ and $\Delta^{7,22}$ sterols (Holden and Patterson 1982), but a few contain mainly Δ^5-unsaturated sterols. In the latter group, 24-methylcholest-5-en-3β-ol and 24-ethyl-cholesta-5,22E-dien-3β-ol usually predominate with moderate amounts of 24-ethylcholest-5-en-3β-ol.

5.1 Class Chlorophyceae

This class is primarily freshwater and includes genera such as *Chlamydomonas*, *Pyramimonas*, *Scenesdemus* and *Oedogonium*. *Chlamydomonas* contains more than 600 species and has been shown to be polyphyletic (Proschold et al. 2001) which makes assessment of the early sterol literature difficult. It has been proposed that species in clades other than that containing *C. reinhardtii* must be transferred to other genera (Proschold et al. 2001).

Miller et al. (2012) have shown that the green alga *Chlamydomonas reinhardtii* synthesizes cycloartenol and converts it to ergosterol and 24-ethyl-5α-cholesta-7,22-dien-3β-ol (7-dehydroporiferasterol) (both having a C24 β-alkyl group) through a highly conserved sterol C24-methylation-C25-reduction pathway that is distinct from the acetate-mevalonate pathway that produces fungal lanosterol and thence to ergosterol by the $\Delta^{24(28)}$-olefin pathway.

Volkman et al. (1994) identified the major sterols in *Pyramimonas cordata* as 24-ethyl-cholesta-5,24(28)Z-dien-3β-ol (88.5 %) with a small amount of the 24(28)E isomer (0.8 %) and an unusual dihydroxylated C_{29} sterol 24-ethylcholesta-5,28(29)-dien-3β,24-diol (saringosterol) probably formed by oxidation of the major sterol.

Dunaliella minuta is unusual in that under stationary phase conditions it produces mainly C_{27} sterols (Ballantine et al. 1979). *Dunaliella salina* has been reported to produce C_{27} sterols at high salinities (4 M) (Kelly 2009) although C_{29} sterols are more commonly associated with this species (Peeler et al. 1989).

The freshwater species *Desmodesmus communis* (*as Scenedesmus quadricauda*) contains only Δ^7 sterols 24-methyl-5α-cholest-7-en-3β-ol, 24-ethyl-5α-cholesta-7,22-dien-3β-ol and 24-ethyl-5α-cholest-7-en-3β-ol (Cranwell et al. 1990). In contrast, freshwater *Eudorina unicocca* contains 5 sterols: 24-methylcholesterol (32 %), cholesterol (27 %), 24-ethylcholesta-5,22E-dien-3β-ol (22 %), 24-ethyl-5α-cholest-7-en-3β-ol and 24-methylcholesterol (4 %) (Cranwell et al. 1990). This species is unusual in that it contains both Δ^5 and Δ^7 sterols.

5.2 Class Ulvophyceae

Very few data are available for microalgal members of this class of green algae. The freshwater species *Ulothrix zonata* contains a complex mix of sterols with 24-methylenecholesterol (41 %), 24-methylcholesterol (23 %), 24-ethylcholesta-5,24(28)Z-dien-3β-ol (16 %) and 24-ethylcholesterol (12 %) as major sterols (Cranwell et al. 1990).

5.3 Class Mamiellophyceae

This class is primarily marine and includes the smallest eukaryotic algal genus *Micromonas* which contains a single species, *M. pusilla*, which is the dominant photosynthetic picoeukaryote in some marine ecosystems (e.g. Throndsen 1976). Unlike many marine algae, it is distributed widely in both warm and cold waters. Under the light microscope it is easily mistaken for a rapidly swimming bacterium. Based on pigment analysis, *Micromonas* shows affinities with the Mamiellales group of the Prasinophyceae, but it lacks the characteristic scales of this group. Note that some still classify this genus in the Prasinophyceae. The sterols of *Micromonas pusilla* (strain CS-98) and a tropical strain *Micromonas* aff. *pusilla* (CS-170) were reported by Volkman et al. (1994) who found the same suite of 4 major sterols, but in very different proportions. The major sterol in CS-98 was isofucosterol (71.7 %), but in CS-170 this was only 19.4 % of total sterols. The second most abundant sterol in CS-98 was 24-methylenecholesterol (15.9 %), but this was the major sterol in CS-170 (54.4 %). This difference seems to reflect a greater extent of alkylation of the 24(28) double bond in CS-98. Both strains contained an unusual dihydroxylated C_{29} sterol 24-ethylcholesta-5,28(29)-dien-3β,24-diol (saringosterol; 3.1 % of total sterols in CS-98 and 14.2 % in CS-170). It seems likely that saringosterol is formed from enzymatic oxidation of 28-isofucosterol.

5.4 Class Chlorodendrophyceae

This class includes freshwater and marine species and now includes the marine flagellate *Tetraselmis* (previously assigned to the Prasinophyceae). Patterson et al. (1993b) examined 11 isolates of *Tetraselmis* and found only 3 sterols. The principal sterol in eight isolates was either 24-methylenecholesterol or 24-methylcholesterol, both C_{28} sterols, with the latter identified as the 24α-isomer campesterol in each case. In the other three isolates, cholesterol was the principal sterol, which is highly unusual for a green alga, together with smaller amounts of 24-methylene-cholesterol and campesterol.

Volkman (1986) and Volkman et al. (1994) reported the sterol composition for *Tetraselmis chui* and found a single major sterol (>96 %) identified as 24-methylcholesterol. In contrast, Ballantine et al. (1979) found a much greater variety of sterols in *Tetraselmis tetrathele* which included 24-methylcholesterol (34 %) and 24-methylcholesta-5,22-dien-3β-ol (57.7 %). Similar data were reported by Lin et al. (1982) for another species *Tetraselmis suecica* where the proportions of these two sterols were 48.1 % and 50.9 % respectively.

Jo et al. (2004) examined the sterol dynamics of a strain of *Tetraselmis suecica* grown both autotrophically and heterotrophically. Six major sterols were found in the photoautotrophically grown cells: cholesta-5,22-dien-3β-ol, 24-methylcholesterol, cholesterol, 24-methyl-cholesta-5,22-dien-3β-ol, 24-methylcholesta-5,24-dien-3β-ol, and 24-ethylchlolesta-5,24-dien-3β-ol in decreasing order of abundance. The total amounts of sterols after 1 week of culture were quite similar as was the composition, but total amounts declined in weeks 2 and 3 and 24-methylcholesterol became the major sterol. Similar changes were found when the cells were grown heterotrophically.

The effect of culture renewal rate (RR) on sterol amounts in *T. suecica* was studied by Fabrégas et al. (1997). The major sterol 24-methylcholesterol ranged from 137 fg cell^{-1} with a 10 % RR to 40 fg cell^{-1} with a 40 % RR and 24-methylenecholesterol ranged from 403 fg cell^{-1} with a 10 % RR to 80 fg cell^{-1} with a renewal rate of 50 %.

5.5 Class Trebouxiophyceae

The Trebouxiophyceae contains the three orders: Chlorellales, Prasiolales and Trebouxiales. *Botryococcus braunii* is perhaps the best known member of this class of microalgae. *Chlorella* was formerly assigned to the Chlorophyceae but most species have now been assigned to the Trebouxiophyceae, although Huss et al. (1999) have proposed that only four species should be kept in the genus *Chlorella* within the Trebouxiophyceae, i.e. *C. vulgaris*, *C. lobophora*, *C. sorokiniana* and *C. kessleri* (= *Parachlorella kessleri*). Since the description of the type species *Chlorella vulgaris*, more than a hundred "Chlorella" species have been established in the literature (Goers et al. 2010), but most have been assigned to other genera.

Extensive data on the sterols in *Chlorella* species have been published by Patterson and coworkers (Patterson 1967, 1969, 1974; Dickson et al. 1972; Dickson and Patterson 1973; Patterson et al. 1974, 1992). These data were collated and expanded by Holden and Patterson (1982) who analysed the sterols in 35 species. From these data they were able to group the algae into six categories according to their sterol distribution. Group Ia contained Δ^5 sterols including cholesterol, 24β-ethylcholesta-5,22-dien-3β-ol (poriferasterol), 24β-methylcholesterol (5-ergostenol) and 24β-ethylcholesterol (clionasterol), all with 24β stereochemistry. The two isolates in Group 1B contained a related composition, but the major product was 24β-methylcholesterol (69–73 % of total sterol) and significant amounts of the C_{29} homolog of ergosterol, 24β-ethylcholesta-5,7,22-trien-3β-ol (7-dehydroporiferasterol), was also found. Group II contained Δ^7 sterols

presumably due to the lack of genes needed for the introduction of the Δ^5 double bond. The major sterol was the C_{29} sterol, 24β-ethylcholesta-7,22-dien-3β-ol (chondrillasterol). Group IIIa contained double bonds at both Δ^5 and Δ^7 presumably due to the lack of a Δ^7 reductase in some species. These isolates lack the ability to introduce a second alkyl group at C-24 resulting in the production, exclusively, of the C_{28} sterols ergosterol with lesser amounts of its mono- and diunsaturated derivatives. Group IIIb was similar, but these species are able to introduce a second alkyl group at C-24, producing the C_{29} homologue of ergosterol, 24β-ethylcholesta-5,7,22-trien-3β-ol, plus 24β-ethyl-5α-cholesta-7,22-dien-3β-ol and 24β-ethyl-5α-cholest-7-en-3β-ol (7-chondrillastenol). The presence of C_{29} sterols in ergosterol-synthesizing organisms is a rare occurrence. The last type of sterol biosynthetic pattern, Group IIIc, was encountered in only one strain of *Chlorella*. This contained unique Δ^8 and $\Delta^{8,22}$ sterols along with ergosterol, 24β-methylcholesta-5,7-dien-3β-ol (5,7-ergostadienol), and 24β-methyl-5α-cholest-7-en-3β-ol (7-ergostenol) as originally published by Patterson et al. (1974). These grouping align well with the various stages in the sterol biosynthetic pathway and this would seem to be a useful way to categorize sterol patterns. However, the taxonomic status of some of these strains is uncertain and it remains to be seen whether they match closely with modern views on *Chlorella* taxonomy.

Chlorella autotrophica (now *Chlorella vulgaris* var. *autotrophica*) contains a complex mixture of C_{28} and C_{29} sterols with Δ^7, $\Delta^{5,7}$ and $\Delta^{5,7,9(11)}$ nuclear double bond systems (Patterson et al. 1992). This alga also contained the rare tetraunsaturated sterols, 24-methylcholesta-5,7,9(11),22-tetraen-3β-olland24-ethylcholesta-5,7,9(11),22-tetraen-3β-ol.

Akihisa et al. (1992) identified ergosterol (51 %) and 7-dehydroporiferasterol (30 %), as the principal sterols of *Chlorella vulgaris*. They also identified the unusual 24β-methyl-$\Delta^{9(11)}$-sterols, 24β-methyl-5α-cholest-9(11)-en-3β-ol and 14α,24β-dimethyl-5α-cholest-9(11)-en-3β-ol, and the same two 24β-alkyl-$\Delta^{5,7,9(11),22}$-sterols, 24β-methylcholesta-5,7,9(11),22E-tetraen-3β-ol (9(11)-dehydroergosterol) and 24β-ethylcholesta-5,7,9(11),22E-tetraen-3β-ol found in *C. autotrophica*.

More recent work by Goers et al. (2010) has confirmed that sterol composition is a reliable chemotaxonomic marker within several groups of *Chlorella* and found high contents of ergosterol in nine species, all from the Chlorellaceae. More distant relatives within the Trebouxiophyceae or representatives of the Chlorophyceae did not contain ergosterol. The sterols in *Mucidosphaerium* (*Dictyosphaerium*) *pulchellum* were reported by Cranwell et al. (1990). The major sterol was cholesterol (90 %) with a small quantity of 24-ethylcholesterol (8 %).

5.6 Class Prasinophyceae

Prasinophytes can be important constituents of the phytoplankton in oceanic waters. Volkman et al. (1994) reported sterol compositions for *Pyramimonas cordata*, and *Pycnococcus provasolii* and found relatively simple distributions of Δ^5-sterols. The major sterols were 24-methylenecholesterol (which also occurs in diatoms), 24-methylcholesterol and 24-ethylcholesta-5,24(28)Z-dien-3β-ol (28-isofucosterol). They proposed that 24-methylcholesterol may be a useful marker for these microalgae. Minor amounts of C_{30} *n*-propylcholestane were produced after hydrogenation of the sterol mixture indicating the presence of C_{30} sterols that were not characterized.

Patterson et al. (1992) found that an un-named strain of *Pyramimonas* contained only 24-methylenecholesterol as a major sterol component (99 %) together with a trace of cholesterol. In marked contrast, *Pyramimonas grossii* contained a complex mixture of C_{28} and C_{29} sterols with Δ^7, $\Delta^{5,7}$ and $\Delta^{5,7,9(11)}$ nuclear double bond systems. Sterols were found both with and without the C-22 side chain double bond; ergosterol and 24β-ethylcholesta-5,7,22-trien-3β-ol (7-dehydroporiferasterol) were the principal sterols. Rare tetraene sterols, 24-methylcholesta-5,7,9(11),22-tetraen-3β-ol and 24-ethylcholesta-5,7,9(11),22-tetraen-3β-ol were found in *P. grossi* as in *Chlorella vulgaris* var. *autotrophica* and *Dunaliella tertiolecta* (Patterson et al. 1992).

6 Sterols in the Phylum Charophyta

6.1 Class Conjugatophyceae

Few data are available for this class. Cranwell et al. (1990) analysed the sterols in the two desmids *Cosmarium bioculatum* and *Xanthidium subhastiferum*. Both contained three main sterols: 24-methylcholesterol, 24-ethylcholesterol and 24-ethylcholesta-5,22E-dien-3β-ol, which are the same three sterols found in higher plants.

7 Sterols in the Phylum Dinophyta

7.1 Class Dinophyceae

Dinoflagellates (Dinophyceae) are generally considered to be the major source of 4-methyl sterols in aquatic environments (e.g. de Leeuw et al. 1983; Volkman 2003 and refs therein), but they are also potential contributors of 4-desmethyl sterols. In most species, 4-methyl sterols predominate (e.g. Leblond and Chapman 2002), but exceptions are known (e.g. Teshima et al. 1980). Complex mixtures of sterols are usually found (Withers et al. 1979; Wengrovitz

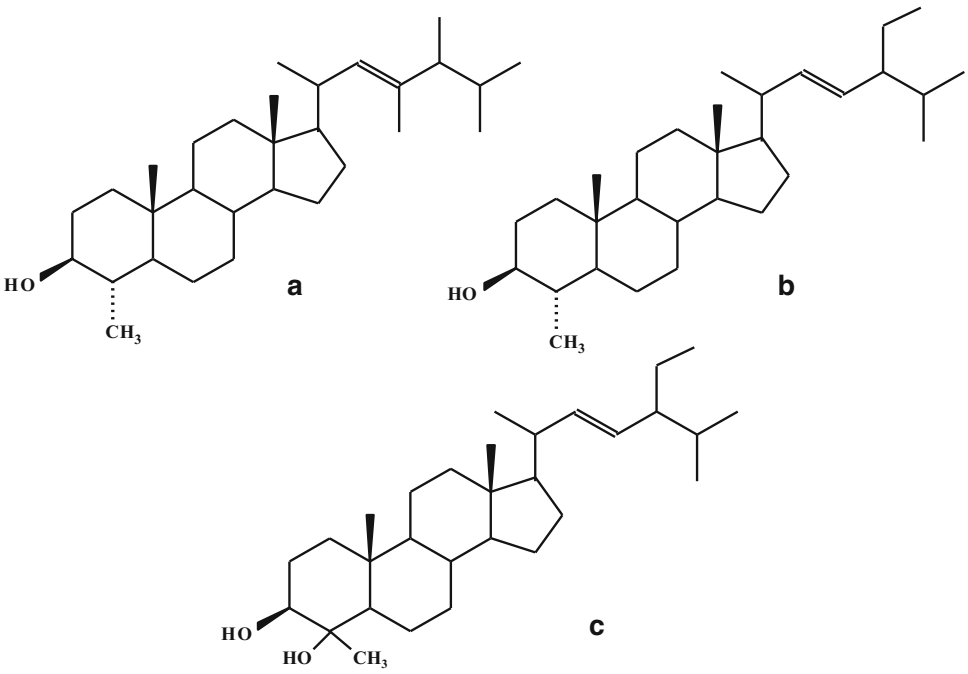


Fig. 5 Comparison of structures of two C_{30} sterols: (**a**) dinosterol (4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol) as found in many dinoflagellates and structurally isomeric 4α-methyl,24-ethyl-5α-cholest-22E-en-3β-ol (**b**) as found in haptophytes from the *Pavlovophyceae*. Also shown is the structure of a dihydroxylated 4-methyl sterol termed a pavlovol (**c**) that appear to be unique to the *Pavlovophyceae*

et al. 1981; Piretti et al. 1997; Amo et al. 2010), and many species contain unusual sterols having Δ^7 or Δ^8 double bonds (e.g. Hallegraeff et al. 1991) and unusual patterns of side-chain alkylation such as 23,24-dimethyl substitution (Leblond and Lasiter 2012 and refs therein).

The major sterol in many dinoflagellates is the 4-methyl sterol dinosterol (4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol; Fig. 5) which has no double bond in the ring system and an unusual 23,24-dimethyl side-chain alkylation (Shimizu et al. 1976; Boon et al. 1979). Because of its specificity as a dinoflagellate marker it has been widely used for paleoclimate studies (e.g. Boon et al. 1979; Makou et al. 2010; Castaneda et al. 2011). Methods to isolate purified dinosterol and other sterols from dinoflagellates for structural or isotopic analysis are now available using reverse phase and normal phase HPLC (Atwood and Sachs 2012). Although dinosterol appears to be a reliable biomarker for dinoflagellates, it should be noted that some dinoflagellates do not contain this sterol at all (Teshima et al. 1980; Kokke et al. 1981; Goad and Withers 1982).

Recently Leblond et al. (2010) published a review of the sterols and steroid ketones in dinoflagellates and related these to taxonomic assignments based on 18 rDNA phylogeny. These authors compiled a database from 102 published analyses of dinoflagellates which contained 58 distinct identified sterols and steroid ketones. Data on both sterols and 18S rDNA were available for 82 of the 102 strains. These data were clustered into six groups:

1. Mainly *Karenia* and *Karlodinium* species containing 4-desmethyl sterols with $\Delta^{8(14),22}$ diunsaturation such as 4α,24-dimethyl-5α-cholesta-8(14),22-dien-3β-ol and 27-nor-4α,24-dimethyl-5α-cholesta-8(14),22-dien-3β-ol;
2. Mainly *Amphidium* species containing 4α-methyl-5α-cholest-8(14)-en-3β-ol and 4α,24-dimethylcholesta-8(14),24(28)-dien-3β-ol. Note that both groups 1 and 2 are distinguished by sterols having a $\Delta^{8(14)}$ double bond which is uncommon in other dinoflagellates;
3. This grouping contained *Polarella glacialis*, *Protoceratium reticulatum*, *Lingulodinium polyedra* and *Gymnodinium simplex*. Major sterols were cholesta-5,22Z-dien-3β-ol, 24-methylcholesta-5,22E-dien-3β-ol, and 4α,24-dimethyl-5α-cholestan-3β-ol;
4. This contained *Akashiwo sanguinea* and prominent sterols 24-methylcholest-5α-cholest-22E-en-3β-ol, 23,24-dimethyl-5α-cholest-22E-en-3β-ol and 4α,24-dimethyl-5α-cholestan-3β-ol;
5. This contained genera such as *Alexandrium*, *Prorocentrum*, and *Symbiodinium*. Major sterols were cholesterol and 4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol (dinosterol);
6. This contained *Alexandrium*, *Gymnodinium*, *Heterocapsa*, *Pfiesteria*, *Pyrocystis* and *Thoracosphaera*. Prominent sterols were 4α,24-dimethyl-5α-cholestan-3β-ol, 4α,23,24-trimethyl-5α-cholestan-3β-ol (dinostanol) and dinosterol.

Several of these groupings matched well with DNA-based phylogeny, but note that some genera occur in two groups as do some sterols. Volkman et al. (1999b) examined the sterols of four species of the dinoflagellate genus *Prorocentrum* and found over 20 sterols which varied considerably in abundance between the species. LeBlond et al. (2010) found that all *Prorocentrum* species were closed related by 18S rDNA analysis, but based on their sterols they were distributed in both clusters 5 and 6. All contained 23,24-dimethylcholesta-5,22E-dien-3β-ol, dinosterol and dinostanol. Those in cluster 5 also produced cholesterol not found in cluster 6 while those in cluster 6 produced 24-methylenecholesterol not found in cluster 5. Similarly, *Pyrocystis lunula* and *Pyrocystis noctiluca* (=*Pyrocystis pseudonoctiluca*), although closely related by 18 S rDNA analysis had quite different sterol distributions with dinosterol abundant in *P. noctiluca* but absent from *P. lunula* (Dahmen and Leblond 2011).

Sterols containing a cyclopropyl ring in the side-chain such as gorgosterol are known from various marine animals, particularly coelenterates and a few dinoflagellates (Withers 1987). Some species of *Gonyaulax* contain large amounts of cholesterol but there does not appear to be a single distribution of 4-desmethyl sterols which characterizes these algae.

Karenia brevis has been shown (Leblond and Chapman 2002) to possess two major sterols, (24S)-4α,24-dimethyl-5α-cholesta-8(14),22-dien-3β-ol (abbreviated ED in their work) and its 27-nor derivative (NED). These novel structures are also found in *Karenia mikimotoi* and *Karlodinium micrum* (=*Karlodinium veneficum*), two dinoflagellates closely related to *K. brevis* (Leblond and Chapman 2002). They are also found as minor components of the more complex sterol profiles of other members of the *Gymnodinium*/*Peridinium*/*Prorocentrum* (GPP) taxonomic group (Leblond and Chapman 2002).

A predominance of the 4-methyl and 4-desmethyl $\Delta^{8(14)}$ sterols and a lack of dinosterol was reported by Mooney et al. (2007) for species in the Karenaceae. Unusual sterols included 23-methyl-27-nor-24-methylcholesta-8(14),22-dien-3β-ol (*Karenia papilionacea*, 59–66 %); 27-nor-(24R)-4α,24-dimethyl-5α-cholesta-8(14),22-dien-3β-ol (brevesterol; *Takayama tasmanica* 84 %, *Takayama helix* 71 %, *Karenia brevis* 45 %, *Karlodinium* sp.? 40 %, *Karenia mikimotoi* 38 %); and 4α,24-dimethyl-5α-cholesta-8(14),22-dien-3β-ol (gymnodinosterol; *K. mikimotoi* 48 %, *Karenia umbella* 59 %, *Karlodinium veneficum* 71–83 %). In *Takayama* species, five steroid ketones were identified, including for the first time the 3-keto form of brevesterol and gymnodinosterol. Steroid ketones have also been reported in *Scrippsiella trochoidea* (Harvey et al. 1988), *Prorocentrum* spp. (Volkman et al. 1999b) and *Crypthecodinium cohnii* (Withers et al. 1978).

8 Sterols in the Phylum Haptophyta

Haptophytes usually contain from one to five major sterols, and commonly cholesterol or 24-methylcholesta-5,22E-dien-3β-ol predominates (Volkman et al. 1981). Moderate amounts of the C_{29} sterols 24-ethylcholesterol and 24-ethylcholesta-5,22E-dien-3β-ol are found in several species (Conte et al. 1994).

8.1 Class Coccolithophyceae

Well known examples of this class include the coccolithophorids *Emiliania huxleyi* and *Gephyrocapsa oceanica*. Both are major sources of organic matter in marine ecosystems and can form large blooms easily visible to satellites due to shedding of their coccolith scales. The sterol distributions of both species are very simple and are dominated (>90 %) by 24-methylcholesta-5,22E-dien-3β-ol (Volkman et al. 1980b, 1995). The principal sterol in *Pleurochrysis carterae* and an unidentified haptophyte strain CCMP1215 was also shown to be 24-methylcholesta-5,22E-dien-3β-ol by Ghosh et al. (1998). In *E. huxleyi*, the stereochemistry at C-24 has been shown to be 24α (Maxwell et al. 1980) as found also in the sterols of *Pleurochrysis carterae* which contains stigmasterol (24α-ethylcholesta-5,22E-dien-3β-ol) and *epi*brassicasterol (Gladu et al. 1990). *Chrysotila lamellosa* (= *Ruttnera lamellosa*) also contains 24α-methylcholesta-5,22E-dien-3β-ol (*epi*brassicasterol) as well as significant amounts of Δ^5- and $\Delta^{5,22}$-C_{29} sterols (Rontani et al. 2004). Goad et al. (1983) identified 24α-methylcholesta-5,22E-dien-3β-ol as the major sterol of the marine haptophyte *Isochrysis galbana*.

Phaeocystis pouchetii is a major phytoplankton species in polar oceans and a major food source for krill (Hamm et al. 2001). Its dominant sterol (93–100 %) is 24-methylcholesta-5,22E-dien-3β-ol irrespective of culture age or life stage (Nichols et al. 1991). Small amounts of cholesterol (8 %) were found in one strain (A1-3) and strain DE10 contained 24-methylenecholesterol (Nichols et al. 1991). In marked contrast to the above results, Ghosh et al. (1998) showed that *Prymnesium parvum* contained only small amounts of sterols consisting solely of cholesterol.

Hymenosulphate, a novel sterol sulphate with Ca-releasing activity has been isolated from the cultured marine haptophyte *Hymenomonas* sp. by Kobayashi (1989).

8.2 Class Pavlovophyceae

A number of papers have reported the unusual sterol compositions of species in the genus *Pavlova*. Volkman et al. (1990) reported the presence of 4-methylsterols, 5α(H)-stanols,

4-desmethyl sterols and unusual dihydroxylated sterols called pavlovols (Fig. 5). The major 4-desmethyl sterol in each of the species analysed by these authors was 24-ethylcholesta-5,22E-dien-3β-ol which occurred with smaller amounts of 24-ethylcholesterol and in some species cholesterol. Two species also contained significant amounts of the 5α(H)-stanol 24-ethyl-5α-cholest-22E-en-3β-ol. The major 4-methyl sterol was a C_{30} sterol identified as 4α-methyl-24-ethyl-5α-cholest-22E-en-3β-ol. This sterol has a similar structure to dinosterol, which occurs in dinoflagellates, except that the side-chain contains a 24-ethyl group rather than 23,24-dimethyl substitution (Fig. 5). Minor 4-methylsterols included 4,24-dimethyl-5α-cholest-22E-en-3β-ol and the fully saturated stanol 4,24-dimethylcholestanol.

Pavlovols have a second hydroxyl group at C-4 and a C-4 methyl group (Fig. 5) in the sterol ring system (e.g. Volkman et al. 1990; Gladu et al. 1991a; Patterson et al. 1992; Véron et al. 1996; Rauter et al. 2005) and have been proposed as a chemotaxonomic marker for this subgroup of haptophytes (Volkman et al. 1997). Mass spectra of the TMSi-ether derivatives of 4α,24-dimethyl-5α-cholestan-3β,4β-diol and 4α-methyl,24-ethyl-5α-cholestan-3β,4β-diol found in *Pavlova pinguis* and 4α,24-dimethyl-5α-cholest-22E-en-3β,4β-diol found in *Diacronema vlkianum* can be found in Volkman et al. (1997).

9 Sterols in the Phylum Euglenophyta

9.1 Class Euglenophyceae

This genus was established by Ehrenberg to accommodate those euglenoid organisms that have eyespots. *Euglena* (*Astasia*) *longa*, a natural mutant of *Euglena* that has lost all potential for photosynthesis contains cycloartenol metabolites indicative of biosynthesis by a plant-type mechanism (Anding et al. 1971; Rohmer and Brandt 1973) since lanosterol was not detected (Anding et al. 1971). Anding and Ourisson (1973) reported the presence of ergosterol in both light-grown and dark-grown *Euglena gracilis* and an unusual 4-methylsterol 4α,24-dimethyl-5α-cholest-8(9)-en-3β-ol has also been reported (Anding et al. 1971).

Brandt et al. (1970) found that free sterols predominate over bound sterols in light-grown green-coloured *E. gracilis* whereas in dark-grown white cells the reverse is true. The free sterols of green cells consist almost exclusively of Δ^7-sterols (98 %) while in white cells Δ^5-sterols make up 31 % of the sterols.

Zielinski et al. (1982) reported that the freshwater *Eutreptia viridis* contained 18 different sterols including a novel sterol with the rare Δ^{23}-unsaturation 24-ethylcholesta-5,7,23Z-trien-3β-ol. The free sterols were dominated by $\Delta^{5,7}$-diunsaturated sterols (ca. 80 %).

10 Sterols in the Phylum Cryptophyta

10.1 Class Cryptophyceae

The Cryptophyceae is a class of algae within the Pyrrhophyta in some systems of classification. Cryptomonads are aquatic unicellular eukaryotes that inhabit both marine and freshwater environments. Most cryptomonads are photosynthetic (and are thus also referred to as cryptophytes) and possess plastids that are very diverse in pigmentation. Cyptomonads are common in freshwater systems but can also be found in marine and brackish habitats. Each cell is around 10–50 μm in size and flattened in shape, with typically two slightly unequal flagella.

Dunstan et al. (2005) examined the sterols of seven cryptophytes. The major sterol in *Rhodomonas* spp. (CS-215, CS-694), *Rhodomonas salina* (CS-174, CS-24), and *Proteomonas sulcata* (CS-412) was 24-methylcholesta-5,22E-dien-3β-ol (91–99 % of total sterols) together with small amounts of cholesterol (1–2.7 %). *Rhodomonas maculata* (CS-85) had the same two sterols, but cholesterol was more abundant (17.7 %). *Chroomonas placoidea* (CS-200) contained in addition the C_{29} sterol 24-ethylcholesta-5,22E-dien-3β-ol (35.5 %).

11 Sterols in the Phylum Glaucophyta

11.1 Class Glaucophyceae

Glaucophytes (or Glaucocystophytes) are freshwater algae that have an almost intact cyanobacterium, referred to as a cyanelle, as the photosynthetic organelle. Heimann et al. (1997) reported the presence of "sitosterol" and an identified sterol in *Cyanophora paradoxa*. A more recent analysis by Leblond et al. (2011) found that *C. paradoxa* and *Glaucocystis nostochinearum* contained very simple sterol distributions consisting of sterols more typically found in higher plants: 24-methylcholesterol, 24-ethylcholesta-5,22E-dien-3β-ol, and 24-ethylcholesterol.

12 Sterols in the Phylum Picozoa

Picoeukaryotes (defined as cells <3 μm) are now known to be ubiquitous in surface waters of all oceans and are likely to be the most abundant eukaryotes in the sea. Most are phototrophic, but some are heterotrophic, especially in oligotrophic coastal sites. In 2007, a novel and widespread picoeukaryotic lineage with affinities to cryptophytes and katablepharids, the "picobiliphytes" was reported from 18S environmental clone library sequences (Not et al. 2007). Until the work of Seenivasan et al. (2013) these heterotrophs

(which may be related to glaucocystophytes) had remained uncultured. These authors described *Picomonas judraskeda* gen. et sp. nov., from marine coastal surface waters, and established a new phylum, Picozoa. No sterol data are available as yet from cultures of these ecologically important organisms.

13 Sterols in the Phylum Cercozoa

13.1 Class Chlorarachniophyceae

Chlorarachniophytes are marine unicellular algae that possess secondary plastids of green algal origin. Although chlorarachniophytes are a small group (the phylum of Chlorarachniophyta contains 14 species in 8 genera), they have variable and complex life cycles that include amoeboid, coccoid, and/or flagellate cells (Hirakawa et al. 2011). They are typically mixotrophic and photosynthetic and have the form of small amoebae, with branching cytoplasmic extensions that capture prey and connect the cells together. The amoeboid morphology may be the result of secondary endosymbiosis of a green alga by a nonphotosynthetic amoeba or amoeboflagellate. The only sterols present in genera *Bigelowiella*, *Gymnochlora*, and *Lotharella* were identified as 24α-methylcholesta-5,22E-dien-3β-ol) and one of the epimeric pair poriferasterol/stigmasterol (24-ethylcholesta-5,22E-dien-3β-ol) (Leblond et al. 2005).

14 Sterols in the Phylum Heterokontophyta (Ochrophyta)

The heterokonts (or stramenopiles) are chromists with chloroplasts surrounded by four membranes. There are more than 100,000 known species. Given the diversity of species present and wide variety of evolutionary paths involved (Leipe et al. 1994) it is not surprising that a great diversity of sterol patterns exist for this Phylum.

14.1 Diatom Classes

Diatoms are photosynthetic secondary endosymbionts found throughout marine and freshwater environments, and are believed to be responsible for around one-fifth of the primary productivity on Earth (Bowler et al. 2008 and refs therein). In spite of the fact that the pennate and centric lineages have only been diverging for 90 million years, their genome structures are dramatically different and a substantial fraction of genes (ca. 40 %) are not shared.

The sterols of diatoms have been the best studied of all the algal classes with data available for more than 100 species (e.g. Orcutt and Patterson 1975; Volkman 1986; Barrett et al. 1995; Rampen et al. 2009a, b, c, 2010). Rampen et al. (2010) analysed the sterols of over 100 diatom strains and detected 44 different sterols of which 11 were considered to be major sterols (i.e. >10 % of total sterols). Two-thirds of the species contained 24-methylenecholesterol, but 24α-methylcholesta-5,22E-dien-3β-ol (*epi*brassicasterol) which is often used as a diatom marker was only the fifth most common sterol. Cholesterol was abundant in a few species, but in *Amphora* species 24-ethylcholesta-5,22E-dien-3β-ol predominates (Gladu et al. 1991b).

Some diatoms have been reported to contain large amounts of Δ^7-unsaturated sterols (e.g. *Thassiosira pseudonana*, *Odontella* (*Biddulphia*) *aurita* and *Fragilaria* sp.; Orcutt and Patterson 1975), but this seems to be rare. Giner and Wikfors (2011) have recently reported Δ^7 sterols in *Ditylum brightwellii*.

A few diatoms lack appreciable amounts of C-24 alkylated sterols. One example is *Biddulphia sinensis* (now *Odontella sinensis*) which contains mostly cholesta-5,22E-dien-3β-ol (Volkman et al. 1980a). In contrast *Biddulphia aurita* (now *Odontella aurita*) analysed by Orcutt and Patterson (1975) contained none of this sterol and among the six identified sterols were the uncommon sterols 24-methylcholest-8(9)-en-3β-ol (22.8 %) and 24-methyl-5α-cholesta-7,22E-dien-3β-ol (18 %).

One interesting report is the presence of the C_{30} sterol gorgosterol in species from the genus *Delphineis* (Rampen et al. 2009c). Figure 6 shows a chromatogram of the sterols found in this alga. This sterol contains a cyclopropyl group in the side-chain which is more commonly associated with the sterols of jellyfish and highlights the diversity of sterol biosynthesis pathways developed in these microalgae. Giner and Wikfors (2011) have confirmed using NMR that the pennate diatom *Delphineis* sp. (CCMP 1095) contains gorgosterol, as well as the 27-nor C_{27} sterol occelasterol.

Volkman et al. (1993) showed that a strain of *Navicula* sp. (CS-146) contained small amounts of the dinoflagellate marker sterol dinosterol plus other 4-methyl sterols including 4α,24-dimethyl-5α-cholest-22E-en-3β-ol (9.7–12.6 %), 4α,24-dimethyl-5α-cholestan-3β-ol (2.0–3.4 %) and 4α,23,24-trimethyl-5α-cholestan-3β-ol (dinostanol or its C-23/C-24 epimer; 0.3–0.6 %). The 4-desmethyl sterol fraction included the common diatom sterol 24-methylcholesta-5,22E-dien-3β-ol (20.2–30.5 %), but the major sterol was 24-ethylcholesterol (31.0–38.6 %) more usually associated with higher plants.

Sterols containing 23-methyl group rather than the more usual 24-methyl group are also found in diatoms. 23-Methylcholesta-5,22E-dien-3β-ol was found in 14 out of 106 diatom cultures studied by Rampen et al. (2009a) thus

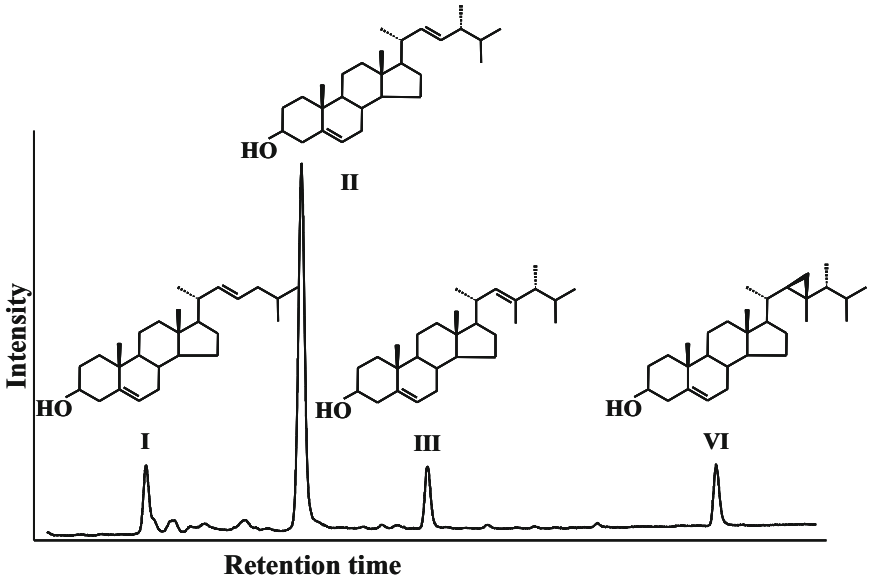


Fig. 6 Chromatogram showing the main sterols present in *Delphineis* sp. CCMP 1095. (*I*) Cholesta-5,22E-dien-3β-ol; (*II*) 24-Methylcholesta-5,22E-dien-3β-ol; (*III*) 23,24-Dimethylcholesta-5,22E-dien-3β-ol; (*IV*) 22,23-Methylene-23,24-dimethylcholest-5-en-3β-ol (gorgosterol) with structures of the major sterols (From Rampen et al., 2009c)

confirming earlier reports of such sterols in diatoms (Barrett et al. 1995). These data confirm that this unusual pattern of side-chain alkylation is not restricted to the dinoflagellates. Rampen et al. (2009b) further showed that diatoms are a likely source of steroidal hydrocarbons containing 23,24-dimethyl alkylation in sediments and petroleum since their precursor 4-desmethyl-23,24-dimethyl sterols were present in 22 of the cultures they studied.

Giner and Wikfors (2011) re-examined the occurrence of sterols with 23,24-dimethyl side-chains in diatoms. The centric diatom *Triceratium dubium* (= *Biddulphia* sp. CCMP 147) contained a high proportion of 23-methylated sterols of which 23,24-dimethylcholesta-5,22E-dien-3β-ol was 37.2 % of total sterols. They also showed that the sterol composition of *Ditylum brightwellii* (CCMP 358) is very complex and includes 5α(H)-stanols and Δ^7-sterols, in addition to the predominant Δ^5-sterols. A pair of previously unknown sterols, 24-ethylcholesta-5,24,28-trien-3β-ol and 24-ethylcholesta-24,28-dien-3β-ol, were also detected and their structures determined by NMR and by synthesis of the former sterol derived from saringosterol. Also detected in *D. brightwellii* was the previously unknown 23-methyl-5α-cholesta-7,22-dien-3β-ol.

14.2 Class Eustigmatophyceae

Eustigmatophytes are a small group of coccoid microalgae. Most are freshwater or live in soils, with the main marine species represented by the genus *Nannochloropsis*. Their colour is distinctive due to the presence of the accessory pigments violaxanthin and β-carotene. Eustigmatophytes contain unusual long chain C_{28}–C_{32} *n*-alkyl-1,15-diols, and the corresponding hydroxy ketones (Volkman et al. 1992, 1999a; Méjanelle et al. 2003).

Sterol data for this algal class are rather limited. Volkman et al. (1999a) analysed three freshwater species *Eustigmatos vischeri*, *Vischeria helvetica* and *Vischeria punctata* and demonstrated that the sterol distributions consisted predominantly of 24-ethylcholesterol with small amounts of cholesterol, 24-methylcholesterol, 24-ethylcholesta-5,22E-dien-3β-ol and isofucosterol.

The high proportion of C_{29} sterols in freshwater species is in marked contrast to marine species from the genus *Nannochloropsis* which contain a dominance of cholesterol (Volkman et al. 1992; Patterson et al. 1994; Méjanelle et al. 2003). Volkman et al. (1992) studied the lipids of *N. oculata*, *N. salina* and an un-named species and found simple distributions of sterols dominated by cholesterol (>75 %) together with small amounts of the C_{29} sterols 24-ethylcholesta-5,24(28) E-dien-3β-ol (fucosterol) and 24-ethylcholesta-5,24(28) Z-dien-3β-ol (isofucosterol). *N. salina* also contained 24-ethylcholesterol. The sterol composition of *N. gaditana* is similar with a predominance of cholesterol and lesser amounts of 24-ethylcholesterol (Méjanelle et al. 2003). Véron et al. (1996) showed that the sterols in *N. oculata* were mostly esterified rather than being present as free sterols.

The value of sterols as a chemotaxonomic tool was demonstrated by Gladu et al. (1995) who studied strain UTEX 2341 which had previously been identified as *Chlorella minutissima*. This strain contained cholesterol as the principal sterol along with 24-methylenecholesterol, fucosterol, and isofucosterol which was inconsistent with any of 35 *Chlorella* strains analyzed at that time. This finding and other data showed that the strain was actually a eustigmatophyte.

14.3 Class Synurophyceae

The Synurophyceae are keterokonts closely related to the Chrysophyceae. Both have a long "flimmer" flagellum and a short "whiplash" flagellum. Fucoxanthin is the main pigment responsible for their "golden brown" colouration. Synurophytes have bristles and scales which are taxon-specific. Silicified resting stages called stomatocysts are unique to the Chrysophyceae and Synurophyceae and may be preserved in sediments facilitating their use as palaeoenvironmental indicators. The oldest silicified scales and bristles are found in Middle Eocene freshwater sediments from northwest Canada, but the oldest stomatocysts have been found in Early Cretaceous marine sediments from the Southern Ocean.

An early study by Collins and Kalnins (1969) of the sterols in *Synura petersenii* (then assigned to the Chrysophyta) identified only two sterols identified as cholesterol and sitosterol.

14.4 Class Chrysophyceae

Chrysophytes have been subject to considerable revision with many species moved to other classes such as the closely related Synurophyceae and Pelagophyceae (Jordan and Iwataki 2012). One of the first lipid studies of *Ochromonas danica* was by Halevy et al. (1966) who found four sterols and identified the C_{28} sterol ergosterol and C_{29} sterol 24-ethylcholesta-5,22E-dien-3β-ol (reported as the 24α isomer stigmasterol). Further work on *Ochromonas danica* and *Poteriochromonas* (*Ochromonas*) *malhamensis* was carried out by Gershengorn et al. (1968) who found cycloartenol and 24-methylenecycloartanol but no lanosterol thus providing evidence of the plant-type biosynthetic pathway used by these algae (Fig. 2). The C_{29} sterol 24β-ethylcholesta-5,22E-dien-3β-ol (poriferasterol) was identified as the major sterol. Melting points were used to define the stereochemistry as 24β which is opposite to the 24α stereochemistry implied by the identification of stigmasterol by Halevy et al. (1966). The C_{28} sterol 24β-methylcholesta-5,22E-dien-3β-ol (brassicasterol) has been identified in an unidentified species assigned to the order Sarcinochrysidales (Chrysophyceae) (Rohmer et al. 1980; Kokke et al. 1984).

The sterols of *Ochromonas danica* have been identified as ergosterol, brassicasterol, 22-dihydrobrassicasterol, clionasterol, poriferasterol, and probably 7-dehydroporiferasterol. By contrast *Poteriochromonas malhamensis* contains only poriferasterol as the major sterol component.

Poteriochromonas (*Ochromonas*) *malhamensis* was one of the first species used to probe the biosynthetic pathways occurring in microalgae. For example, Knapp et al. (1971) were able to show that the three 24-ethylidene sterols fucosterol, iso-fucosterol and 24-ethyl-5α-cholesta-7,24(28)-dien-3β-ol could all be transformed to poriferasterol, by *P. malhamensis*, but with varying degrees of efficiency,

Billard et al. (1990) showed that the C_{29} sterols 24-ethylcholesta-5,22E-dien-3β-ol, 24-ethylcholesterol and fucosterol predominated in the genera *Chrydoderma*, *Chrysowaernella*, *Chrysomeris* and *Giraudyopsis*. The status of the first genus is uncertain, but the latter three are now classified in the class Chrysomerophyceae. This is in marked contrast to the sterols found in *Sarcinochrysis* and *Nematochrysopsis* which contain the C_{30} sterol 24-*n*-propylidenecholesterol (previously assigned to the Chrysophyceae, but now considered to be part of the Pelagophyceae).

A paleoenvironmental application by Soma et al. (2007) linked the profile of 24-ethylcholesta-5,22E-dien-3β-ol in steryl chlorin esters (SCEs) in sediments of Lake Baikal over the past 28,000 years with the reported distribution of chrysophyte cysts during the Holocene.

14.5 Class Chrysomerophyceae

Chrysomeris ramosa contains 24-ethylcholesterol as the major sterol together with 24-ethylcholesta-5,22E-dien-3β-ol, 24-methylcholesterol and cholesterol (Billard et al. 1990), but percentage data were not provided. *Giraudyopsis stellifer* also contained 24-ethylcholesterol as the major sterol, but other sterols included fucosterol, 24-ethylcholesta-5,22E-dien-3β-ol, 24-methylenecholesterol, 24-methylcholesta-5,22E-dien-3β-ol and cholesterol (Billard et al. 1990).

14.6 Class Pelagophyceae

These small unicellular algae were formerly grouped with Chrysophyceae and are classified into two orders: Pelagomonadales and Sarcinochrysidales (Giner et al. 2009). These are the only algae known to date that synthesize the C_{30} sterol 24-propylidene cholesterol. In a comprehensive study, Giner et al. (2009) analysed the sterol compositions of 42 strains of pelagophyte algae including 17 strains of *Aureococcus anophagefferens* using a combination of GC and HPLC techniques. ^{1}H-NMR data were obtained for 17 strains. All strains analyzed contained 24-propylidenecholesterol. All strains from the order Sarcinochrysidales contained the (24E)-isomer, while all strains in the order Pelagomonadales contained the (24Z)-isomer, either alone or together with the (24E)-isomer. The occurrence of Δ^{22} and 24α-sterols was limited to the Sarcinochrysidales. The first occurrence of 24-n-propylcholesta-5,22E-dien-3β-ol in an alga, CCMP

1410, was reported. Traces of the rare sterol 26,26-dimethyl-24-methylenecholesterol were detected in *Aureococcus anophagefferens*, and the (25R)-configuration was proposed, based on biosynthetic considerations. Traces of a novel sterol, 24-propylidenecholesta-5,25-dien-3β-ol, were detected in several species. Raederstorff and Rohmer (1984) analysed the sterols of *Nematochrysopsis roscoffensis* (=*N. marina*) and also found the C_{30} sterols 24(E)-24-*n*-propylidenecholesterol and 24-*n*-propylcholesterol.

14.7 Class Xanthophyceae

There are about 600 species and 100 families of Xanthophyceae, but most are very rare. Common genera include *Botrydium*, *Tribonema* and *Vaucheria*. These yellow-green microalgae are mostly found in fresh water or wet soil, but a few are marine. They can be confused with green algae because of their pigmentation, but they are actually secondary endosymbionts that evolved from protists that engulfed a microalga and assimilated its chloroplasts. Most xanthophytes are coccoid or filamentous, but some are siphonous. 18S ribosomal RNA gene analysis indicates that the Xanthophyceae is most closely related to the Phaeophyceae and that the class may be paraphyletic (Potter et al. 1997).

Mercer et al. (1974) studied the sterols of *Botrydium granulatum*, *Tribonema aequale* and *Monodus subterraneus* (= *Monodus subterranea*). In each case, the major sterols were cholesterol and 24β-ethylcholesterol (clionasterol), and their proportions did not vary with age of the cultures. Small amounts of cycloartenol and 24-methylenecycloartanol were also found in all three algae. The biosynthesis of clionasterol in *M. subterranea* was studied by Mercer and Harries (1975). Clionasterol grown in the presence of labelled methionine showed the participation of a 24-ethylidene sterol intermediate in its biosynthesis. Data from cells incubated with labelled mevalonic acid showed that the 24-ethylidene sterol intermediate is reduced directly to clionasterol and not isomerized to a Δ^{24}-sterol which is then reduced.

14.8 Class Dictyochophyceae

Dictyocha is the generic name for all extant silicoflagellates, or for species in which the basket-shaped skeleton consists of a single ring with several connecting bars (Kristiansen and Preisig 2001). Few data are available for these microalgae. Patterson and Van Valkenburg (1990) analysed the sterols from cultured *Dictyocha fibula* and found only 4-methyl-cholesta-5,22E-dien-3β-ol and 24-methylenecholesterol.

14.9 Class Raphidophyceae

Marine and freshwater raphidophytes form a monophyletic group and 18S ribosomal RNA gene sequences suggest that the Raphidophyceae is a sister taxon to the Phaeophyceae-Xanthophyceae clades, but the bootstrap value was only 40 % (Potter et al. 1997). These microalgae are of interest for their propensity to form harmful algal blooms in both marine (brown pigmented species) and freshwater (green pigmented species) environments. The lipids of Raphidophyceae microalgae still remain understudied and the taxonomy is not clearly established. Fatty acids and pigments have been used as chemotaxonomic markers for these algae (Mostaert et al. 1998) as well as sterols (Nichols et al. 1987; Patterson and Van Valkenburg 1990; Marshall et al. 2002; Giner et al. 2008; Leblond et al. 2013).

Nichols et al. (1987) reported data on the sterols and fatty acids of the red tide flagellates *Heterosigma akashiwo* and *Chattonella antiqua* (=*Chattonella marina var. antiqua*) and this work was expanded by Marshall et al. (2002) who reported the sterols and fatty acids in six marine raphidophyte algae. The dominant sterol in *Chattonella* spp., *H. akashiwo* and *Fibrocapsa japonica* was identified as 24-ethylcholesterol, but the configuration at C-24 was not specified at that time (Nichols et al. 1987; Marshall et al. 2002); a reinvestigation of the sterols of *C. marina* using ^{1}H NMR spectrometry indicated the presence of the 24α-configuration (sitosterol) (Giner et al. 2008). The *Chattonella* species also contained small amounts of the uncommon sterol cholest-8(9)-en-3β-ol (1.8–5 %). *F. japonica* contained the highest proportion of 24-ethylcholesterol (84–92 %) with small amounts of cholesterol (5–11 %) and isofucosterol (24-ethylcholesta-5,24(28)Z-dien-3β-ol; ca. 2 %; Marshall et al. 2002).

Giner et al. (2008) also analysed the sterols and fatty acid compositions of three harmful algal species previously classified in the genus *Chattonella* (Raphidophyceae). These were "*Chloromorum toxicum*" (ex a North American strain identified originally as *Chattonella* cf. *verruculosa*, and probably a member of the Raphidophyceae; Edvardsen et al 2007), *Verrucophora farcimen* (= *Pseudochattonella farcimen*; Dictyochophyceae), and *Verrucophora verruculosa* (= *Pseudochattonella verruculosa*; Dictyochophyceae). *C. toxicum* contained the 24β-ethyl sterols, poriferasterol (24β-ethylcholesta-5,22E-dien-3β-ol) and clionasterol (24β-ethylcholesterol), as its major sterols. In contrast, the stereochemistry of the 24-ethyl sterols of *Chattonella marina* and *Heterosigma akashiwo*, was determined to be 24α and 24β, respectively (Giner et al. 2008). Both *Pseudochattonella* (*Verrucophora*) strains contained the 27-nor C_{27} sterol occelasterol as the only detected sterol.

Patterson and Van Valkenburg (1990) reported that *Olisthodiscus luteus* contained 24-ethylcholesterol,

24-ethylcholestanol, 24-methylcholesterol and cholesterol. The presence of a fully saturated stanol in microalgae is unusual. The only sterol analysis of a freshwater species is by Leblond et al. (2013) who reported sterol compositions for 21 isolates of the green-pigmented, raphidophyte *Gonyostomum semen* from Scandinavian lakes. All contained the C_{29} sterols 24-ethylcholesta-5,22E-dien-3β-ol and 24-ethylcholesterol as major components together with smaller amounts of 24-methylcholesterol. The same three sterols occur in higher plants.

15 Conclusions

The published literature on the sterol compositions of microalgae continues to increase but it is still far from comprehensive. Extensive data are now available for green algae, diatoms and dinoflagellates from which it has been possible to assemble groupings of species containing common sterol distributions. In some cases these align with taxonomic groupings but in others species that are only distantly related can have similar compositions. Unfortunately, sterol data for some algal classes are still restricted to just a handful of species and some supposed characteristics may be challenged as additional compositional data are obtained. It is apparent that some earlier sterol identifications are now known to be in error (particularly with regard to C-24 stereochemistry) and many sterols are more widely distributed than had been thought. This should not be a surprise since sterol biosynthesis is an ancient microalgal trait and variations in biosynthesis are to be expected over such geologically long time periods. As sequence data become more readily available it will be useful to test these sterol groupings against taxonomies defined from genetic data and to discover what changes in genetic makeup are responsible for variations in sterol compositions.

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Carotenoids

Einar Skarstad Egeland

1 Introduction

This chapter intends to give an overview and an update of most aspects of microalgal carotenoids. Due to the limitations of a chapter format, only a brief description including selected examples can be given here, but the reader is referred to several books, chapters and articles, giving the possibility to look up more examples and details on the various topics.

Carotenoids are a group of natural compounds, easily recognised by their colour, as the orange β,β-carotene (= β-carotene) colouring carrots, the astaxanthin giving salmon flesh its distinct pinkish colour, or the red ψ,ψ-carotene (= lycopene) in tomatoes (see Fig. 1). The colours of carotenoids vary in general from bright yellow to dark red, although rare purple or colourless carotenoids have also been isolated from natural sources (Britton et al. 2004).

Carotenoids are synthesised by all kinds of photosynthetic bacteria, plants and algae, but also some non-photosynthetic bacteria and some fungi. Animals of any kind cannot produce carotenoids, but might absorb and metabolise carotenoids from their diet, e.g. algae (Britton et al. 1998).

Approximately 750 natural carotenoids are known (Britton et al. 2004), out of these, about 200 from algal sources, many of these encountered only in a very limited number of species (Egeland 2011a).

Despite their stability within living cells, carotenoids are unstable compounds when they are extracted from the cells, as they are not tolerant to oxygen from the air, to light, room temperature and acids; some are also not tolerant to bases. To work with carotenoids in the laboratory requires special equipment and very careful laboratory practice. The advantage is that carotenoids are brightly coloured; therefore they are easy to follow during extraction, separation, identification or other laboratory work.

1.1 Molecular Structure of Carotenoids

Carotenoids consist of 40 carbon atoms, which make a sort of backbone (Fig. 1). Regularly, both ends of the backbone end in a hexagon, although also open chained carotenoids such as ψ,ψ-carotene are widely encountered. If only carbon and hydrogen atoms are present in the molecule, these carotenoids, as a group, are called carotenes, while xanthophylls are carotenoids also having oxygen atom(s).

1.2 Nomenclature of Carotenoids

The carotenoids are named as a 'carotene' (IUPAC Commission on the Nomenclature of Organic Chemistry and IUPAC–IUB Commission on Biochemical Nomenclature 1975), proceeded by two Greek letters in alphabetical order describing the form of rings (or lack of ring) at each end of the backbone (Fig. 2).

To be able to describe the molecule, each carbon atom in a carotenoid structure is numbered. The numbering starts at both ends of the molecule and goes to the middle of the molecule, from 1 to 15 from the left end and from 1′ to 15′ from the right end. Finally, the methyl (CH_3) groups pointing out from the backbone are numbered, from 16 to 20 (16′–20′). Any stereochemistry, oxygen function, additional double (or triple) bonds or lack of double bonds are designated specifically by the use of prefixes, although the main oxygen function is given as a suffix after the stem name 'carotene'. Also, any shortening of the main chain is described by prefixes.

As an example, the main carotenoid in brown seaweeds, fucoxanthin, has the following scientific name: (3*S*,5*R*,6*S*,3′*S*,5′*R*,6′*R*)-3′-acetyloxy-5,6-epoxy-3,5′-dihydroxy-6′,7′-didehydro-5,6,7,8,5′,6′-hexahydro-β,β-caroten-8-one

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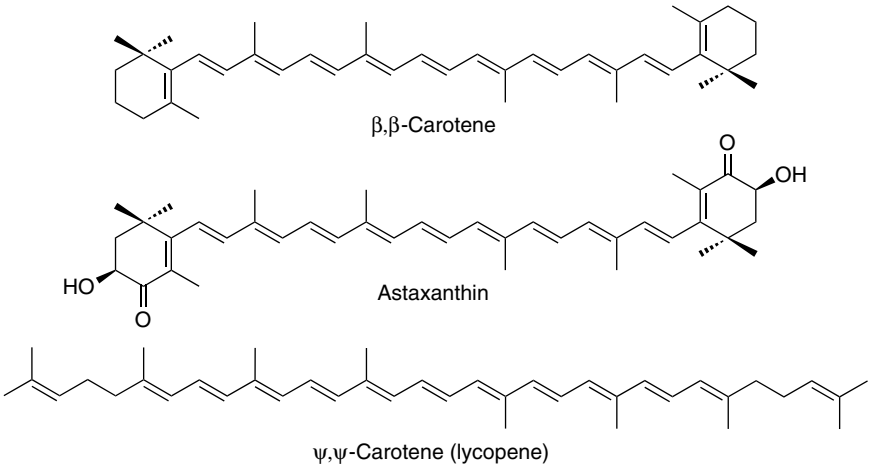


Fig. 1 Some common carotenoids

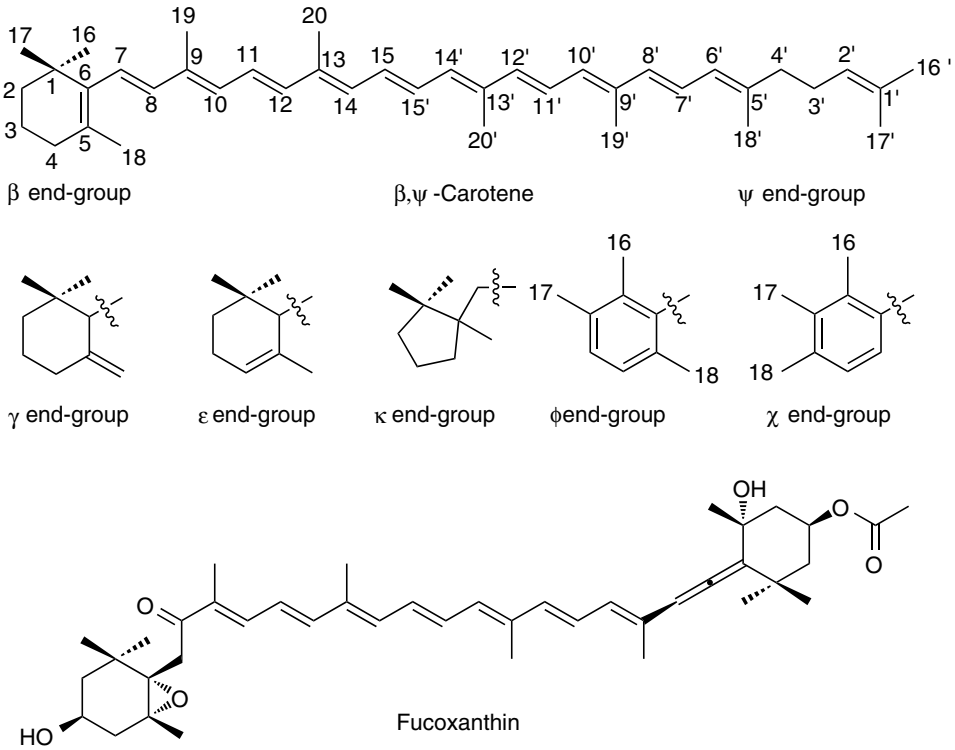


Fig. 2 Carotenoid end groups, numbering of carotenoid carbons; the structure of fucoxanthin = (3*S*,5*R*,6*S*,3′*S*,5′*R*,6′*R*)-3′-Acetyloxy-5,6-epoxy-3,5′-dihydroxy-6′,7′-didehydro-5,6,7,8,5′,6′-hexahydro-β,β-caroten-8-one

(Britton et al. 2004; Egeland 2011a; IUPAC 2014); see Fig. 2 for structure. Since the scientific names of xanthophylls are usually very long, the common names are used almost exclusively in the carotenoid literature, while the systematic names are commonly used for carotenes in modern literature.

1.3 Functions of Carotenoids in Algae

The main function of carotenoids in algae is, of course, to participate in light harvesting and transfer of the harvested light energy to chlorophylls in photosynthesis. Linked to the light harvesting in photosynthesis, the carotenoids also act as "sunglasses" when the algal cells are exposed to high irradiances, and as the carotenoids are antioxidants, they may also protect the cell from oxidation due to reactive singlet oxygen and radicals. A detailed description of the function of carotenoids in algal photosynthesis and light protection can be found in Young and Britton (1993), Larkum et al. (2003), and Falkowski and Raven (2007) and chapter "Photosynthesis and Light Harvesting in Algae" in this book (Larkum 2016).

Some algal species are famous for their production of secondary carotenoids (carotenoids produced only under certain occasions) when exposed to extreme conditions, e.g. the production of β,β-carotene by *Dunaliella salina* and astaxanthin

by *Chromochloris* (*Chlorella*) *zofingiensis*[1] or *Haematococcus pluvialis*, (Sun et al. 2008; Dufossé 2009; Jeffrey and Egeland 2009; Borowitzka 2010). These secondary carotenoid producers are popular for commercial purposes. Some examples are given later in this chapter.

The ability of carotenoids stabilising cell walls and cell membranes (Gruszecki 2010) has been described in bacteria (Britton 2008a and refs. therein), including the blue-green alga (cyanobacterium) *Synechocystis* (Mohamed et al. 2005).

Apocarotenoids (carotenoids with fewer than 40 carbon atoms) and carotenoid metabolites (metabolic cleaved carotenoids) act as hormones on some green algae (Moewus 1938, 1940; Nakanishi 1991; Boonyareth et al. 2009). Seaweeds have been found to release carotenoids (Saha et al. 2011; Grosser et al. 2012), and reported to act as an antimicrobial agent (Saha et al. 2011).

A general overview of various functions of carotenoids, not limited to algal cells, can be found in Britton et al. (2008).

1.4 Commercial Carotenoids

Various companies offer carotenoids for sale. For example, some companies produce a small number of carotenoids in large quantities for use as food and feed additives. The carotenoids may be offered in the pure form, or more often, as a formulated additive where the unstable carotenoids are supplied in gelatine or oil, protecting the carotenoid from air oxidation. These formulations may also help dissolving the carotenoid in water, so they may be used as additives to e.g. soft drinks. These carotenoids usually are made by chemical synthesis, although some companies are instead offering carotenoids isolated from algae, yeasts or flowers. The number of carotenoids offered is limited, due to the lack of good chemical syntheses or natural sources to isolate the carotenoids from. Furthermore, carotenoids, like other chemicals, to be used in food and feed must through a rigorous process to be accepted by health or animal welfare authorities. For further information, see Borowitzka (2013a) and references herein.

A few companies have instead specialised to offer carotenoids to be used in a laboratory scale, either in powder or crystalline form (usually 1 mg) for various laboratory experiments, or dissolved in an appropriate solvent (about 3 µg of carotenoid in 2.5 mL solvent) for high performance liquid chromatography (HPLC) calibration; see Egeland and Schlüter (2011) for details.

[1]Wherever possible the currently accepted names for species are used. The name used in the paper cited is also indicated. For details of names see chapter "Systematics, Taxonomy and Species Names: Do They Matter?" of this book (Borowitzka 2016).

2 Carotenoid Distribution in Algae

The "tree of life", describing the evolution of organisms and their connections, has recently been revised based on new insights provided by molecular studies, see Adl et al. (2005), Keeling et al. (2005), and Cavalier-Smith (2007).

Algae were earlier thought have evolved from common ancestors, but modern systematics is founded on the endosymbiont theory, where various colourless cells have incorporated algal cells, degrading them to chloroplasts. This lead to various algal groups having similar pigmentation, despite the algal cells not being evolutionary related to each other. The endosymbiont theory has been beautifully illustrated by Delwiche (1999) and expanded by Jeffrey et al. (2011) (Fig. 3).

This chapter gives an overview of carotenoids in the algae, covering the prokaryote blue-green algae (= cyanobacteria), eukaryote microalgae and seaweeds, and both freshwater and marine algal groups. For more details see the overviews given by Bjørnland and Liaaen-Jensen (1989), Liaaen-Jensen (1998), Liaaen-Jensen and Egeland (1999), and Jeffrey et al. (2011).

2.1 Prokaryotes: Cyanobacteria (Blue-Green Algae)

This group of prokaryotes has traditionally been recognised as blue-green algae, and is therefore included in this chapter, despite their modern names cyanobacteria or chloroxybacteria (Graham et al. 2009).

Cyanobacteria in general have chlorophyll *a* as their only chlorophyll. Several carotenoids are unique for cyanobacteria, e.g. the glycosidic carotenoids oscillatoriaxanthin and myxoxanthophyll, see Fig. 4 for structures. Besides the traditional cyanobacteria, some so-called prochlorophytes have distinct chlorophylls. According to Jeffrey et al. (2011), the cyanobacteria can be divided into five pigment groups, based on their chlorophyll and/or carotenoid content.

Traditional cyanobacteria (CYANO-1 and CYANO-2 in Jeffrey et al. 2011) are generally recognised by the carotenoid glycosides (CYANO-1), e.g. myxoxanthophyll, oscillaxanthin and aphanizophyll, although these are absent (CYANO-2) in some coccoid species (Hertzberg et al. 1971; Six et al. 2004; Jeffrey et al. 2011). A mixture of sugars bound to the parent carotenoid is commonly encountered (Hertzberg et al. 1971; Aakermann et al. 1992). Figure 4 shows carotenoids common in CYANO-1 and CYANO-2 cyanobacteria.

Other characteristic pigments include zeaxanthin, caloxanthin and nostoxanthin, the latter two being recognised by two hydroxygroups on the same β-ring, one of the hydroxyl

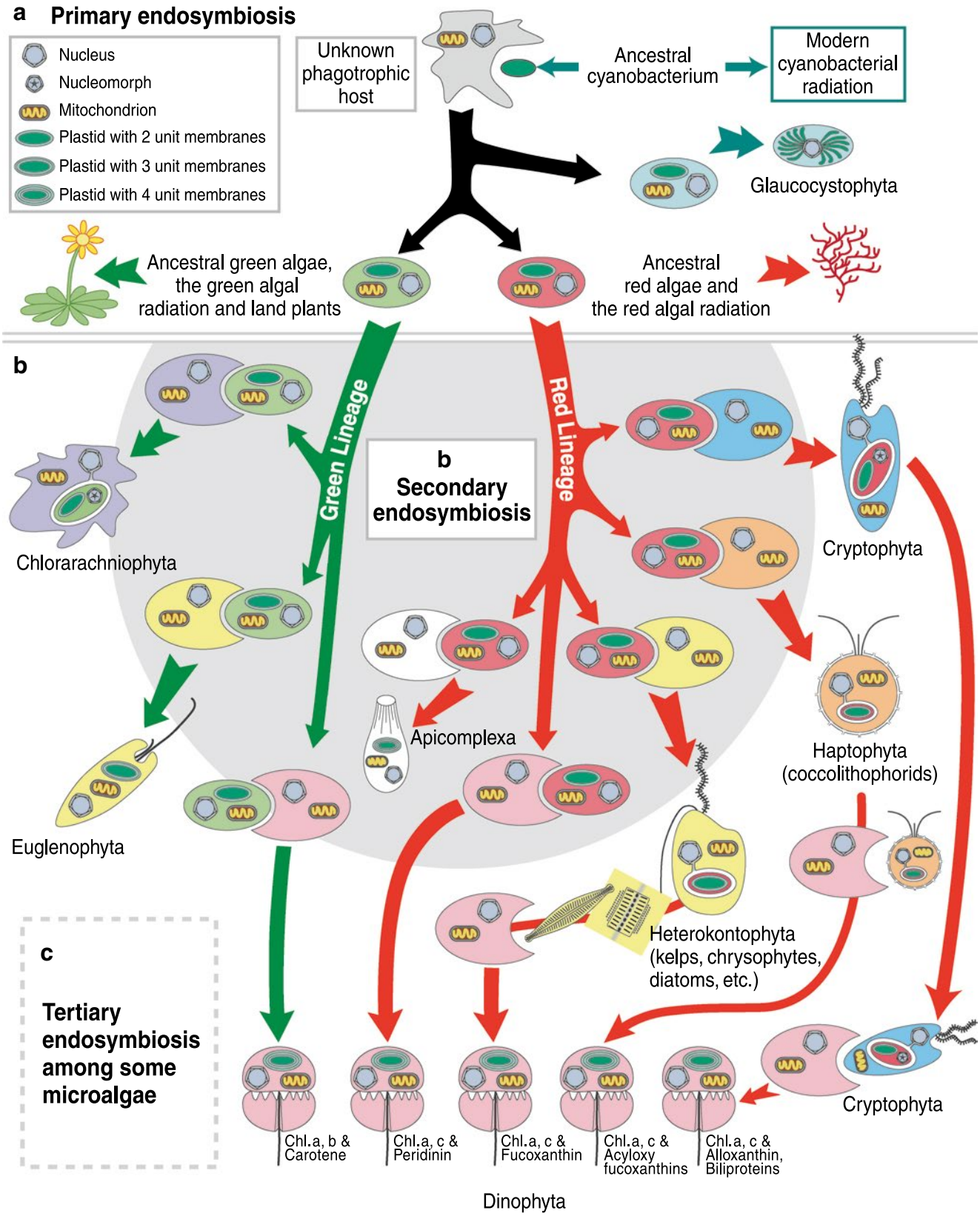


Fig. 3 The endosymbiont theory illustrated (**a**) Primary endosymbiosis (**b**) Secondary endosymbiosis (**c**) Tertiary endosymbiosis among some microalgae (From Jeffrey et al. (2011), based on Delwiche (1999) with permision)

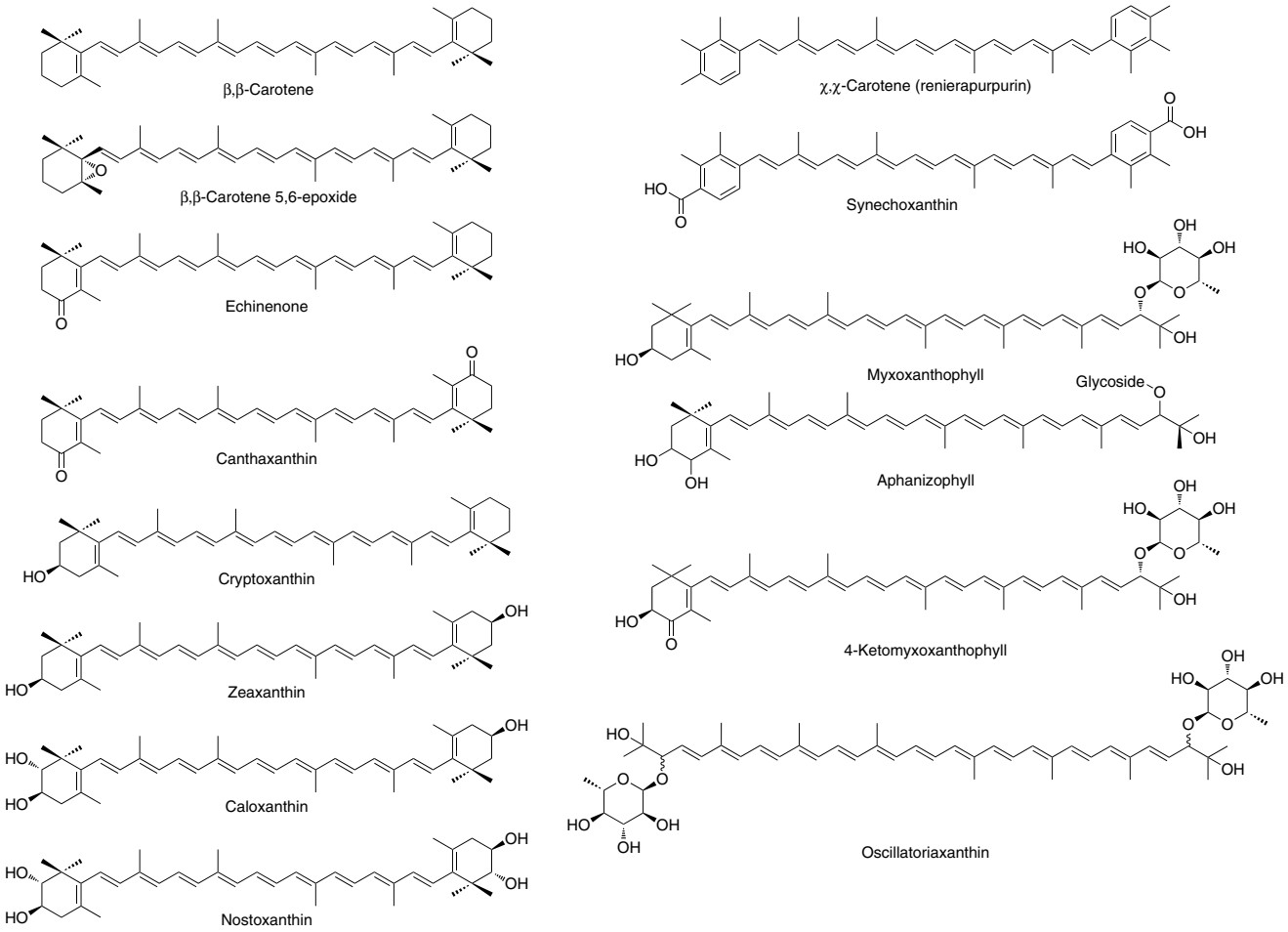


Fig. 4 Carotenoids of common cyanobacteria

groups are positioned in the rare 2-position. Ketocarotenoids like echinenone, canthaxanthin and 4-ketomyxoxanthophyll are common. Only one carotenoid with epoxidic end group, β,β-carotene-5,6-epoxide, generally isolated as its furanoxide derivative, may be present in minor amounts.

Although most species have many of these carotenoids present, only β,β-carotene has been reported from all species, often as the major carotenoid (Hertzberg et al. 1971; Aakermann et al. 1992; Palinska et al. 2011).

A variety of the CYANO-1 cyanobacteria pigmentation is encountered in the isolate *Synechococcus* sp. PCC 7002. This isolate produces the rare, aromatic χ end group in the carotenoids χ,χ-carotene (= renierapurpurin) and the novel synechoxanthin (Graham et al. 2008). These two χ,χ-carotenoids occurs together with common CYANO-1 carotenoids as myxoxanthophyll, zeaxanthin, cryptoxanthin and β,β-carotene. Chlorophyll *a* is reported to be the only chlorophyll.

The carotenoid content may vary within the same isolate when cultivated under diverse conditions; e.g. *Geitlerinema amphibium* cultivated under various irradiances and temperatures showed an almost inverse content of β,β-carotene and myxoxanthophyll (Jodłowska and Latała 2013) with β,β-carotene being dominant at low irradiance and high temperature, while myxoxanthophyll was dominant at high irradiance and low temperature.

Except for trace amounts, only chlorophyll *a* is encountered in CYANO-1 and -2; biliproteins are also present (Jeffrey et al. 2011).

CYANO-3 cyanobacteria, one of the so-called prochlorophyte groups, contain both chlorophyll *a* and *b*, along with carotenoids as zeaxanthin, cryptoxanthin and β,β-carotene (Jeffrey et al. 2011; Takaichi et al. 2012). For carotenoid structures, see Fig. 5.

CYANO-4 has the diethenyl (= divinyl) forms of chlorophylls *a* and *b*, together with zeaxanthin as the major carotenoid, besides β,β-carotene, β,ε-carotene (= α-carotene) and sometimes also minor amounts of ε,ε-carotene (= ε-carotene) (Jeffrey et al. 2011; Takaichi et al. 2012).

CYANO-5 have chlorophyll *d* as the main chlorophyll (Jeffrey et al. 2011), zeaxanthin and β,ε-carotene are major carotenoids, together with traces of zeinoxanthin, β,β-

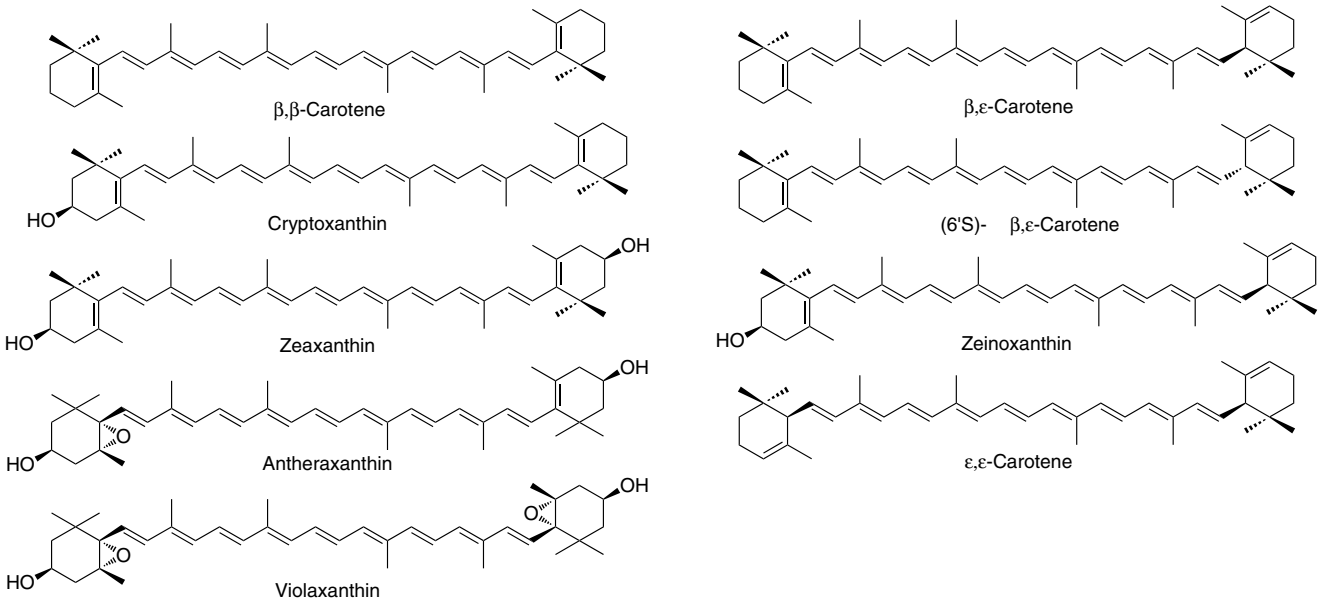


Fig. 5 Carotenoids of uncommon cyanobacteria (so-called 'prochlorophytes')

carotene and sometimes also cryptoxanthin (Takaichi et al. 2012). Surprisingly, the β,ε-carotene isolated from *Acaryochloris* strains was identified as the (6′*S*)-epimer (the mirror-image) of the common (6′*R*)-β,ε-carotene found in CYANO-4 and elsewhere (Takaichi et al. 2012).

Also, a chlorophyll *f* has been isolated together with chlorophyll *a* from the novel *Halomicronema hongdechloris*, accompanying chlorophyll *a* and the carotenoids violaxanthin, antheraxanthin, zeaxanthin and β,β-carotene have been claimed (for structures, see Fig. 5), carotenoids which are common in various algal groups, but not in any other cyanobacteria (Chen et al. 2010, 2012). Violaxanthin and antheraxanthin are the only hydroxylated epoxidic carotenoids to be reported from cyanobacteria, and only from this species; but the 'identification' was based on HPLC alone, see Sects. 4.4 and 4.5, this chapter. This pigmentation, if correct, would form a CYANO-6 group.

2.2 Glaucophytes and *Paulinella*

The carotenoids of the rare glaucophytes (glaucocystophytes) are little studied. β,β-Carotene, cryptoxanthin and zeaxanthin have been reported (see Fig. 5 for structures). Beside carotenoids, biliproteins and chlorophyll *a* are found (Jeffrey et al. 2011 and refs. therein). The pigmentation indicates a cyanobacterium as an endosymbiont.

The parasitic *Paulinella* species are other organisms that have incorporated cyanobacteria as endosymbionts, but no information about pigmentation has been found in the literature.

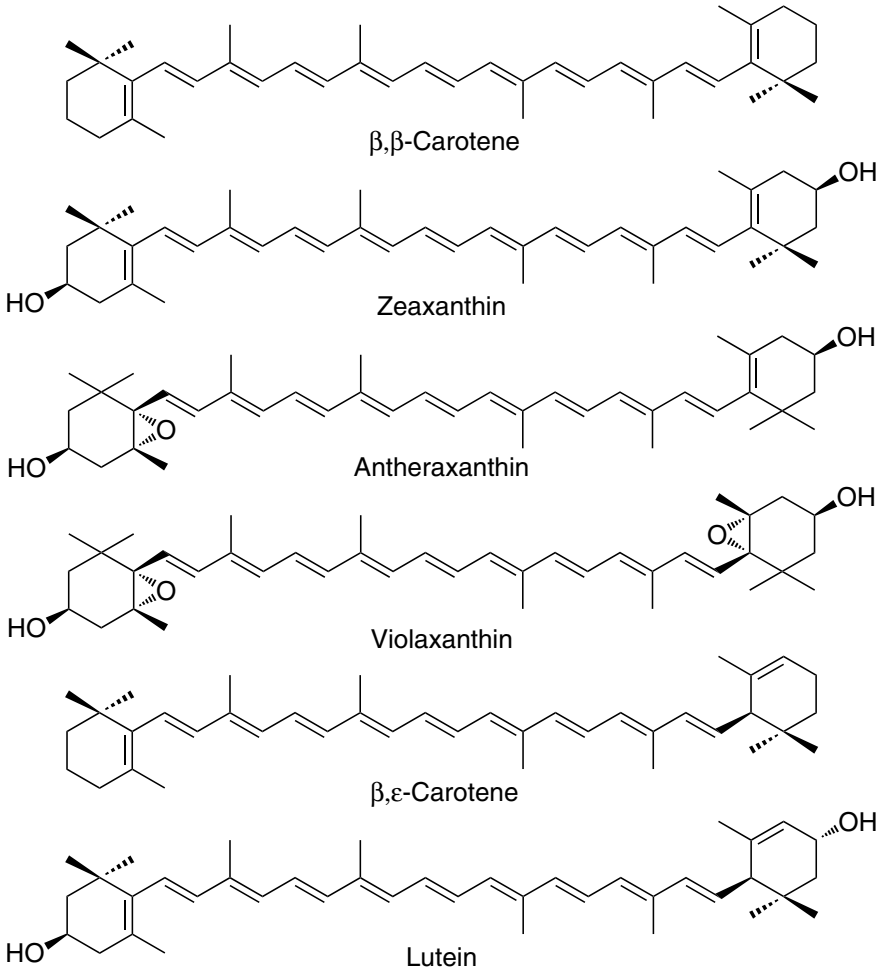
2.3 Red Algae

Red algae have biliproteins and chlorophyll *a* as major pigments. Common carotenoids are β,β-carotene, β,ε-carotene, lutein and zeaxanthin (Fig. 6), although some of them may well be absent in some species (Bjørnland and Aguilar-Martinez 1976; Schubert et al. 2006). The carotenoids with ε end group are in its common (6′*R*) form (Bjørnland et al. 1984). The epoxidic carotenoid antheraxanthin is the major carotenoid in Corallinaceae and Gracilariaceae species, present together with small amounts of violaxanthin; here lutein, the major carotenoid in other red algae, is minor (Esteban et al. 2009) or absent (Schubert et al. 2006).

A study by Schubert et al. (2011) reported high amounts of antheraxanthin together with minor amounts of violaxanthin, zeaxanthin and β,β-carotene in the basal section of the red seaweed *Gracilaria damaecornis*, compared to the apical section, where zeaxanthin and β,β-carotene were dominant, with minor amounts of antheraxanthin. In *Eucheuma isiforme*, the carotenoids lutein, zeaxanthin, β,β-carotene and β,ε-carotene were all more abundant in the apical section than in the basal section, but their relative amounts were similar.

Analysis of carotenoids from red macroalgae is difficult, due to common growth of epiphytes having their own pigmentation (Bjørnland and Aguilar-Martinez 1976).

Fig. 6 Carotenoids of *red* algae



2.4 Green Algae and Other Algae Having the Same Carotenoids

According to traditional systematics, genomic sequencing and pigmentation, the green alga and higher plants are closely related (e.g. Christensen 1980–1994; Adl et al. 2005; Keeling et al. 2005). As for pigmentation, the plants, green algae, and 'green' prasinophytes contain the same carotenoids and are described together.

The chlorarachniophytes, although biologically very different from green algae, have probably obtained a green alga as endosymbiont and have the same pigmentation as green algae (Sasa et al. 1992; Keeling et al. 2005).

Photosynthetic tissue in plants, green algae and 'green' prasinophytes (type 1, see Egeland et al. 1997) have commonly lutein as a major carotenoid, together with minor amounts of the hydrocarbons β,β-carotene and β,ε-carotene and varying amounts of zeaxanthin, the epoxidic antheraxanthin and violaxanthin and the allenic epoxide neoxanthin, the latter in the 9′-*cis* form (Goodwin 1980; Liaaen-Jensen and Egeland 1999; Jeffrey et al. 2011), see Fig. 7. Chlorophylls *a* and *b* are always present.

The major difference between the pigmentation in green algae and red algae is not the carotenoids, but the other pigments encountered, biliproteins in red algae and chlorophyll *b* in green algae, green prasinophytes and plants, although neoxanthin is generally present only in the green group.

Some green algae and prasinophytes (type 2, see Egeland et al. 1997) have additional carotenoids, loroxanthin, loroxanthin ester, siphonaxanthin and/or siphonaxanthin ester (= siphonein); these are encountered mostly in deep-water species (Yokohama 1981, 1983; Yokohama et al. 1992). The species *Mesostigma viride* is recognised by the *trans* variety of neoxanthin, besides saturated esters of siphonaxanthin, in opposition to the monounsaturated siphonaxanthin esters commonly observed elsewhere (Yoshii et al. 2003). The only analysed chlorarachniophyte contained loroxanthin ester beside common green algal carotenoids (Sasa et al. 1992).

In the macroalga *Chara canescens*, the male plants produced large amounts of carotenes (β,β-carotene, β,ε-carotene and some β,ψ-carotene) in addition to common green algal carotenoids, while the female plants had only minor amounts of carotenes (Küster et al. 2005). Other green algae are also known to produce large amounts of secondary carotenoids

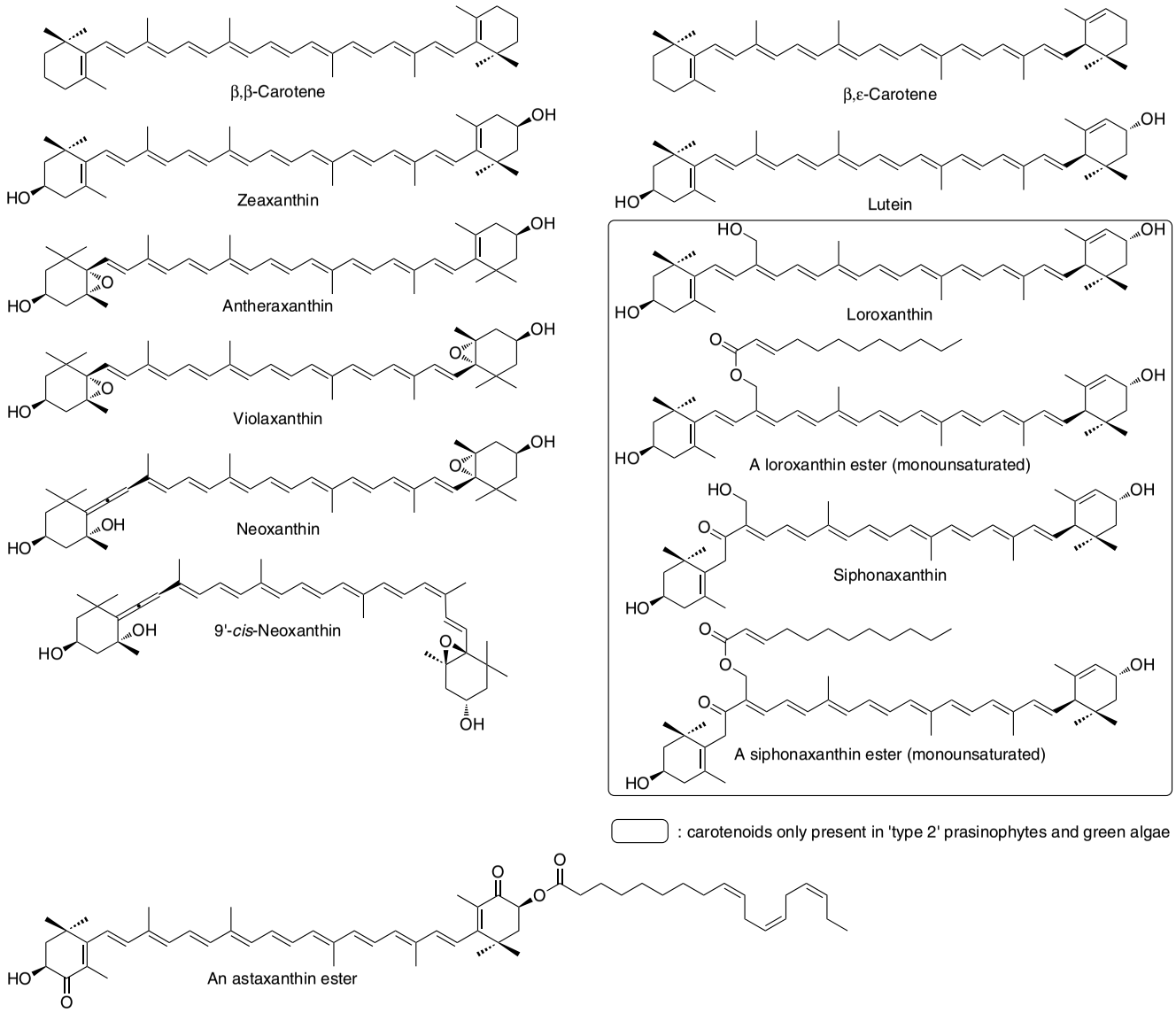


Fig. 7 Carotenoids of plants, green algae and prasinophytes

under various stresses, e.g. β,β-carotene and astaxanthin esters – see later in this chapter.

2.5 Olive-Green Algae

Some prasinophytes have prasinoxanthin instead of lutein as the main pigment. Accompanying carotenoids, having a rare 7′,8′-saturated bond next to an ε end group, include dihydrolutein, micromonol, micromonal and uriolide (Egeland et al. 1997), see Fig. 8 for structures. A noteworthy carotenoid, although minor, is preprasinoxanthin, having the same left end group as fucoxanthin, the main carotenoid in diatoms and brown seaweeds. Also, the peculiar cyclic ester of uriolide is elsewhere only seen in dinoflagellates, e.g. peridinin and pyrrhoxanthin; see below. The common green algal pigments are generally present also here (see above and Fig. 7).

Division of this pigment group based on which pigments accompany prasinoxanthin is not recommended, as the accompanying uriolide and micromonal were only present in *Pseudoscourfieldia marina* cultivated under low irradiance (Egeland et al. 1996).

The content of prasinoxanthin is given in the description of the novel algal class, the Mamiellophyceae (Marin and Melkonian 2010), although this new class does not include all prasinoxanthin-producing species from the old Prasinophyceae (Egeland et al. 1997; Marin and Melkonian 2010).

2.6 Green Algae with Exceptional Pigmentation

A few species of green algae have been reported to contain 'abnormal' carotenoid combinations, having diatoxanthin, diatoxanthin and alloxanthin reported together with common green algal pigments in *Picocystis salinarum* (Roesler et al. 2002) and *Coccomyxa* sp. (Crespo et al. 2009). For structures, see Fig. 9 (for the 'abnormal' carotenoids) and Fig. 7 (for common green algal carotenoids).

The snow alga, *Chlamydomonas nivalis*, is reported to produce a special kind of esterified astaxanthin, astaxanthin diglucoside diesterified with fatty acids, (Fig. 9) (Řezanka et al. 2008, 2013).

2.7 Euglenophytes

The euglenophytes are recognised by their content of chlorophyll *a* and *b*, but the carotenoids show little resemblance between the euglenophytes and most green algae. Only a few articles are available on detailed carotenoid analysis of euglenophytes, so the information about the pigment content of this class is limited. The pigmentation shows similarities to the 'abnormal' green algae mentioned above.

Eutreptiellanone and derivates (Fig. 10) has so far only encountered in *Eutreptiella gymnastica*, recognised by its rare 3,6-oxygen bridge group (Fiksdahl et al. 1984a; Bjørnland et al. 1986).

Common carotenoids in euglenophytes include the acetylenic carotenoids diatoxantin and diadinoxanthin, besides heteroxanthin, neoxanthin (in its 9′-*cis* form), β,β-carotene and β,ε-carotene. Also, some highly unsaturated carotenoids like 3,4,7,8,3′,4′,7′,8′-octadehydro-β,β-carotene and a siphonaxanthin dodecenoate has been encountered (Fiksdahl and Liaaen-Jensen 1988), the latter being the same siphonaxantin ester (= siphonein) as in the green macroalga *Codium fragile* (Egeland 1996). Secondary carotenoids such as astaxanthin diesters may be produced under nitrogen starvation (Grung and Liaaen-Jensen 1993).

2.8 Cryptomonads

The characteristic pigment of cryptomonads is the diacetylenic alloxanthin, the major carotenoid, accompanied by crocoxanthin, monadoxanthin and (6′*R*)-β,ε-carotene, see Fig. 11 (Pennington et al. 1985), although other carotenes may also be present. Other pigments include chlorophyll *a* and c_2, and biliproteins (Jeffrey et al. 2011).

Despite being encountered also in a few other species, alloxanthin is a good marker pigment for cryptomonads.

2.9 Golden-Brown Algae

Haptophytes consists mainly of coccolithophytes (alternative name prymnesiophytes), although some primitive species has been placed in a separate class, the Pavlovophyceae. Also here, chlorophyll *a* and c_2 are widespread, sometimes together with other chlorophyll *c* forms (Zapata et al. 2004). The main carotenoid is fucoxanthin or 19′-hexanoyloxyfucoxanthin, with or without accompanying fucoxanthin derivatives as 19′-butanoyloxyfucoxanthin, 19′-hexanoyloxy-4-ketofucoxanthin and 4-ketofucoxanthin. Other common carotenoids are β,β-carotene, diadinoxanthin and diatoxanthin, see Fig. 12 for structures. The allenic, acetylenic carotenoid gyroxanthin diester is encountered in some species as a minor carotenoid. The only carotenoid with ε end group is β,ε-carotene, sometimes present in minor amounts.

The golden-brown algae have been divided into eight pigment groups based on the content of various chlorophylls and fucoxanthin derivatives (Zapata et al. 2004). Despite some groups are satisfactory documented, others seem to be based on pigments that might or might not be present. Seoane et al. (2009) discusses the carotenoid content of several

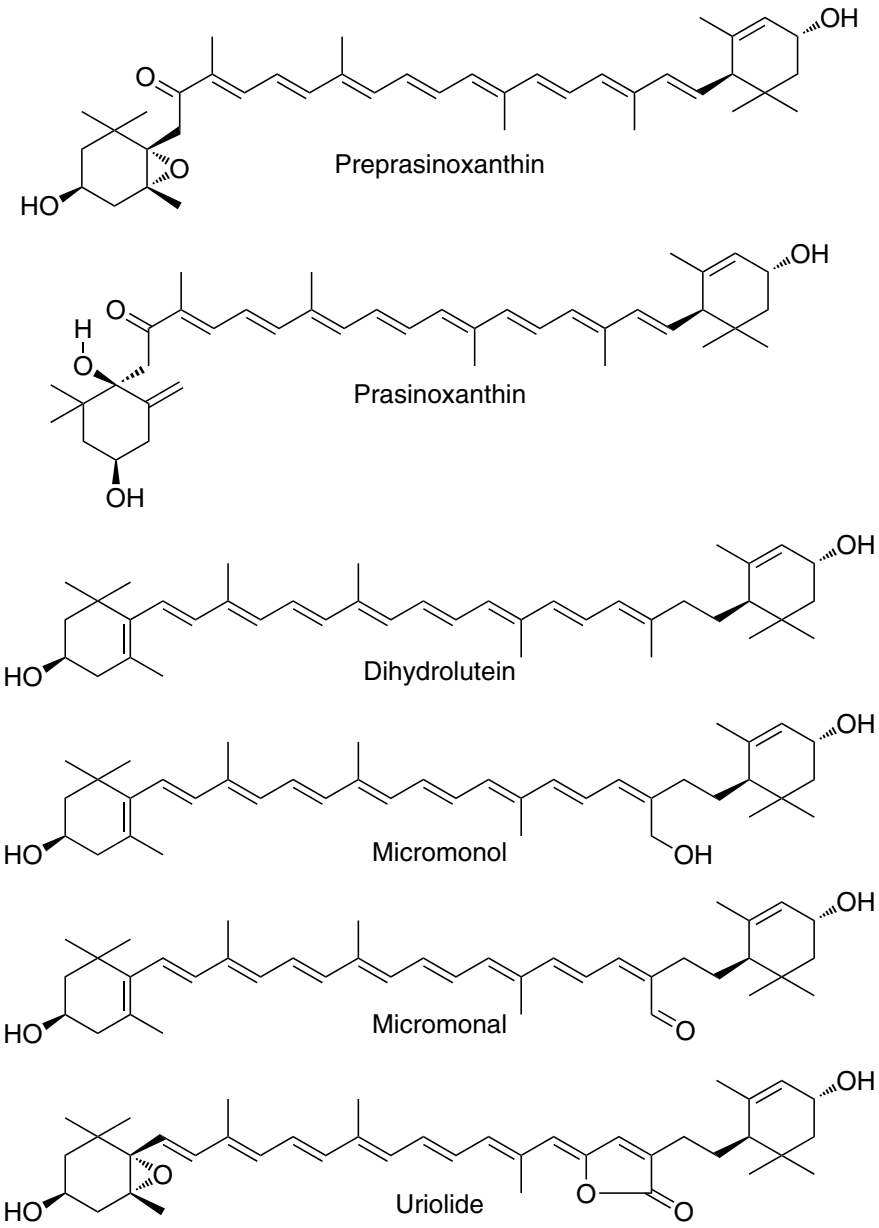


Fig. 8 Carotenoids characteristic for certain prasinophytes (pigment type 3), e.g. the mamiellophytes

golden-brown algae cultivated under different irradiances, showing differences in the number of pigments for some species.

2.10 Diatoms, Bolidophytes and Phaeothamniophytes

Diatoms are a huge algal class, containing several thousand species. Although many species exist, the pigmentation of diatoms is quite simple, having fucoxanthin as the main carotenoid, sometimes also 19′-butanoyloxyfucoxanthin, accompanied by the acetylenic diadinoxanthin and diatoxanthin, and β,β-carotene (Pennington et al. 1988; Stauber and Jeffrey 1988). In a few studies, other carotenoids have been described, such as fucoxanthinol (Pennington et al. 1988) and the presence of violaxanthin in *Phaeodactylum tricornutum* cultivated at high irradiance (Lohr and Wilhelm 1999). For carotenoid structures, see Fig. 13.

As for chlorophylls, chlorophyll *a*, chlorophyll c_2, and chlorophyll c_1/or chlorophyll c_3 are regularly present (Jeffrey et al. 2011 and references therein).

The pigmentation of bolidophytes is identical to the pigmentation of diatoms (Guillou et al. 1999). As for the phaeothamniophytes, little work on carotenoids has been published, but the pigmentation is similar to the diatoms (Bailey et al. 1998).

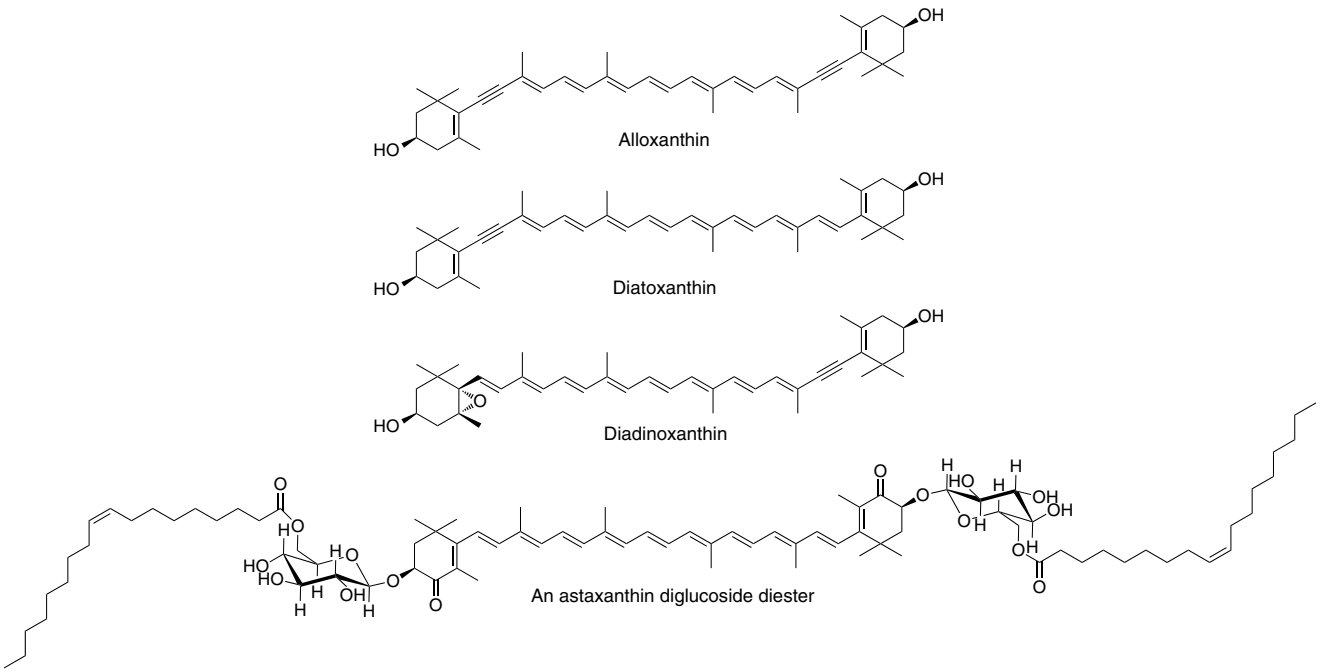


Fig. 9 Uncommon carotenoids from atypical green algae

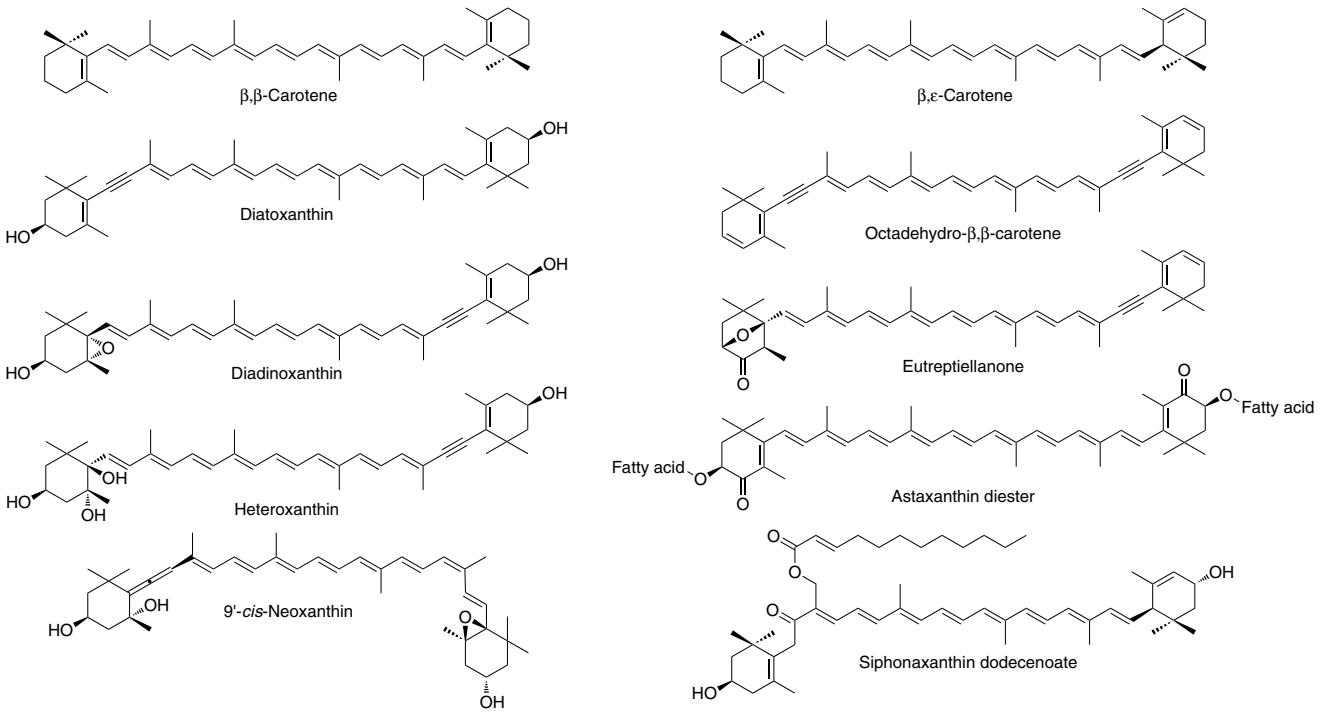


Fig. 10 Carotenoids of euglenophytes

2.11 Pelagophytes

The pelagophytes have mainly the same carotenoids and chlorophylls as diatoms (Bjørnland et al. 1989), (Fig. 14). Notable is the presence of small amounts of (6*S*,6′*S*)-ε,ε-carotene, having the same uncommon stereochemistry at the ε end group as reported for (6′*S*)-β,ε-carotene from the cyanobacterium *Acaryochloris* (see above).

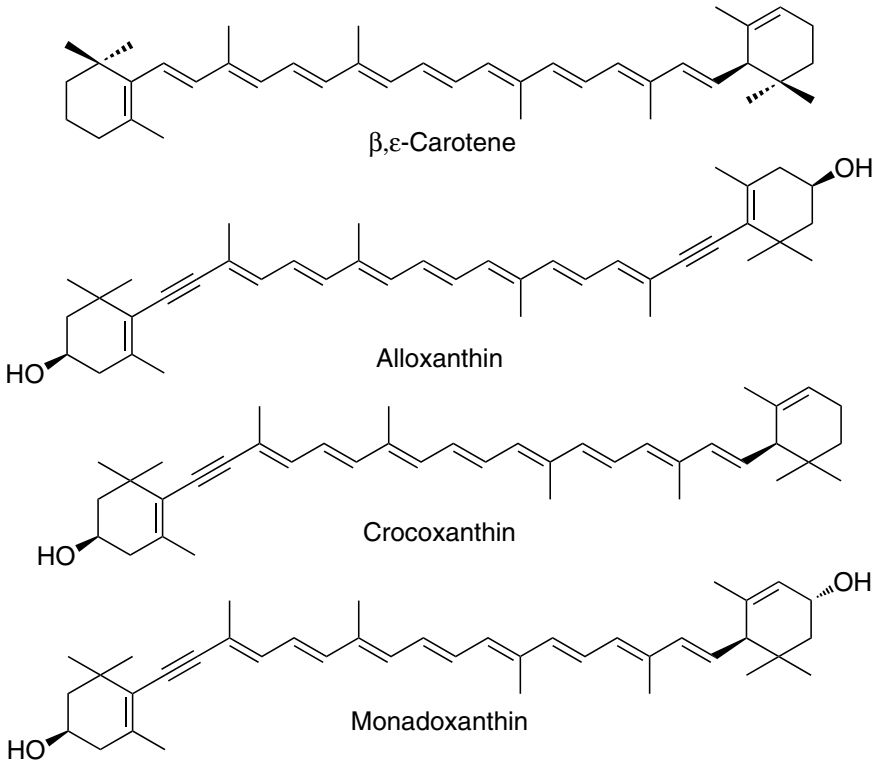


Fig. 11 Carotenoids of cryptomonads

2.12 Aurearenophytes

This recently erected class (Aurearenophyceae), although very little studied, is notable, as the only species, *Aurearena cruciata* contains fucoxanthin as the major carotenoid, reported to be accompanied by violaxanthin, antheraxanthin and zeaxanthin, together with the acetylenic diatoxantin and diadinoxanthin (Kai et al. 2008) – see Fig. 15 for structures. Only chlorophyll *a* was found, chlorophyll *c*s were lacking. This may resemble the production of violaxanthin in diatoms at high irradiance; see above. A more detailed analysis, using mass spectrometry, is needed to confirm the pigment identities.

2.13 Golden Algae, Chloromonads, Chrysomerophytes, Pinguiophytes, Schizocladiophytes, Synchromophytes, Synurophytes and Brown Algae (Brown Seaweeds)

The Chrysophyceae have recently been divided into several classes, so some publications presenting the pigments of golden algae are referring to species no longer being included of this class (Jeffrey et al. 2011 and references therein). For the remaining golden algae, fucoxanthin is the major carotenoid, accompanied by violaxanthin, antheraxanthin, zeaxanthin and β,β-carotene. Chlorophyll *a* is the main chlorophyll, together with one or more chlorophyll *c*s (Jeffrey et al. 2011). This pigmentation pattern is also valid for the chloromonads (raphidophytes) (group II in Fiksdahl et al. 1984b; Guidi-Rontani et al. 2010), chrysomerophytes (Lichtlé et al. 1995), pinguiophytes (Kawachi et al. 2002), synchromophytes (Horn et al. 2007; Schmidt et al. 2012) and synurophytes (Andersen 1987; Jeffrey et al. 2011). The carotenoid structures are shown in Fig. 16.

Note that fucoxanthin is accompanied with the acetylenic carotenoids diatoxanthin and diadinoxanthin in diatoms, bolidophytes and pelagophytes, while in golden algae, chloromonads, pinguiophytes, synurophytes and brown seaweeds, fucoxanthin occurs together with violaxanthin, antheraxanthin and zeaxanthin, carotenoids elsewhere seen in plants and green algae.

Brown seaweeds contain the same carotenoids as golden algae, and no acetylenic carotenoids have been found, despite being reported in the older literature (Haugan and Liaaen-Jensen 1994a). Colombo-Pallotta et al. (2006) have interestingly reported the content of pigments in *Macrocystis pyrifera* blades related to depth, finding twice the amount of fucoxanthin in blades at 18 m, compared to at the surface, while both violaxanthin and zeaxanthin had the maximum concentration in the surface blades.

As the recently described Schizocladiophyceae shows resemblance with brown algae, it might be expected that they also have the same pigmentation (Kawai et al. 2003).

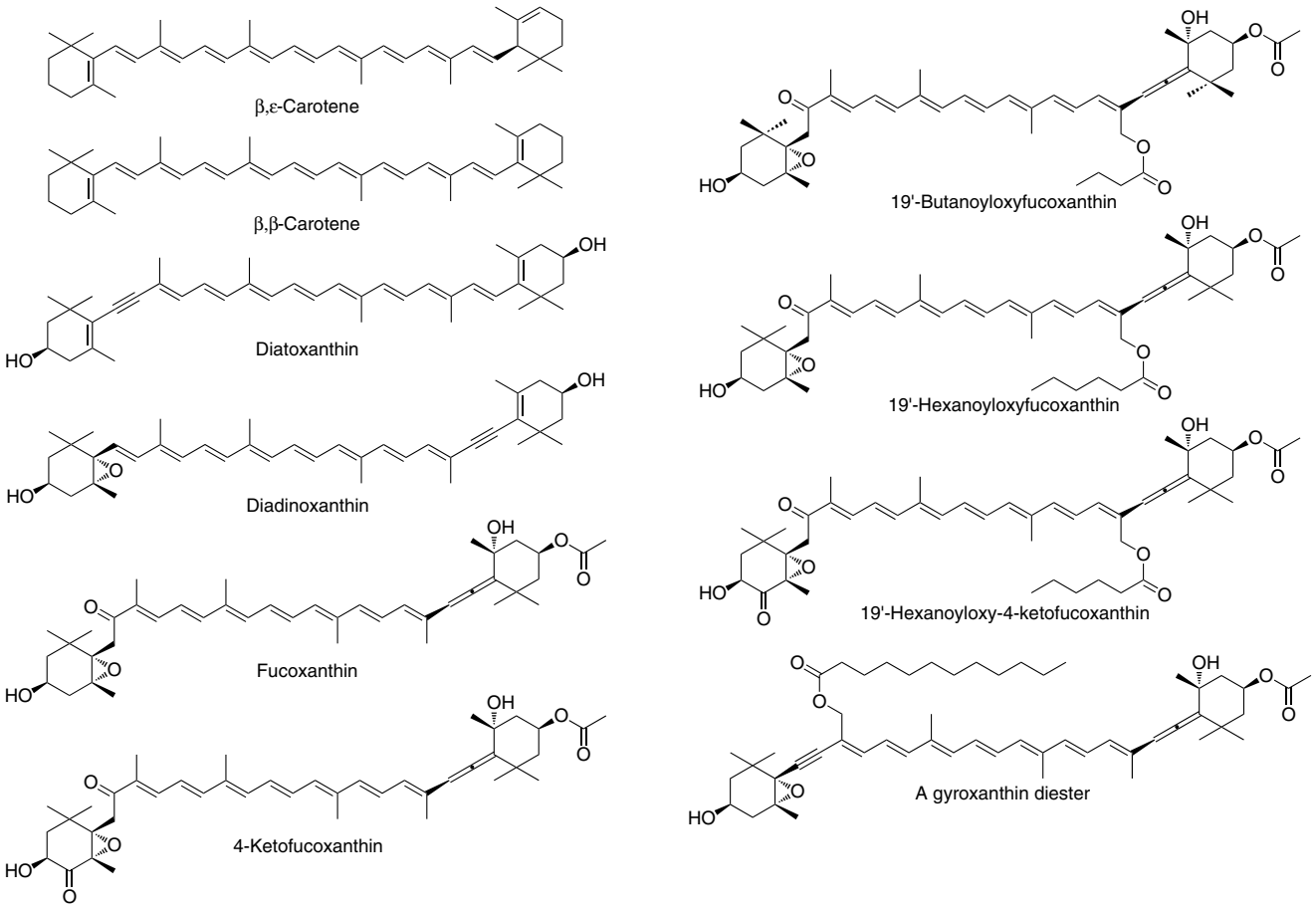


Fig. 12 Carotenoids of *golden-brown* algae

2.14 Silicoflagellates

The silicoflagellates (Dictyochophyceae) contain algae that have fucoxanthin (alone or with 19′-butanoyloxyfucoxanthin) as a characteristic carotenoid, accompanied by diadinoxanthin and diatoxanthin (Eikrem et al. 2004; Edvardsen et al. 2007). For structures, see Fig. 17. Only a few species have been analysed with regards to pigmentation, and good quality data (with at least using mass spectroscopy) are lacking.

2.15 Eustigmatophytes

The characteristic carotenoid in eustigmatophytes is vaucheriaxanthin, commonly in the diesterified form, e.g. as a mixture of vaucheriaxanthin 3-acetate 19′-octanoate and vaucheriaxanthin 3-acetate 19′-decanoate in *Nannochloropsis salina* (Egeland 1996). Accompanying pigments are chlorophyll *a*, β,β-carotene, zeaxanthin, antheraxanthin and violaxanthin (Jeffrey et al. 2011), and sometimes ketocarotenoids like canthaxanthin (Norgård et al. 1974); see Fig. 18 for carotenoid structures.

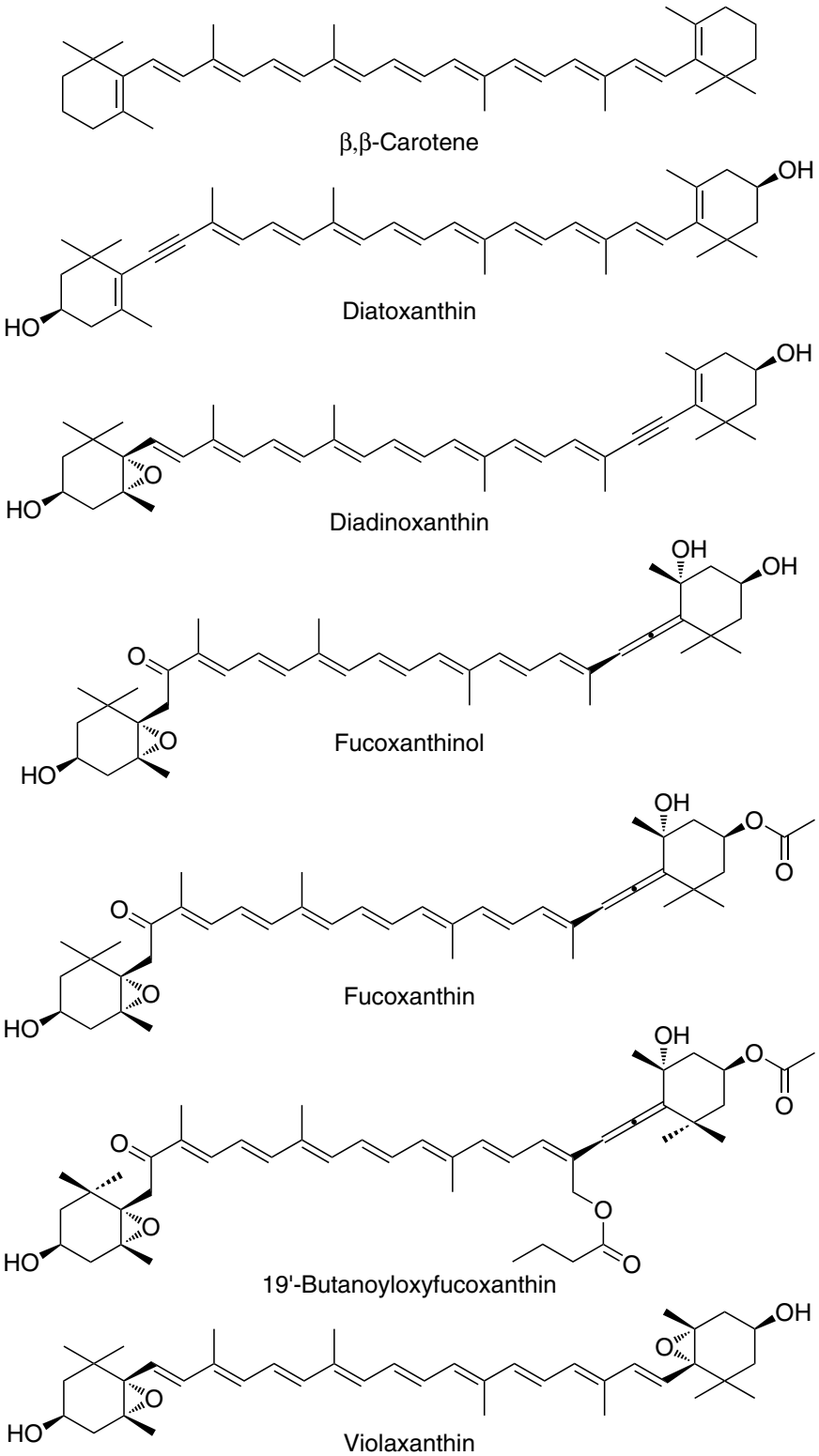
2.16 Xanthophytes

As for the eustigmatophytes, vaucheriaxanthin diester(s) is the characteristic carotenoid, but in xanthophytes, this is accompanied by diadinoxanthin, diatoxanthin, heteroxanthin and β,β-carotene (Stransky and Hager 1970; group I in Fiksdahl et al. 1984b); see Fig. 19 for structures. Chlorophyll *a* is accompanied with minor amounts of chlorophyll *c*s (Jeffrey et al. 2011).

2.17 Dinoflagellates of Many Kinds

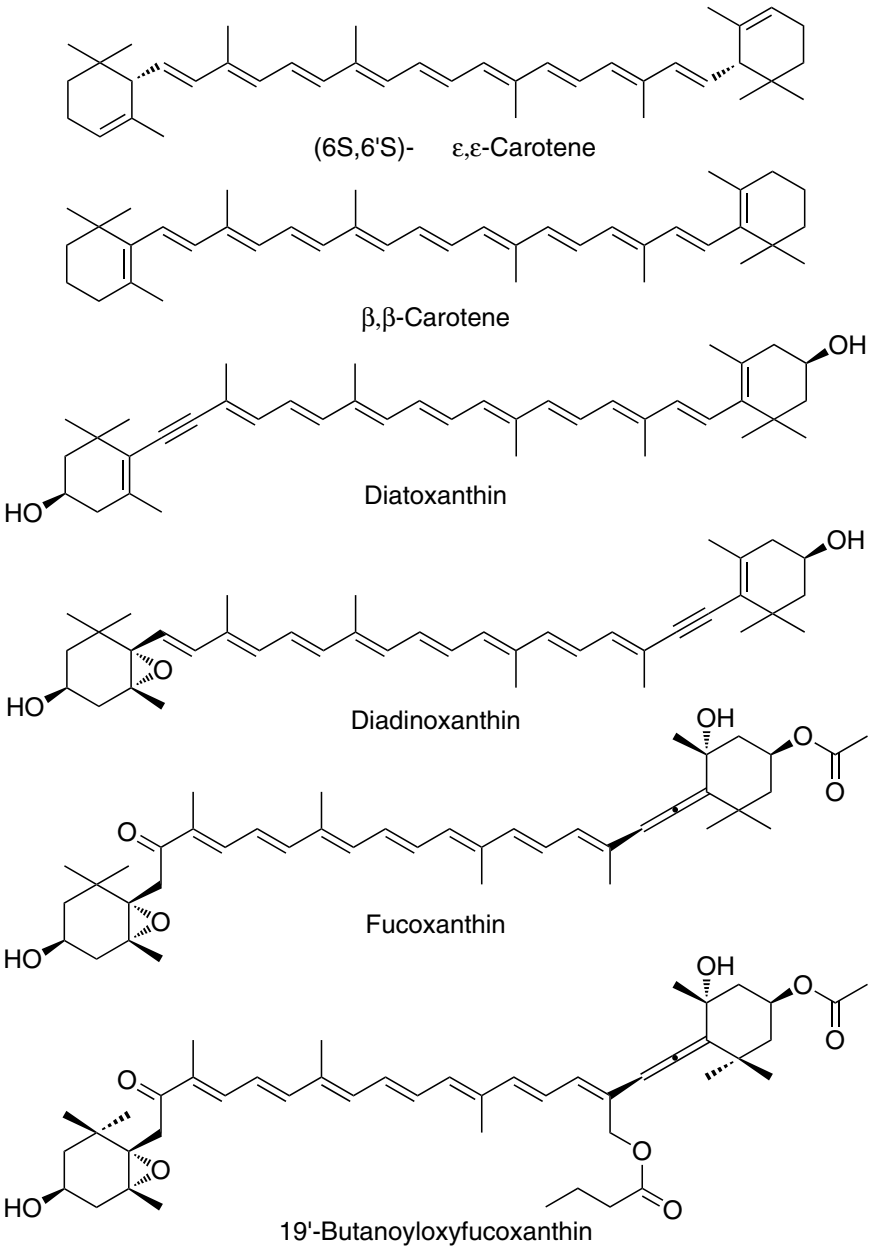
The dinoflagellates, partly existing as colourless heterotrophic ‘animals’, partly as photosynthetic algae, have probably arisen from colourless species incorporating various algal cells with various carotenoids into their body (see Fig. 3),

Fig. 13 Diatom carotenoids



and are the most diverse algal class when pigments are considered. The diversity makes this algal class most interesting, but it also makes chemical analyses of water samples more confusing. For common HPLC analyses of water samples, it is not clear whether the dinoflagellates or their 'mother algae' are present, as they have the same pigmentation; therefore, use of microscopy to aid the HPLC analyses is recommended.

Fig. 14 Carotenoids of pelagophytes



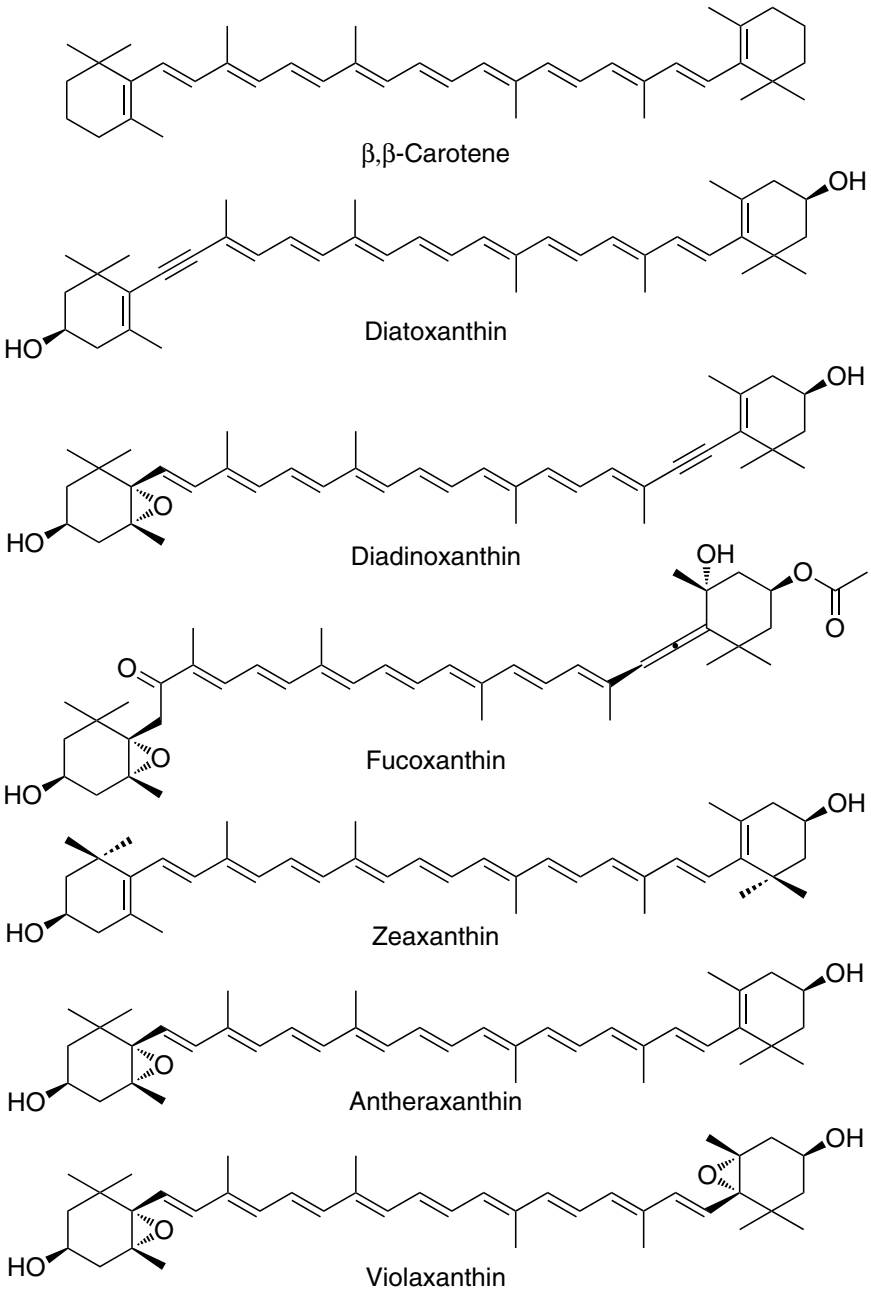
Jeffrey et al. (2011) presents five different pigment groups:

DINO-1 has the nor-carotenoid cyclic ester peridinin as the main carotenoid, commonly accompanied by β,β-carotene, diadinoxanthin, diatoxanthin, dinoxanthin, pyrrhoxanthin, peridininol and the peculiar dihydrocarotenal diglycoside known as P-457 (Johansen et al. 1974; Aakermann et al. 1993; Wakahama et al. 2012), see Fig. 20 for structures. Common chlorophylls are chlorophyll *a* and c_2 (Jeffrey et al. 2011). This pigment group is unique to dinoflagellates; peridinin, pyrrhoxanthin and P-457 have never been encountered in any other algae, making the endosymbiont source unknown (Bodył and Moszczyński 2007).

DINO-2 have a haptophyte endosymbiont, therefore the main pigments are, besides chlorophyll *a* and *c*s, the carotenoids fucoxanthin and derivatives 19′-butanoyloxyfucoxanthin and 19′-hexanoyloxyfucoxanthin as major carotenoid(s), accompanied by diatoxanthin, diadinoxanthin and the uncommon acetylenic allenic carotenoid gyroxanthin diester (dodecanoate ethanoate and others). β,ε-carotene is the only carotenoid with an ε end group, and β,β-carotene; see Fig. 21 for structures (Bjørnland and Liaaen-Jensen 1989; Bjørnland et al. 2003).

Zapata et al. (2012) have divided the DINO-2 group, based on the presence or absence of 19′-butanoyloxy-4-ketofucoxantin and 19′-hexanoyloxy-4-ketofucoxanthin.

Fig. 15 Carotenoids of aurearenophytes



DINO-3 has a diatom endosymbiont, having besides chlorophyll *a* and *c*s the carotenoids fucoxanthin (no acetyloxyfucoxanthins), diadinoxanthin, diatoxanthin and β,β-carotene, and β,ε-carotene may also be present (Jeffrey et al. 2011; Zapata et al. 2012); see Fig. 22 for structures.

DINO-4 have a cryptomonad endosymbiont, so the major carotenoid is alloxanthin with β,ε-carotene and crocoxanthin (Fig. 23), accompanied by chlorophyll *a* and c_2, and biliproteins (Meyer-Harms and Pollehne 1998; Jeffrey et al. 2011; Zapata et al. 2012).

The last group, DINO-5 have a prasinophyte endosymbiont; therefore the chlorophylls *a* and *b* are present. At least one species has prasinoxanthin, see Jeffrey et al. (2011), others have typical green algae carotenoids (Fig. 24) as β,β-carotene, lutein, zeaxanthin, antheraxanthin, violaxanthin and 9′-*cis*-neoxanthin (Jeffrey et al. 2011; Matsumoto et al. 2012; Zapata et al. 2012). As a curiosity, the dinoflagellate *Noctiluca scintillans* (*miliaris*) has intact cells of *Pedinomonas noctilucae* within its body (Sweeney 1976), and therefore, typical green algal carotenoids (Furuya and Lirdwitayaprasit 2000).

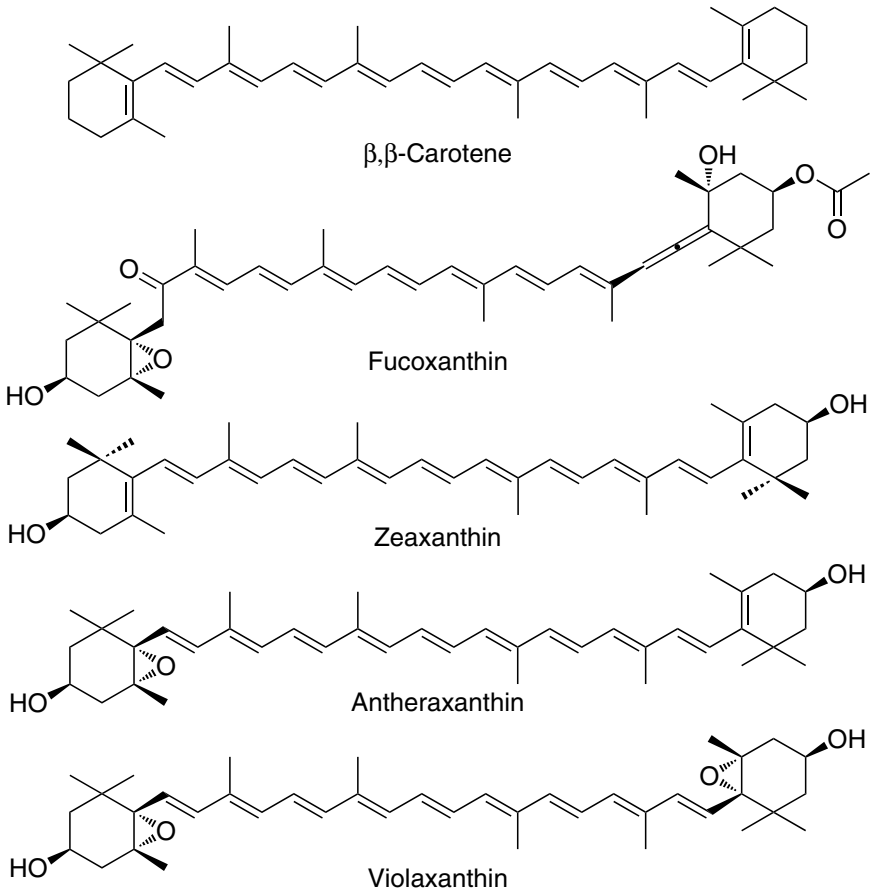


Fig. 16 Carotenoids of *brown* seaweeds and *golden* algae

2.18 Carotenoids in Shifting Algal Classes

Algal systematics is discussed in chapter "Systematics, Taxonomy and Species Names: Do They Matter?" of this book (Borowitzka 2016). Modern genomic methods will lead to further reshuffling of species and genera in the traditional algal classes, erecting new ones and maybe combining others. Usually, the described classes have homogenous pigmentation; known exceptions are the prasinophytes (where prasinoxanthin and accompanying carotenoids are present in some species, while the rest have green algal pigments) and the dinophytes (see above).

3 Biosynthesis of Carotenoids in Algae

The biosynthesis of carotenoids will be briefly outlined in this chapter. A detailed description, referring to a large number of articles, can be found in Lohr (2011). As the biosynthesis of carotenoids is studied mainly in bacteria and in green plants, lack of knowledge is obvious for the biosynthesis of many characteristic algal carotenoids, even the biosynthesis of major carotenoids such as fucoxanthin. The biosynthesis will be described mainly through figures showing the chemical reactions to the various compounds involved, adding numbers to indicate the various enzymes known to catalyse the reactions studied in detail.

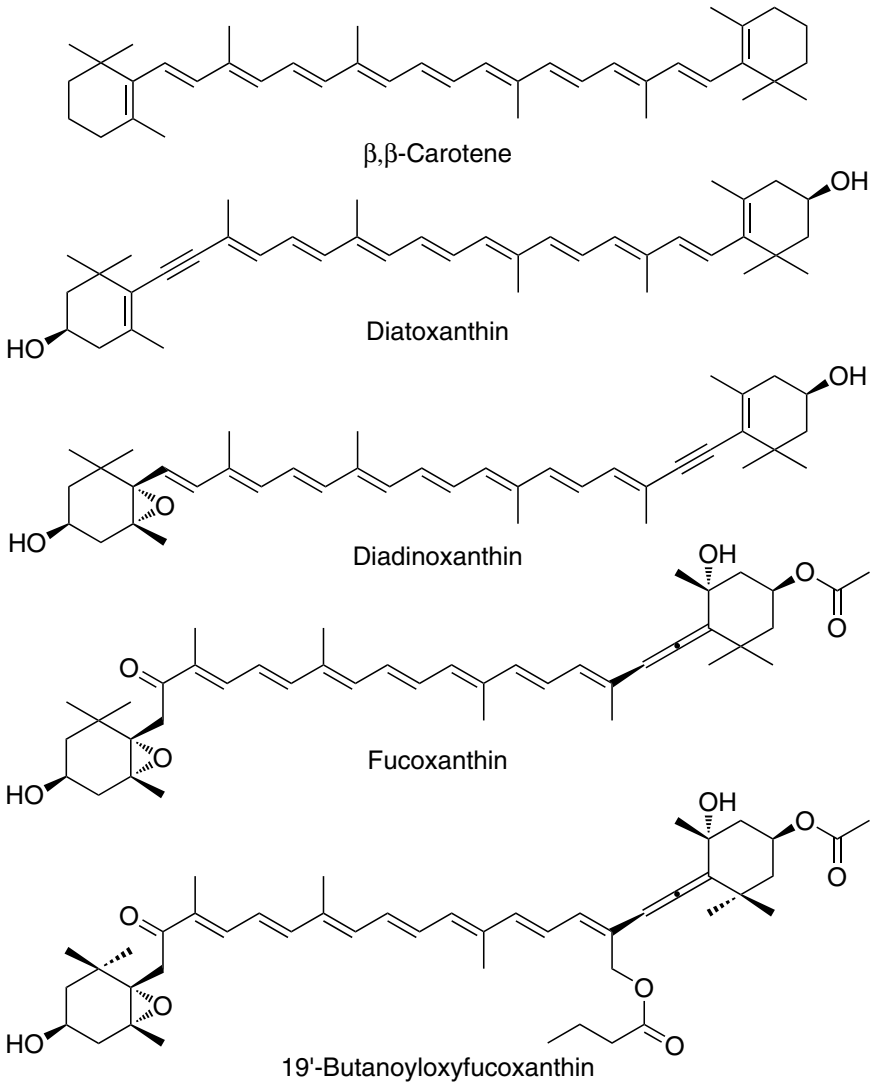
A nice illustration by Cazzonelli and Pogson (2010) of the higher plant and green algal carotenoid biosynthetic pathway is shown in Fig. 25.

3.1 Biosynthesis of 3-Methylbut-3-Enyl Diphosphate, the Building Block of Carotenoids

The synthesis of 3-methylbut-3-enyl diphosphate (= isopentenyl diphosphate) was long thought to occur only from acetyl-coenzyme A via the 3,5-dihydroxy-3-methylpentanoate (= mevalonate) to 3-methylbut-3-enyl diphosphate (Fig. 26). The letter (or number) given on arrows refers to the enzyme catalysing each reaction and the genes encoding them (Table 1).

This biosynthetic route occurs in a large range of algae (Table 2), but is surpassed by the 3-methyl-2,3,4-trihydroxybutyl phosphate (=2-*C*-methyl-*D*-erythritol phosphate) biosynthetic route (see Fig. 27), occurring in all

Fig. 17 Carotenoids of silicoflagellates



studied algal species, from all classes (Lichtenthaler et al. 1997; Schwender et al. 1997; Lohr et al. 2012).

3.2 To Phytoene, the First Carotenoid

The isoprene unit 3-methylbut-3-enyl diphosphate isomerises to 3-methylbut-2-enyl diphosphate (= dimethylallyl diphosphate) and combines to 3,7-dimethylocta-2,6-dienyl diphosphate (= geranyl diphosphate), adding another 3-methylbut-3-enyl diphosphate to 3,7,11-trimethyldodeca-2,6,10-trienyl diphosphate (= farnesyl diphosphate), and once again adding a 3-methylbut-3-enyl diphosphate and becomes 3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraenyl diphosphate (= geranylgeranyl diphosphate) (Fig. 28). Two such molecules will so combine "head to head" and become the first carotenoid, 15-*cis*-phytoene (Fig. 29).

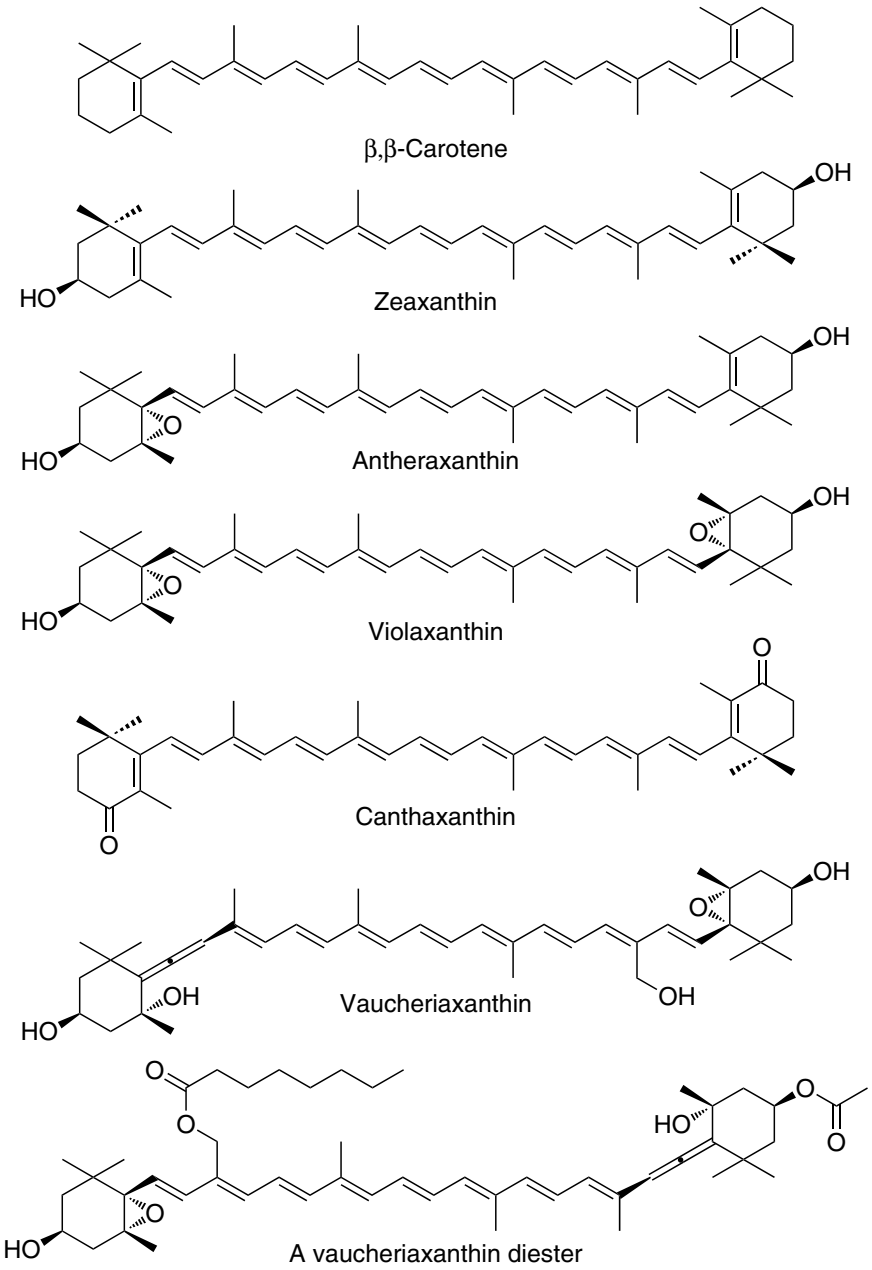
3.3 To β,β-Carotene and Other Carotenes

When the first carotenoid, 15-*cis*-phytoene has been synthesised, the carotenoid is isomerised and desaturated consecutively until ψ,ψ-carotene (= lycopene), then cyclised at one or both ends, commonly to β,β-carotene, β,ε-carotene and ε,ε-carotene (Fig. 30). The uncommon χ,χ-carotene in an *Synechococcus* isolate is biosynthesised from β,β-carotene (Graham and Bryant 2008).

3.4 To Major Xanthophylls

From the carotenes, oxygen functions are inserted, giving us the xanthophylls described above. Some examples are highlighted here.

Fig. 18 Carotenoids of eustigmatophytes



3.4.1 To Lutein

Lutein is biosynthesised from β,ε-carotene through β,ε-caroten-3-ol (= zeinoxanthin) and/or β,ε-caroten-3′-ol (= α-cryptoxanthin), by adding hydroxyl groups at both ends of the molecule (Fig. 31). Various enzymes have been encountered to hydroxylise the β end group, while only one enzyme so far is reported for the formation of the 3-hydroxy-ε end group. For details, see Hirschberg (1998) and Lohr (2011).

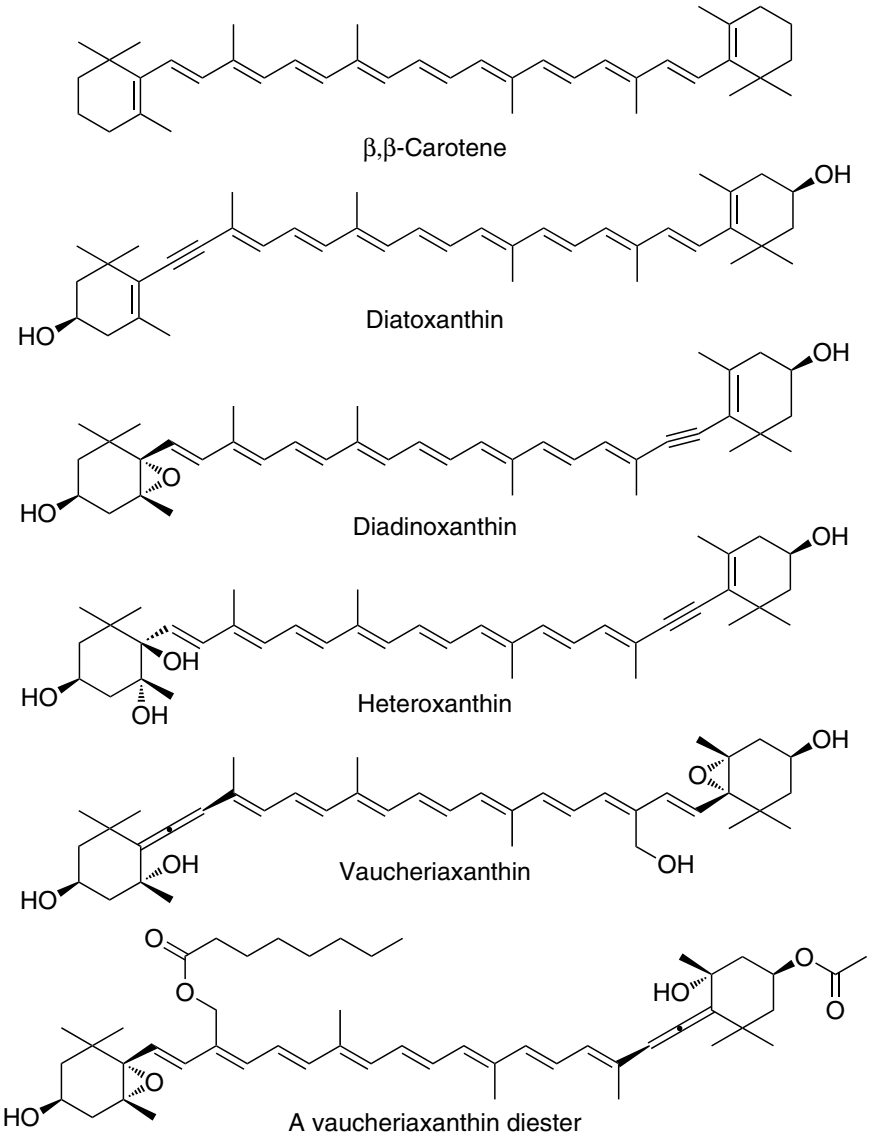
3.4.2 To Prasinoxanthin

Prasinoxanthin is probably synthesised from lutein, although the two carotenoids only exceptionally occurs together. Preprasinoxanthin was thought to be the probable precursor based on chemical analogy (Foss et al. 1984), and this carotenoid has indeed been isolated from a prasinophyte (Fig. 32). So far, no enzymes or genes controlling this process have been reported.

3.4.3 To Uriolide and Derivatives

The synthesis of dihydrolutein is suggested to go from lutein (Böhme et al. 2002), although more economically, it could be made through a diverging route from neurosporene to 7′,8′-dihydro-β,ε-carotene and further to dihydrolutein (Egeland et al. 1997) (Fig. 33). From dihydrolutein, structural resemblance has been used to hypothesise the biosynthesis through micromonol and micromonal to uriolide and

Fig. 19 Carotenoids of xanthophytes



derivatives (Fig. 34). Also here, no enzymes or genes have been published.

3.4.4 To Canthaxanthin

From β,β-carotene, oxygen are incorporated in the 4-position to echinenone, and in 4′-position of echinenone to give canthaxanthin (Fig. 35). Different enzymes are found in different algae (Lohr 2011, see Table 1).

3.4.5 To Zeaxanthin

β,β-Carotene are added a hydroxy group in the three-position to become cryptoxanthin, and similar, on the other end, to become zeaxanthin (Fig. 36). The enzymes involved are supposed to be the same as for 3-hydroxylation of the ε end group of lutein (Lohr 2011).

3.4.6 To Astaxanthin

The biosynthesis to astaxanthin can occur along various pathways. One pathway goes from β,β-carotene to canthaxanthin as described above. Canthaxanthin is then added a hydroxy group in three-position, becoming adonirubin, and once again, on the other end, becoming astaxanthin. Alternatively, β,β-carotene can be transformed into zeaxanthin (as above), next, oxygen can be added in the four-position to give rise to adonixanthin, and further to astaxanthin. Also, it is possible to imagine a combination of these routes, e.g. first adding both oxygens to one end, next on the other end (Fig. 37) (Harker and Hirschberg 1997). The biosynthesis of astaxanthin in various algae has been discussed by Lohr (2011) and for non-algae by Cunningham and Gantt (2011). In *Haematococcus pluvialis*, research indicates that astaxanthin

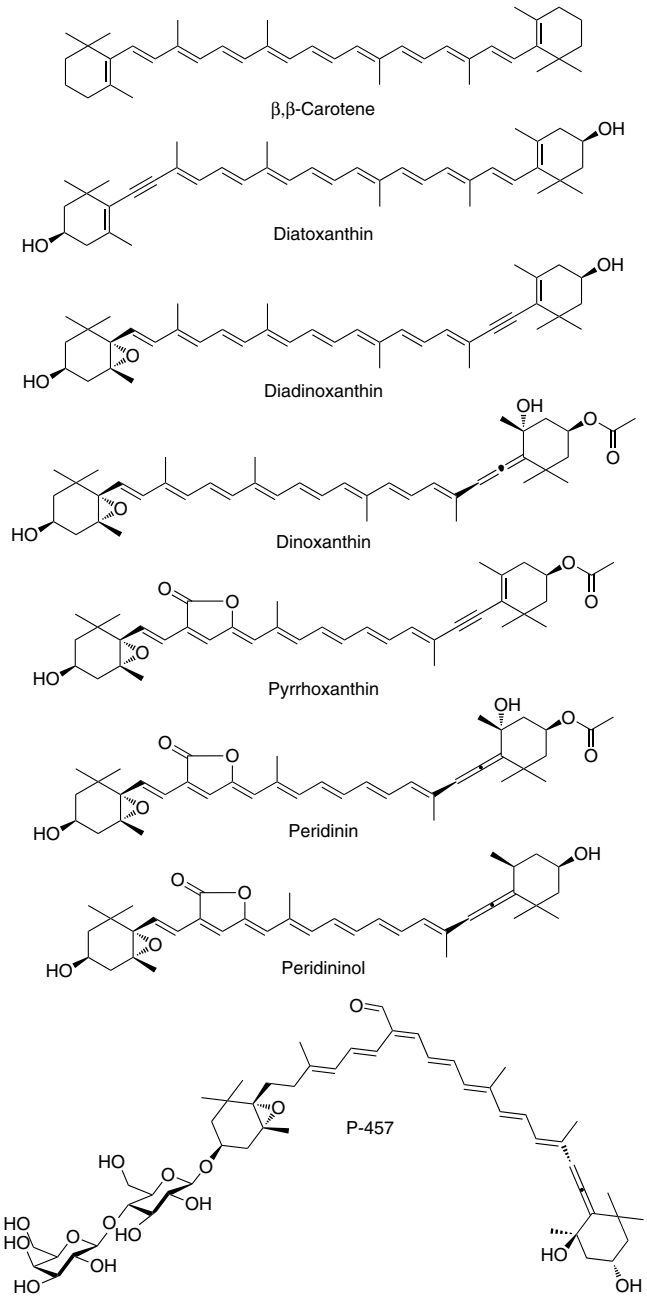


Fig. 20 Carotenoids of dinoflagellates, type DINO-1 (pigmentation unique to dinoflagellates)

is syntesised from β,β-carotene with canthaxanthin as the central intermediate (Boussiba 2000). Lemoine and Schoefs (2010) have summarised the biosynthesis of astaxanthin and metabolic and regulation networks in *Haematococcus pluvialis* under various stress conditions.

3.4.7 To Neoxanthin

Neoxanthin is synthesised from zeaxanthin (see above). Next, an oxygen atom is added to one end of zeaxanthin, giving antheraxanthin, and to the other end, giving violaxanthin. Violaxanthin will so be transformed into *trans*-neoxanthin and further isomerised to 9′-*cis*-neoxanthin, although the isomerisation can theoretically be performed before the transformation to neoxanthin (Fig. 38). For details about the enzymes involved, see Lohr (2011).

3.4.8 To Diatoxanthin and Diadinoxanthin

The synthesis of diatoxanthin and diadinoxanthin has been discussed, but evidence of the biosynthetic routes has yet been published. The suggested biosynthetic routes are shown in Fig. 39.

3.4.9 To Fucoxanthin

Fucoxanthin occurs partly together with zeaxanthin and violaxantin, partly together with diatoxanthin and diadinoxanthin. Biosynthetic routes has been hypothesised, (Fig. 40), but no evidence in favour of one or the other route has been published so far.

3.4.10 To Peridinin

The biosynthesis of peridinin has been suggested to go from neoxanthin through dinoxanthin (= neoxanthin acetate) (Fig. 41). Also here, no enzymes have been reported.

3.4.11 To Gyroxanthin

The rare carotenoid gyroxanthin has structural elements suggesting biosynthesis from diadinoxanthin, but also from neoxanthin (Fig. 42).

3.5 Combining the Puzzle Pieces to the Whole Picture

The various biosynthetic routes pictured above are combined to show the full picture of biosynthetic routes to algal carotenoids (including the cyanobacteria) – see Fig. 43 for carotenoids synthesised from ψ,ψ-carotene (= lycopene) through β,ε-carotene, Fig. 44 for carotenoids synthesised from β,β-carotene, and Fig. 45 for other carotenoids.

3.6 Xanthophyll Cycles and 'Sunshade Pigments'

When seaweeds, green algae, plants and other violaxanthin-containing algae are exposed to high irradiances, the biosynthesis is promptly reversed, and violaxanthin is re-biosynthesised to antheraxanthin and further to zeaxanthin. When the irradiance drops, zeaxanthin is once again used to synthesise violaxanthin, completing the xanthophyll cycle (Fig. 46). This rapid transfer of carotenoids act light protection in the cells, and zeaxanthin is therefore the 'sunglasses' of the cell (Brunet et al. 2011).

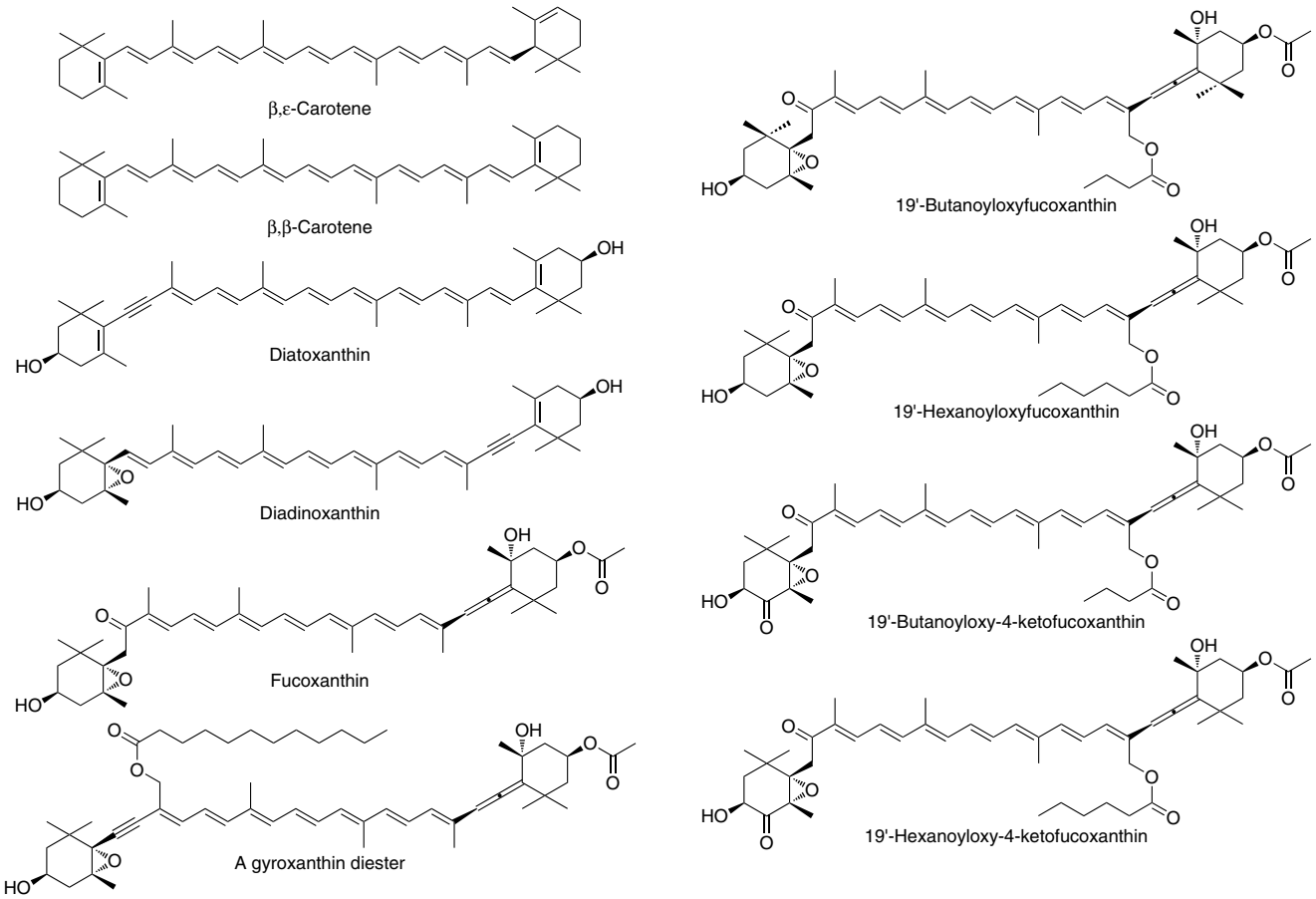


Fig. 21 Carotenoids of dinoflagellates, type DINO-2 (haptophyte pigmentation)

Note that the content of violaxanthin not necessarily is highest at low light. For example, in *Macrocystis pyrifera*, both violaxanthin and zeaxanthin were present in highest concentration in the blades at the sea surface (Colombo-Pallotta et al. 2006; Ocampo-Alvarez et al. 2013).

As described in Sect. 2 above, only some species produce violaxanthin. Those algae having diadinoxanthin, e.g. diatoms, dinoflagellates and euglenophytes, protect themselves by re-synthesising diatoxanthin from diadinoxanthin under high irradiance, and re-re-synthesising diadinoxanthin when again exposed to low light intensities. This xanthophyll cycle is containing only these two carotenoids, not three as in the plant/green algae xanthophyll cycle above (Fig. 46).

A xanthophyll cycle is not encountered in all kinds of algae, but in some, zeaxanthin is accumulated at high light intensities (Egeland et al. 1996; Brunet et al. 2011). In the dinoflagellate *Peridinium gatunense*, diadinoxanthin was accumulated in a natural bloom (Yacobi 2003), and the xanthophyll cycle was observed only in culture. The increase of β,β-carotene in some *Dunaliella* species at high irradiances is well-known (e.g. Lamers et al. 2010) and the carotene is harvested commercially, see later in this chapter.

Sigaud-Kutner et al. (2005) have described diel changes in carotenoid content in *Tetraselmis gracilis*, finding an increased amount of lutein and zeaxanthin at daylight, while the content of prasinoxantin was increasing at night, and decreasing during the day. Despite the fact that violaxanthin, antheraxanthin and zeaxanthin were all present, all of them showed the same pattern: at the highest during the day, and the lowest during the night. It is noteworthy that Guaratini et al. (2009) did not find any prasinoxanthin, despite cultivating the same clone at the same culturing conditions.

Details about the xanthophyll cycle phenomena have recently been described elsewhere (Brunet et al. 2011); see also Demmig-Adams and Adams (1993) and Telfer et al. (2008).

3.7 Stress-Induced Synthesis: Secondary Carotenoids

Some algal species are of great commercial interest, as they produce large amount of certain carotenoids, so-called secondary carotenoids, when the algal cells are exposed to non-optimal growth conditions (Goodwin 1980; Young 1993;

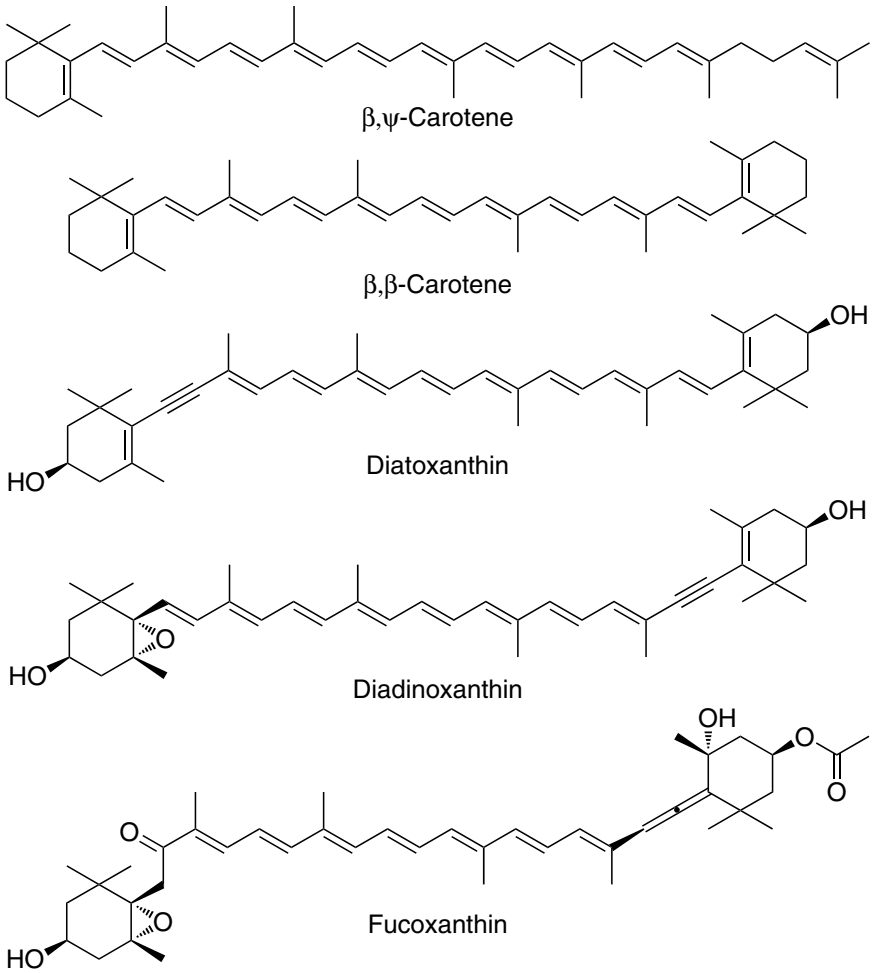


Fig. 22 Carotenoids of dinoflagellates, type DINO-3 (diatom pigmentation)

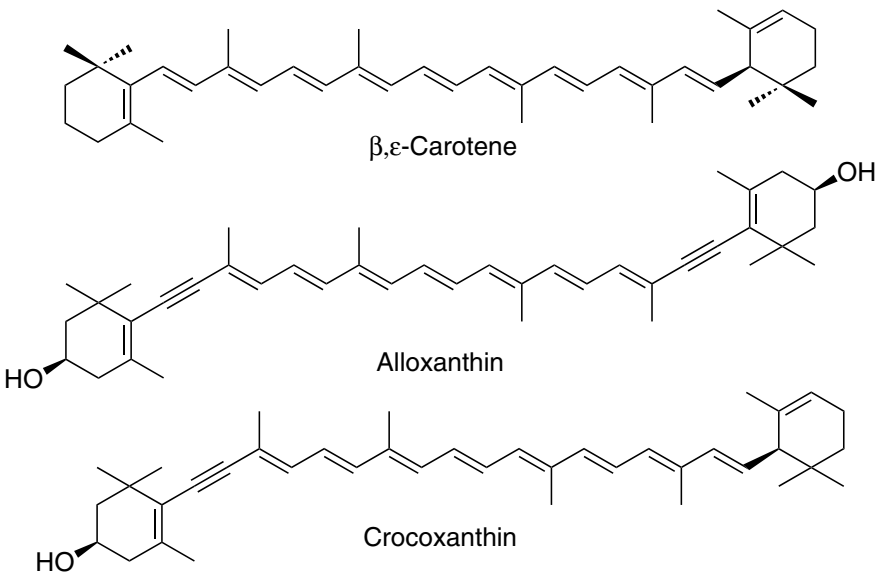


Fig. 23 Carotenoids of dinoflagellates, type DINO-4 (cryptomonad pigmentation)

Borowitzka 2010). The synthesis of secondary carotenoids can depend on a long range of environmental factors, e.g. high irradiance, high salinity, or low nutrient availability. The carotenoids produced as secondary carotenoids are usually of two types, either carotenes, e.g. β,β-carotene, or ketocarotenoids, e.g. canthaxanthin. The synthesis of

Fig. 24 Carotenoids of dinoflagellates, type DINO-5 (prasinophyte pigmentation)

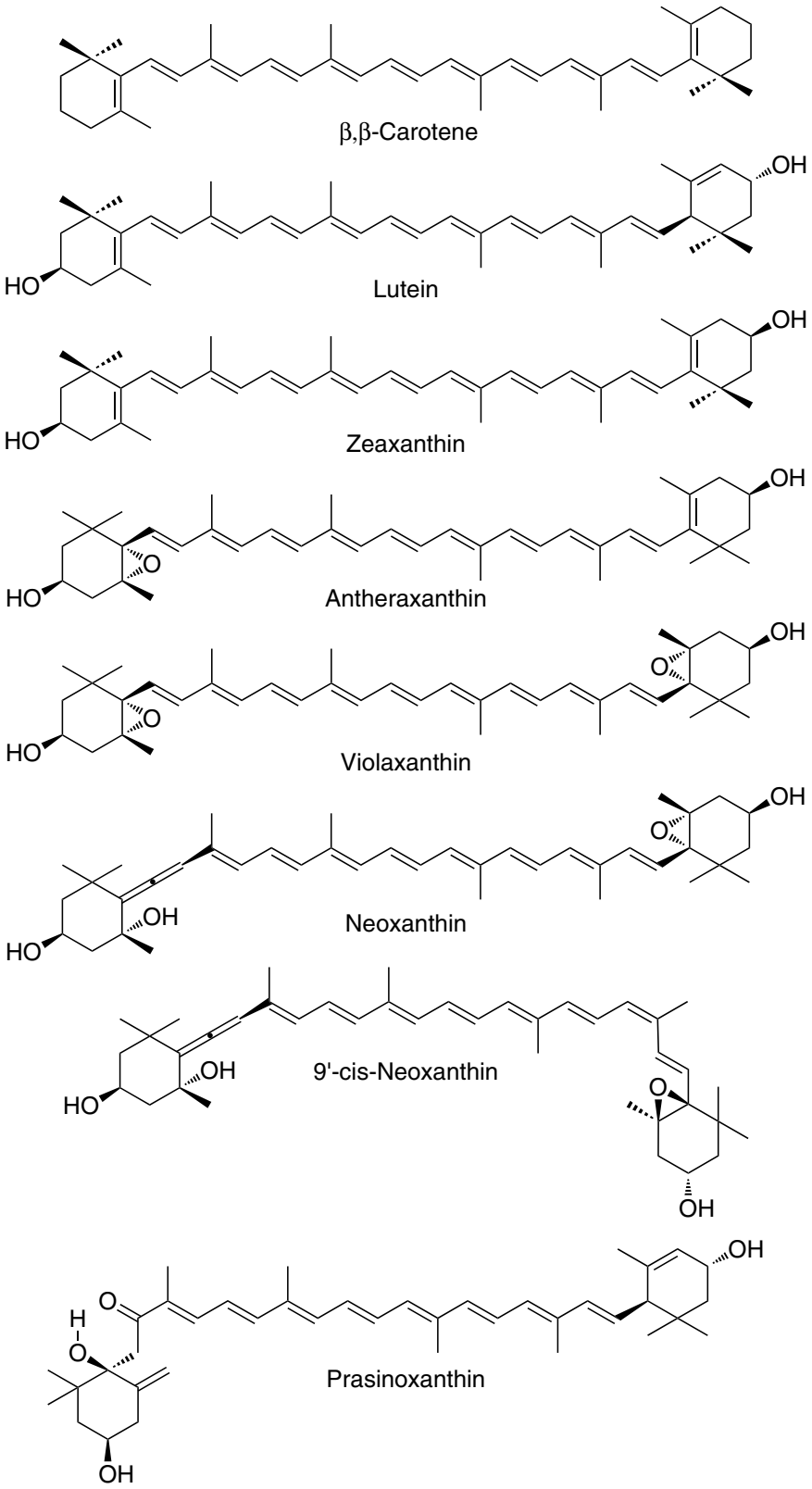


Fig 25 (continued) β-hydroxylase, *CCD* carotenoid cleavage dioxygenase, *CRTISO* carotenoid isomerase, *DXR* 1-deoxy-D-xylulose 5-phosphate reductoisomerase, *DXS* 1-deoxyxylulose-5-phosphate synthase, *εLCY* ε-cyclase, *εOHase* ε-hydroxylase, *GGPP* geranylgeranyl diphosphate, *HDR* 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase, *NCED* 9-*cis*-epoxycarotenoid dioxygenase, *NXS* neoxanthin synthase, *PDS* phytoene desaturase, *PSY* phytoene synthase, *SDG8* histone methyltransferase, *VDE* violaxanthin de-epoxidase, *ZDS* ζ-carotene desaturase, and *ZE* zeaxanthin epoxidase (From Cazzonelli and Pogson (2010) with permission)

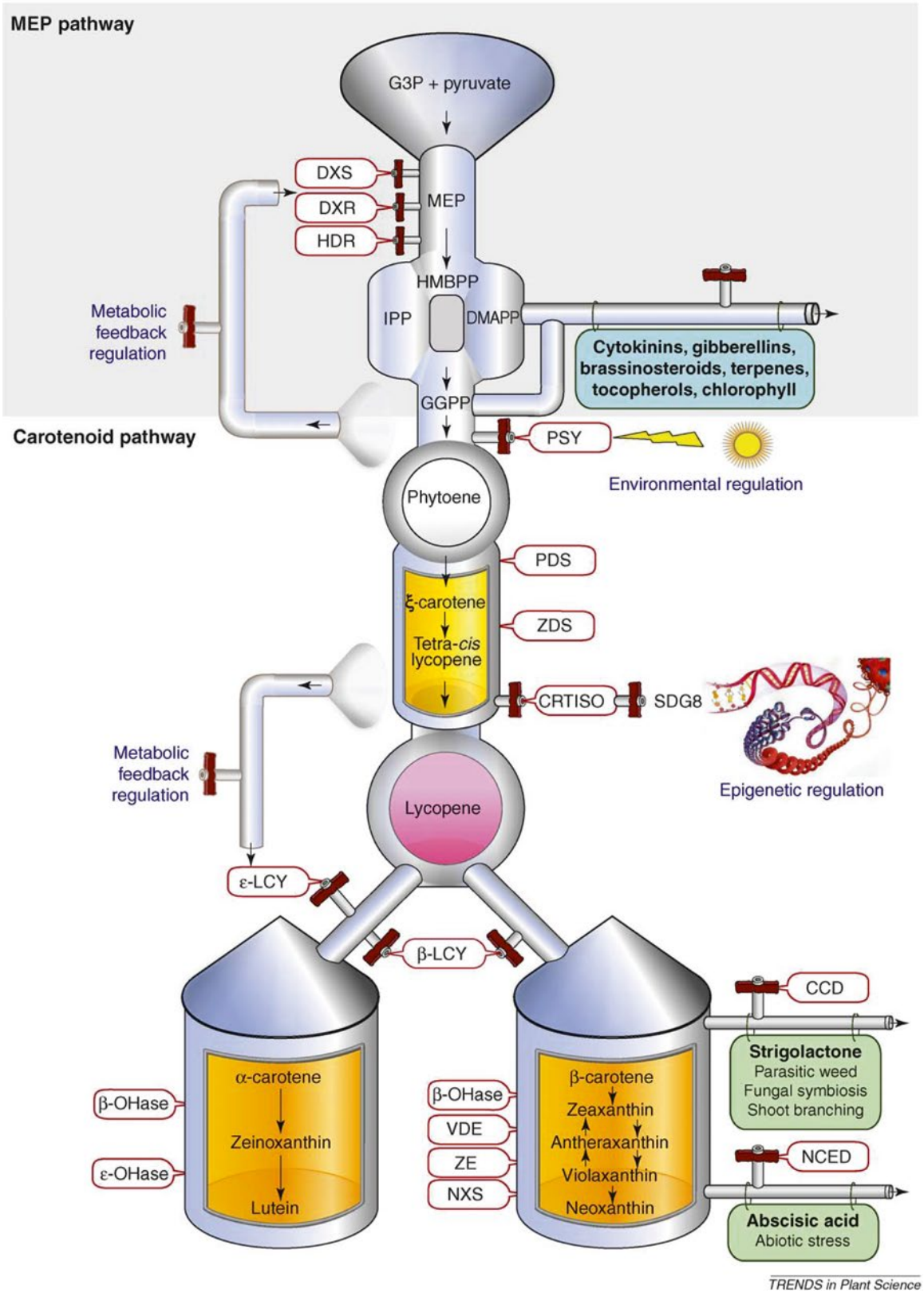


Fig. 25 Major reactions in the higher plant carotenoid biosynthetic pathway showing enzymes, carotenoids and their precursors (*pipes*), carotenoid sinks (*barrels*), carotenoid-derived signalling hormones (*green signs*) and other MEP isoprenoid-derived metabolites (*blue sign*). The windows displayed within the chrome pipes indicate abundant carotenoid pigments found in photosynthetic tissues and also represent key nodes for regulation in the pathway. Carotenoid biosynthesis is modulated by environmental factors (*light*), chromatin modification and metabolic feedback regulation. The side funnels represent examples of metabolic feedback control mechanisms acting upon biosynthetic gene expression as a result of altered PSY and CRTISO enzymatic activity, respectively. First, the bottleneck in phytoene biosynthesis is regulated by PSY and its overexpression increased *DXS* and *DXR* mRNA levels post-transcriptionally in etiolated tissues. Second, loss-of-function CRTISO mutants show reduced eLCY transcript levels in etiolated tissues. Abbreviations: *βLCY* β-cyclase, *βOHase*

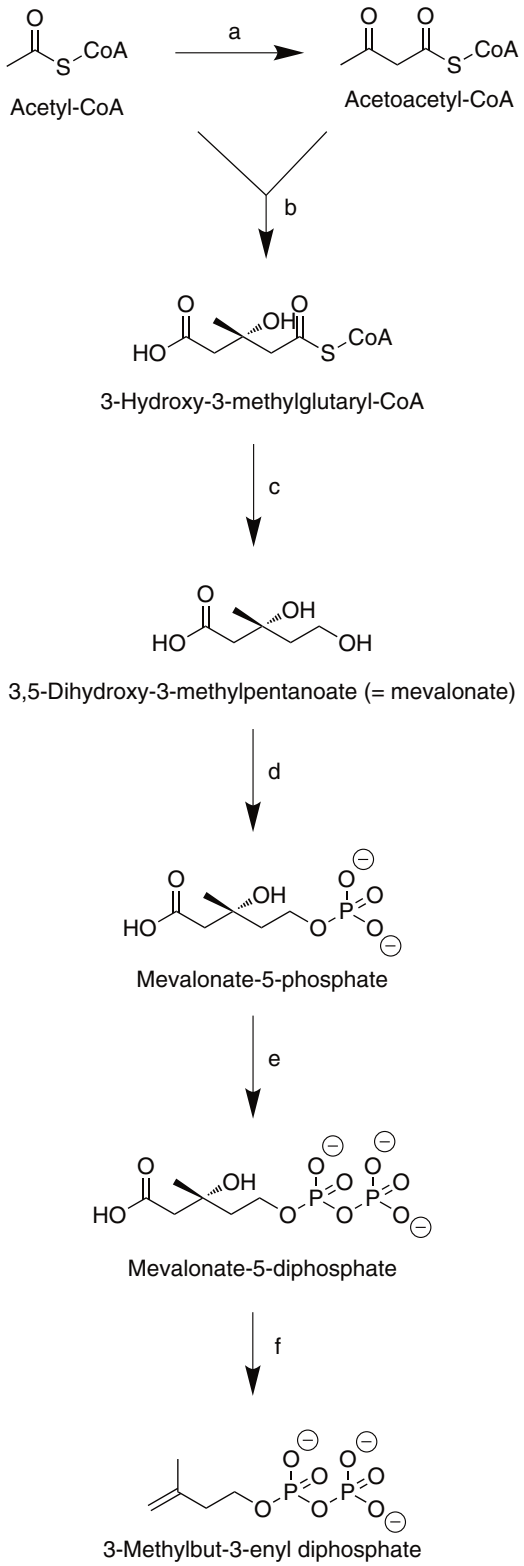


Fig. 26 Biosynthesis of 3-methylbut-3-enyl diphosphate (= isopentenyl diphosphate) – the 3,5-dihydroxy-3-methylpentanoate (= mevalonate) pathway

secondary carotenoids is mostly reported to occur in green algae (Goodwin 1980; Solovchenko 2013), although not exclusively (Young 1993). Some examples are given below.

3.7.1 Light Stress

Besides the normal biosynthetic regulation by the xanthophyll cycles (see above), high irradiances may induce production of secondary carotenoids. The secondary carotenoids probably protect the algal cells, simply by shading it from critical irradiances, which could damage or kill the cells.

In the green alga *Chromochloris* (*Chlorella*) *zofingiensis*, the synthesis of astaxanthin was induced by high irradiance; combined nitrogen deprivation and high irradiance increased the astaxanthin content even further (Cordero et al. 2010), while cells cultivated heterotrophically on sugar at darkness also produced astaxanthin (e.g. Sun et al. 2008). In *Haematococcus pluvialis*, Li et al. (2008) reported elevated astaxanthin production induced by high irradiance, but even more combined with addition of sodium acetate and iron (II) sulphate; see also Lee and Zhang (1999) for a discussion of how light intensity affects the production of astaxanthin in *Haematococcus*. Also, for *Dunaliella*, light intensity is a factor in the production of secondary carotenoids; see e.g. Jeffrey and Egeland (2009) and references therein, Borowitzka (2013b), and Mogedas et al. (2009).

Large cytoplasmic oil bodies of triglycerides form a depot for β,β-carotene in the freshwater green alga *Lobosphaera* (*Parietochloris*) *incisa* cultivated under high irradiance (Solovchenko 2012).

3.7.2 Nutrient Stress

The secondary carotenoids are often produced under nitrogen deficiency (Goodwin 1980; Jeffrey and Egeland 2009 and references therein), although other factors may also influence, e.g. light intensity (see above).

Both nitrogen and sulphur starvation gave increased production of carotenoids in *Dunaliella salina* (Salguero et al. 2003), even more by the combination of nitrogen starvation and high irradiance. Abe et al. (2007) have described the synthesis of secondary carotenoids in the aerial green microalga *Coelastrella striolata* var. *multistriata* under nitrogen deficiency, canthaxanthin being the main secondary carotenoid, in addition to some β,β-carotene and astaxanthin. Similarly the aerial green alga *Trentepohlia aurea* and *T. arborum* have a higher carotenoid (mainly β,β-carotene) productivity when N-limited (Abe et al. 1998; Chen et al. 2015).

3.7.3 Other Kinds of Stress-Induced Production of Secondary Carotenoids

A wide range of compounds may also induce production of secondary carotenoids. A few examples will be given here, to illustrate the diversity of such compounds.

The most common example of stress-inducing compounds is salt, increasing the production of β,β-carotene in *Dunaliella salina* (e.g. Loebich 1982; Borowitzka et al. 1990). Another example is increased astaxanthin accumulation in *Chromochloris zofingiensis* when exposed to moderate amounts of iron (II) ions (Wang et al. 2013). In *Haematococcus*

Table 1 Distribution of known carotenogenic genes/enzymes in the genomes of various algae and cyanobacteria (The table with its text and data is taken from Lohr (2011) with additions from Lohr et al. (2012))

Step	Enzyme (*gene*)[a]	Full name	EC	*C.r. V.c.*	*O.l. O.t.*	*C.m. G.s.*	*P.t. T.p.*	*E.h.*	7421	6803	7002	7942	9313
1	DXS	1-Deoxy-D-xylulose 5-phosphate synthase	2.2.1.7	+	+	+	+	+	+	+	+	+	+
2	DXR/IspC	1-Deoxy-D-xylulose 5-phosphate reductoisomerase	1.1.1.267	+	+	+	+	+	+	+	+	+	+
3	MCT/IspD	2-*C*-Methyl-D-erythritol 4-phosphate cytidyltransferase	2.7.7.60	+	+	+	+	+	+	+	+	+	+
4	CMK/IspE	4-(Cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol kinase	2.7.1.148	+	+	+	+	+	+	+	+	+	+
5	MDS/IspF	2-*C*-Methyl-D-erythritol 2,4-cyclodiphosphate synthase	4.6.1.12	+	+	+	+	+	+	+	+	+	+
6	HDS/IspG	4-Hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	1.17.4.3.	+	+	+	+	+	+	+	+	+	+
7	HDR/IspH	4-Hydroxy-3-methylbut-2-en-1-yl diphosphate reductase	1.17.1.2	+	+	+	+	+	+	+	+	+	+
8a	IDI type I	Isopentenyl diphosphate:dimethylallyl diphosphate isomerase type I	5.3.3.2	+	+	+	2	+	–	–	–	–	–
8b	IDI type II	Isopentenyl diphosphate:dimethylallyl diphosphate isomerase type II	5.3.3.2	–	–	–	–	–	–	+	+	+	–
9	GGPS/CrtE	Geranylgeranyl diphosphate synthase	2.5.1.29	+	+	+	+	+	+	+	+	+	+
10	PSY/CrtB	Phytoene synthase	2.5.1.32	+	2	+	+/2	+	+	+	+	+	+
11a	PDS/CrtP	Phytoene desaturase		+	2	+	2	+	–	+	+	+	+
11b	CrtI	Phytoene desaturase		–	–	–	–	–	+	–	–	–	–
12	Z-ISO	ζ-Carotene isomerase		n.i.	n.i.	n.i.	n.i.	n.i.	–	n.i.	n.i.	n.i.	n.i.
13	ZDS/CrtQb	ζ-Carotene desaturase	1.14.99.30	+	n.o.	+	+	+	–	+	+	+	+
14	CRTISO/CrtH	Carotenoid isomerase		+	+	n.o.	n.o.	n.o.	n.o.	+	+	+	+
15a	LCYB/CrtL-b	Lycopene β-cyclase		+	+	+	+	+	–	–	–	+	+
15b	CruA	Lycopene β-cyclase		–	–	–	–	–	+	+	+	–	–
15c	CruP	Lycopene β-cyclase		+[b]	+[b]	+[b]	+[b]	+[b]	+	+	+	+	–
16	LCYE/CrtL-e	Lycopene ε-cyclase		+	+	–	–	–	–	–	–	–	+
17a	CrtR	Carotene β-hydroxylase (non-haem iron)		–	–	+	–	–	–	+	+	+	+
17b	CHYB/CrtZ	Carotene β-hydroxylase (non-haem iron)		+	+	–	–/+[c]	–	–	–	–	–	–
17c	CYP97A	Carotene β-hydroxylase (cytochrome P450)		2/+	+	–	–	–	–	–	–	–	–
18	CYP97C	Carotene ε-hydroxylase (cytochrome P450)		+	+	–	–	–	–	–	–	–	–
19	ZEP	Zeaxanthin epoxidase	1.14.13.90	+	2	–	3/2	2	–	–	–	–	–
20	VDE	Violaxanthin de-epoxidase	1.10.99.3	n.o.	+	–	+	+	–	–	–	–	–

Table 1 (continued)

Step	Enzyme (*gene*)[a]	Full name	EC	*C.r. V.c.*	*O.l. O.t.*	*C.m. G.s.*	*P.t. T.p.*	*E.h.*	7421	6803	7002	7942	9313
21a	BKT/CrtW	Carotene β-ketolase		+	−	−	−	−	+	−	+	−	−
21b	CrtO	Carotene β-ketolase		−	−	−	−	−	+	+	−	−	−
22	NSY	Neoxanthin synthase	5.3.99.9	+	+	−	+	+	+[d]	+[d]	+[d]	+[d]	−
23	CruF	Carotenoid ψ-1-hydroxylase		−	−	−	−	−	+	+	+	−	−
24	CruG	Carotenoid ψ-2′-*O*-glycosyltransferase		−	−	−	−	−	+	+	+	−	+
25	CrtG	2,2′-β-Hydroxylase		−	−	−	−	−	−	+	−	+	−
26	CruE	Carotenoid β-ring desaturase/ methyltransferase		−	−	−	−	−	+	+	+	−	−
27	CruH	Carotenoid χ-ring C18-hydroxylase		−	−	−	−	−	+	+	+	−	−
a	AACT	Acetoacetyl-CoA thiolase	2.3.1.9										
b	HMGS	3-Hydroxy-3-methylglutaryl-CoA synthase	2.3.3.10										
c	HMGR	3-Hydroxy-3-methylglutaryl-CoA reductase	1.1.1.34										
d	MVK	Mevalonate kinase	2.7.1.36										
e	PMK	5-Phosphomevalonate kinase	2.7.4.2										
f	MVD	Mevalonate-5-diphosphate decarboxylase	4.1.1.33										

Data were compiled by Dr. Martin Lohr (2011) from the references in Lohr (2011) and were complemented by BLAST searches for homologues in GenBank (http://www.ncbi.nlm.nih.gov/GenBank) and the Genome Portal of the Joint Genome Institute (http://genome.jgi-psf.org). The presence of a unique homologue in a genome is indicated by '+', multiple highly similar homologues are denoted by the respective copy number; *n.i.* not identified, *n.o.* no orthologue. See the figures in this chapter for the function of the enzymes. Abbreviations of organisms are: *C.m. Cyanidioschyzon merolae, C.r. Chlamydomonas reinhardtii, E.h. Emiliania huxleyi, G.s. Galdieria sulphuraria, O.l. Ostreococcus lucimarinus, O.t. Ostreococcus tauri, P.t. Phaeodactylum tricornutum, T.p. Thalassiosira pseudonana, V.c. Volvox carteri*; 6803 = *Synechococcus* sp. PCC 6803; 7002 = *Synechococcus* sp. PCC 7002; 7421 = *Gloeobacter violaceus* PCC 7421; 7942 = *Synechococcus elongatus* PCC 7942; 9313 = *Prochlorococcus marinus* MIT 9313

The addition given with small letters is added from Lohr et al. (2012)

[a]Abbreviations of enzymes from eukaryotic/prokaryotic (if available) phototrophs. Abbreviations of eukaryotic enzymes are according to Hirschberg, Cunningham and Phillips et al. (see Lohr 2011 for refs.). In cases in which there are multiple synonyms, abbreviations indicative of catalytic functions of the proteins were preferred. For carotenogenic enzymes from bacteria, a different nomenclature is commonly used (see Lohr 2011 for ref.). Here, affiliation of a gene locus/enzyme to the pathway of carotenoid biosynthesis is indicated by the abbreviation 'Crt' for 'carotenoid' (or 'Isp' for 'isoprene', in the case of enzymes of the isoprene-forming MEP pathway), followed by a capital letter in which is assigned in alphabetical order to newly discovered genes. Meanwhile, the number of known carotenogenic genes has become larger than the size of the alphabet. Consequently, by advancing the third letter in 'Crt' by one, new genes have been labelled with 'Cru' plus a capital letter.

[b]The function of the CruP gene in eukaryotic phototrophs is not known

[c]CHYB in *T. pseudonana* is probably a pseudogene

[d]As cyanobacteria do not synthesise neoxanthin, the function of the NSY orthologue in these organisms is unknown

Table 2 Distribution of the MVA and the MEP pathways in examples of different oxygenic phototrophic species (The table with its text and data is taken from Lohr et al. (2012). For reference literature for evidence, see Lohr et al. (2012))

Phylogeny, organism	Pathway		Evidence
	MVA	MEP	
Cyanobacteria	–	+	Various genomic
Synechocystis sp. PCC 6714	–	+	Biochemical
Primary endosymbiosis			
Glaucophyta – glaucocystophytes			
Cyanophora paradoxa	+	+	Cloning, EST[a]
Rhodophyta – red algae			
Cyanidioschyzon merolae	–	+	Genomic
Galdieria sulphuraria	+	+	Cloning, biochemical
Cyanidium caldarium	+	+	Biochemical
Viridiplantae – green lineage			
Chlorophyta – green algae			
Chlorophyceae			
Chlamydomonas reinhardtii	–	+	Genomic
Acutodesmus (*Scenedesmus*) *obliquus*	–	+	Biochemical, EST
Trebouxiophyceae			
Chlorella fusca (=*Scenedesmus fuscus*)	–	+	Biochemical
Coccomyxa sp. *C-169*	–	+	Genomic
Prasinophyceae			
Ostreococcus lucimarinus	–	+	Genomic
Tetraselmis striata	–	+	Biochemical
Streptophyta – streptophytes			
Mesostigma viride	+	+	Biochemical, genomic
Embryophytes – land plants	+	+	Biochemical, various genomic
Secondary endosymbiosis			
Euglenophyta			
Euglena gracilis	+	+	Biochemical, EST
Chlorarachniophyta			
Bigelowiella natans	+	+	EST
Heterokontophyta – stramenopiles			
Chrysophyceae			
Ochromonas danica	+	+	Biochemical
Bacillariophyceae – diatoms			
Phaeodactylum tricornutum	+	+	Biochemical, genomic
Nitzschia ovalis	+	+	Biochemical
Pelagophyceae			
Aureococcus anophagefferens	+	+	Genomic
Haptophyta – haptoflagellates			
Isochrysis galbana	?	+	EST
Emiliania huxleyi	+	+	Genomic
Cryptophyta – cryptomonads			
Guillardia theta	?	+	EST
Alveolata – alveolates			
Dinophyta – dinoflagellates	?	+	Various EST
Apicomplexa (non-photosynthetic, apicoplast)			
Plasmodium falciparum	–	+	Biochemical, genomic

This table is not comprehensive in that for chlorophytes only exemplary species were listed. '+' indicates proof of existence of pathway. '–' indicates absence of the pathway. '?' indicates unknown if present

[a]*EST* expressed sequence tag

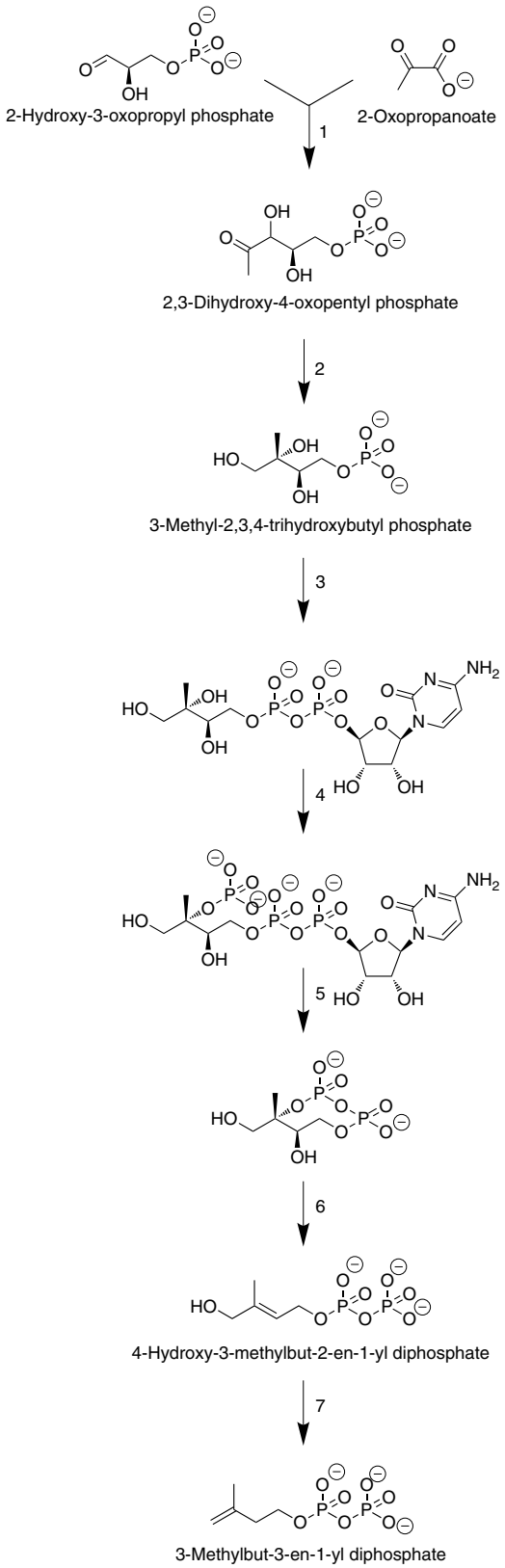


Fig. 27 Biosynthesis of 3-methylbut-3-enyl diphosphate (= isopentenyl diphosphate) – the 3-methyl-2,3,4-trihydroxybutyl phosphate (=2-*C*-methyl-D-erythritol phosphate) pathway

pluvialis, the plant hormone jasmonic acid increases the production of astaxanthin (Gao et al. 2012a), the same effect was found with salicylic acid (Raman and Ravi 2011; Gao et al. 2012b). Orosa et al. (2005) have reported elevated amounts of astaxanthin esters upon addition of malonate, as well as acetate. FInally, the reduction or increase of pH caused increased synthesis of β,β-carotene in both *Chloroidium ellipsoideum* and *Dunaliella salina* (Khalil et al. 2010).

3.8 Carotenoid Metabolites

Carotenoids may be the basis for the synthesis of carotenoid metabolites, apo-carotenoids (where at least the middle double bond system of the conjugated chain is retained) or end group metabolites. Although some work has been done on carotenoid metabolites, this has been with focus on metabolites from plant or animal carotenoids, and not on metabolites from algal carotenoids. An overview has been published by Britton (2008b). From algae, an apo-carotenone (not an apo-carotenoid as such, as the middle part of the carotene conjugated double bond system is lacking) has been reported from the green alga *Chaetomorpha basiretrorsa* (Shi et al. 2005). Watson (2003) has given an overview of algal odour compounds and their effects of signal compounds; a couple of these are probable carotenoid metabolites. The effect of β-cyclocitral from *Microcystis* on *Daphnia magna* is described by Jüttner et al. (2010); the lytic effect on *Microcystis* and *Phormidium* is described by Harada et al. (2009).

4 Chemical Analysis of Algal Carotenoids

Carotenoids may be handled in the lab in far smaller amounts compared with work with colourless compounds, due to their bright colours, making it very easy to follow them from one step to another, e.g. from algal cells to solvent extract. It is also pleasant to work with such nice colours!

4.1 Precautions for Work

The laboratory to be used must be free from all strong oxidation agents and strong acids, as these will destroy the carotenoid pigments. Sunshine must be avoided; a laboratory without any windows is perfect, but also thick curtains may be used to block the sunlight from the room. The light in the laboratory should be as low as possible, but enough to work safely, e.g. turn off the light near you, but keep a desk lamp lightening up a wall to give indirect light or have the light turned on in the other end of the laboratory room. If low light is not possible, sheets of black cloth may cover equipment

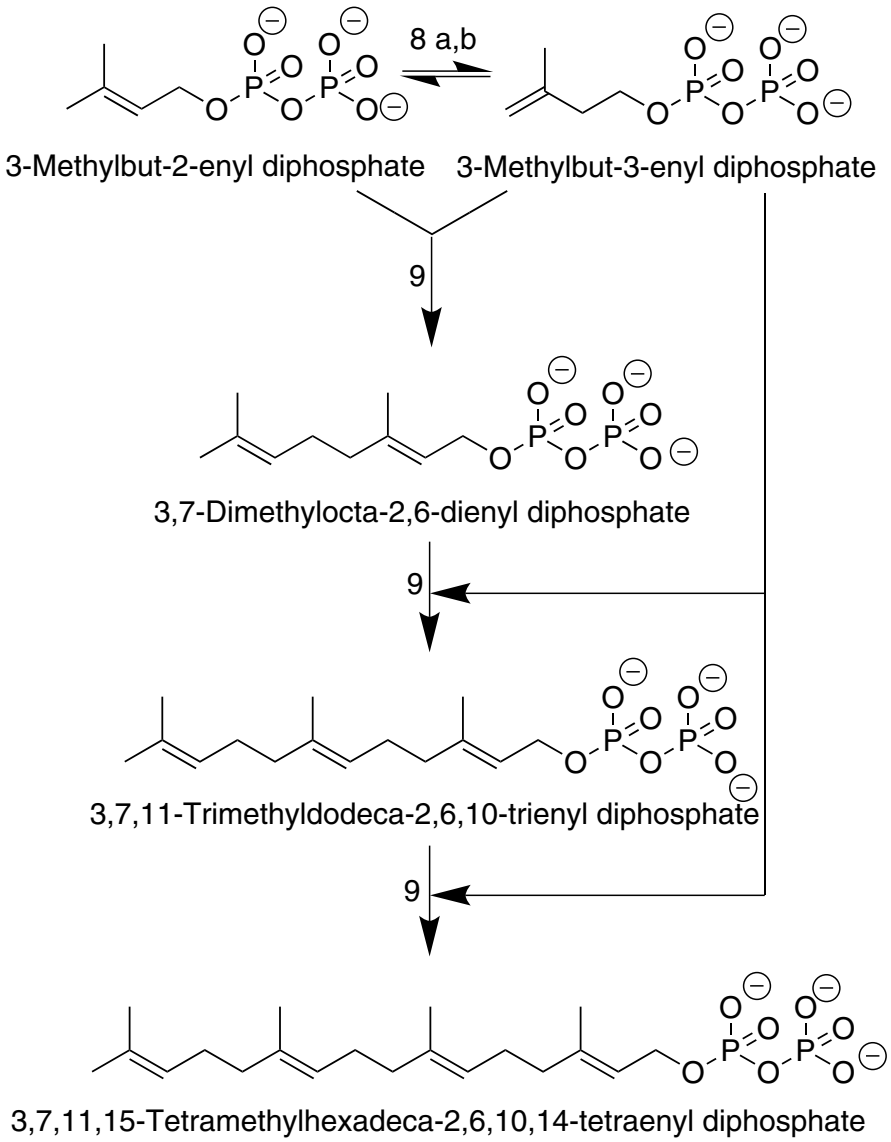


Fig. 28 Biosynthesis of 3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraenyl diphosphate (= geranylgeranyl diphosphate)

and all kinds of flasks or vials containing carotenoids, hiding the carotenoids from exposure to light. Ordinary beakers can be 'lined' with a piece of a thick black cloth (make sure to have large enough cloth to use some as a lid), such are excellent for holding flasks and tubes, e.g. when carrying from one room to another.

Oxygen degrades carotenoids, so nitrogen or argon gas (preferably 99.999 % pure or better) must be available to add to all flasks and vials containing carotenoids, for removal of oxygen from air. All samples must be flushed with nitrogen every time they are handled; always keep the carotenoids under nitrogen! Bubbling gas through solutions of carotenoids is generally not necessary; replacing the air above the solution with nitrogen (or argon) is usually sufficient.

Ethers should in general be avoided, as these may contain peroxides, degrading the carotenoids. Whenever ether is used, it should be purified just before use to remove the peroxides. All other solvents should be of high quality (preferably 99 % pure), bulk quality solvents might be distilled to obtain pure solvents to a more affordable price.

Some prefer to add antioxidants to prevent oxidation of the carotenoids, but this will influence the analysis by a light absorption spectrophotometer, and any antioxidant must be removed before a mass spectrum or an NMR spectrum is recorded.

Any storage of carotenoid-containing extracts, etc., in room temperature should be avoided; all samples should be stored under nitrogen in a freezer, e.g. a common household freezer kept as cold as possible. A refrigerator may be suitable for short-time storage, but should not be used for any overnight or long-time storage. Of course, to work within a freezer is not possible, but make sure to keep the carotenoids out in the lab as short time as possible. Be aware that stoppers usually 'pop' off any tube or vial, when the sample is

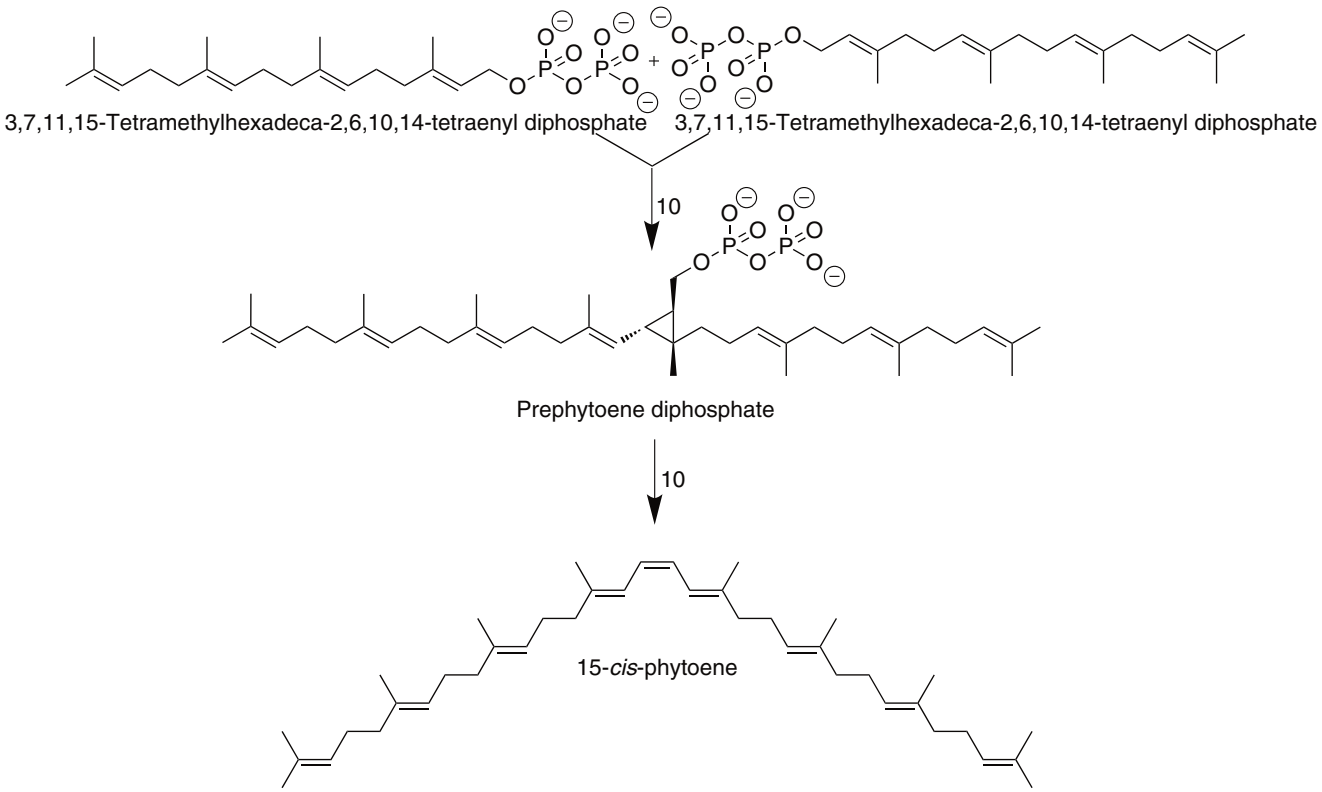


Fig. 29 Biosynthesis of 15-*cis*-phytoene, the first carotenoid

warmed up by room temperature. Screw caps are preferred, but any common stopper can be used, just be ready to replace any stopper popping off the flask due to evaporation of solvents, and flush with nitrogen.

All carotenoids will be degraded by any kind of acid, and the epoxidic carotenoids like antheraxanthin and violaxanthin reacts even with any trace of acid, turning them into carotenoid furanoxides (see below). This is why strong acids should never be used in the same room as the carotenoids. Prasinophyte extracts contains naturally enough acid (or enzymes?) to degrade all epoxidic carotenoids, so prior to extraction, the cells should be added small amounts of a common buffer at pH 7.2.

A good advice is to never use all your algae material at once, instead keep some in case of accidents or unexpected degradation of the carotenoids.

For general literature on handling of carotenoids, see e.g. Schiedt and Liaaen-Jensen (1995) or Pinckney et al. (2011).

Plastic equipment is generally not recommended, as the plasticisers may leak into the pigment extracts. Glassware washed with a good, solid detergent and water, next rinsed with plenty of pure water and dried in a warm cupboard (60–120 °C) is preferred. Make sure not to use any acid-washed equipment for cultivation! Common automated pipettes should generally not be used, as these are calibrated for water, having a different surface tension than solvents. Common glass pipettes are recommended.

As you are working with harmful solvents, you may want to use gloves. Special chemical gloves can well be used, but be sure to use the right glove for the right solvent in use; it is impossible to find gloves that protect against all kinds of solvents. Do not use any type of glove that does not protect against the solvent you are handling, or disposable gloves! The solvents penetrate through the glove material if the glove is not made for the right solvent. The pores of your skin is more open in a glove, so the solvents will enter your body more easily when using e.g. common disposable gloves. Instead, wash your hands with soap and water whenever you notice any spill of solvent.

Always wear safety glasses, and always work in a fume hood with the door lowered, so you are exposed as little as possible to the harmful solvent vapour. A common cotton laboratory coat is recommended, but keep it closed, and leave to dry in a fume hood if wetted by solvent spill.

Also have common safety equipment available, as eye-wash, emergency shower and fire extinguisher, preferably with CO_2.

4.2 Storage of Algae Prior to Extraction

Algae should be stored frozen, either on a folded filter (the filter enclosing the algal cells) or in a paste, free from as much water as possible. Be aware when filtering the cells, to use

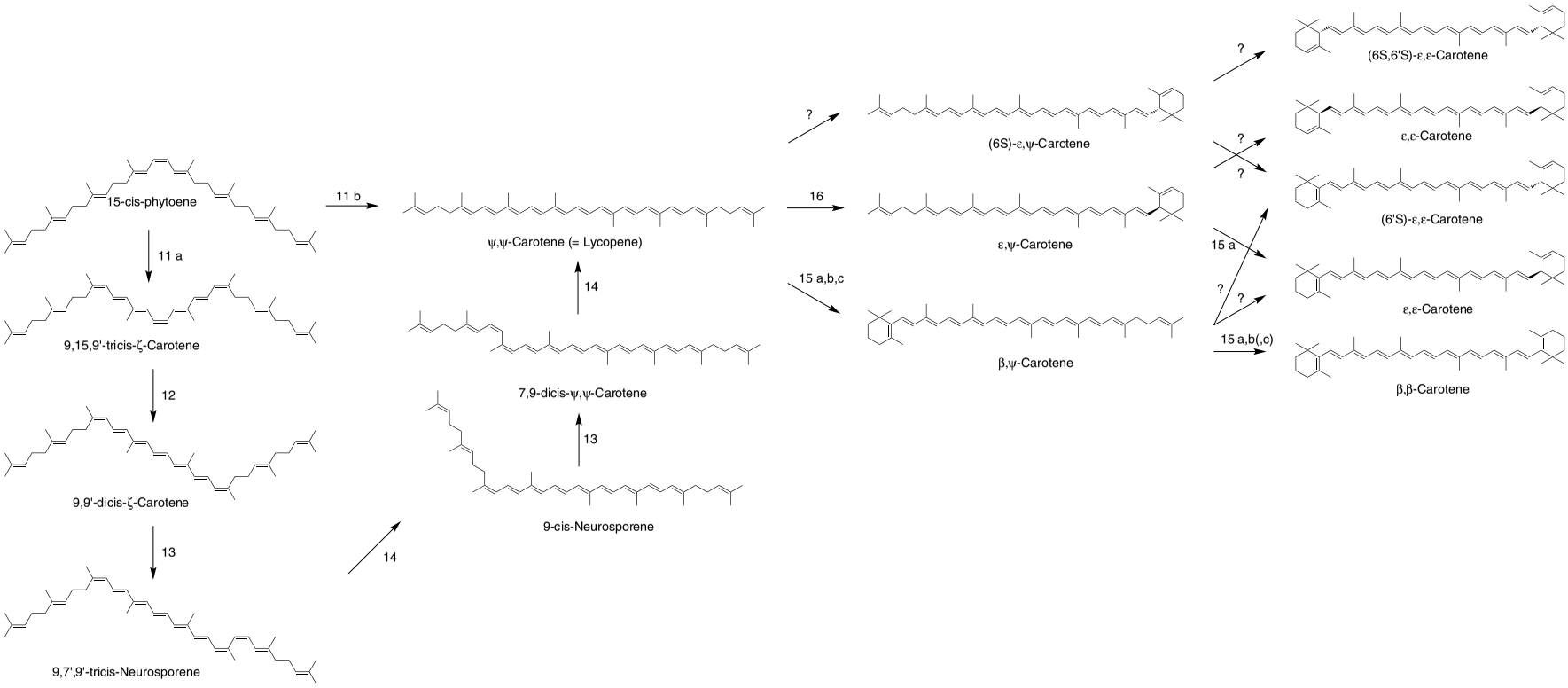


Fig. 30 Biosynthesis of the carotenes

gentle suction, not crushing the cells. If the cells are intact, they will protect the carotenoids from degrading. Keep the filter or the paste in a closed container, preferably free from air (by nitrogen or by flattening e.g. a small plastic bag).

Whenever algal cells need to be sent from one lab to another, the cells should be wrapped well, packed in a thick Styrofoam box with plenty of cooling elements and dry ice. Dry ice alone is not recommended, if the parcel gets stuck somewhere during transport, the dry ice might disappear, even though the parcel is placed in a freezer at the transport hub.

Sending frozen algal cells is risky businesses, as the parcels regularly are delayed or misplaced on the way, ruining the sample if thawed. A more secure way of sending cells is by lyophilisation (freeze-drying) prior to sending, but lyophi-

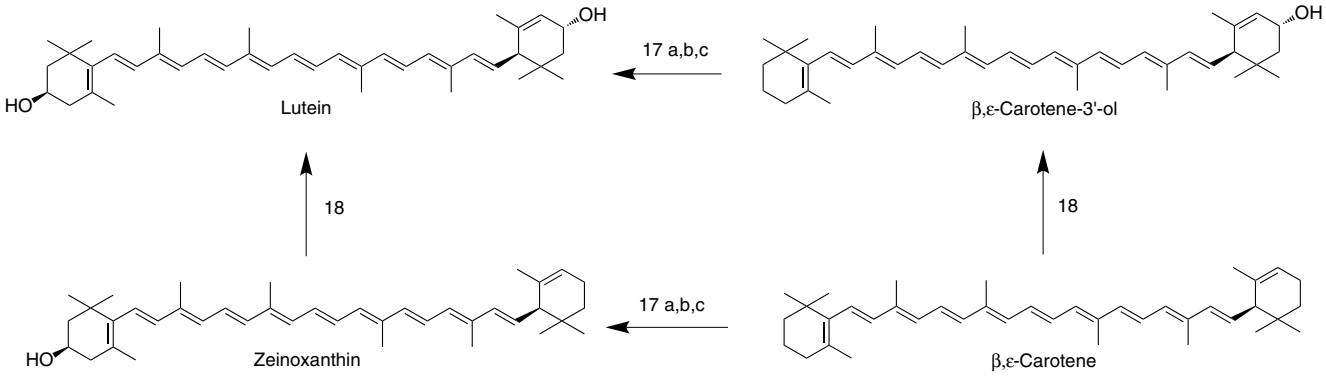
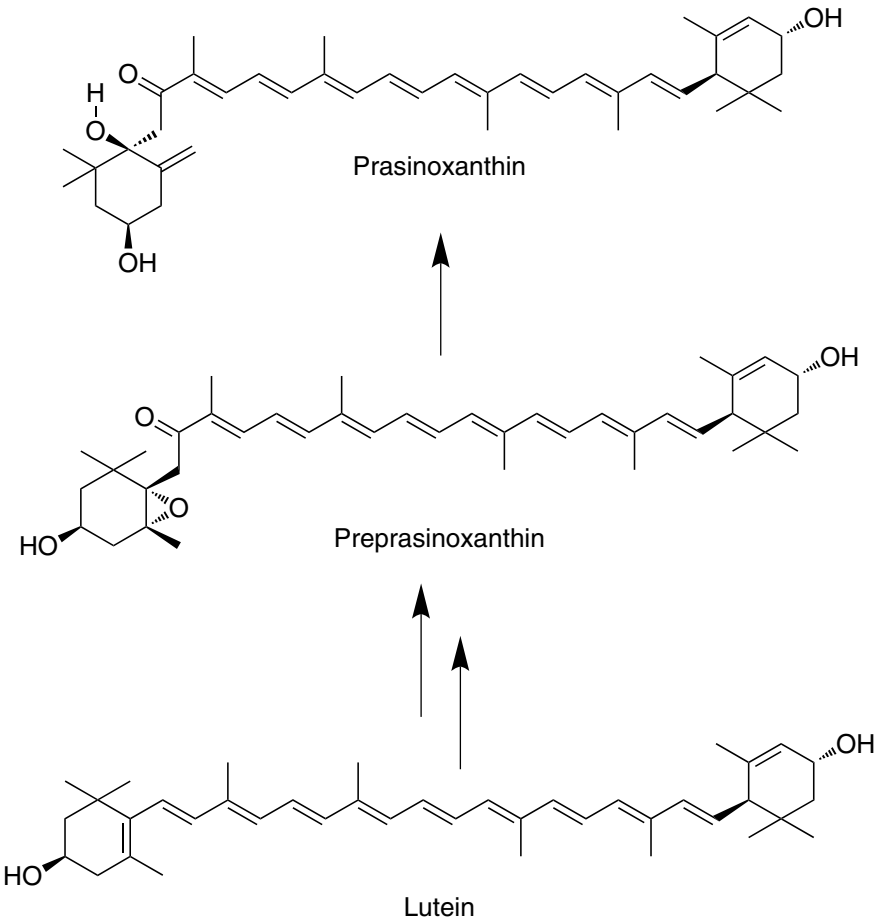


Fig. 31 Biosynthesis of lutein

Fig. 32 Suggested biosynthesis of prasinoxanthin



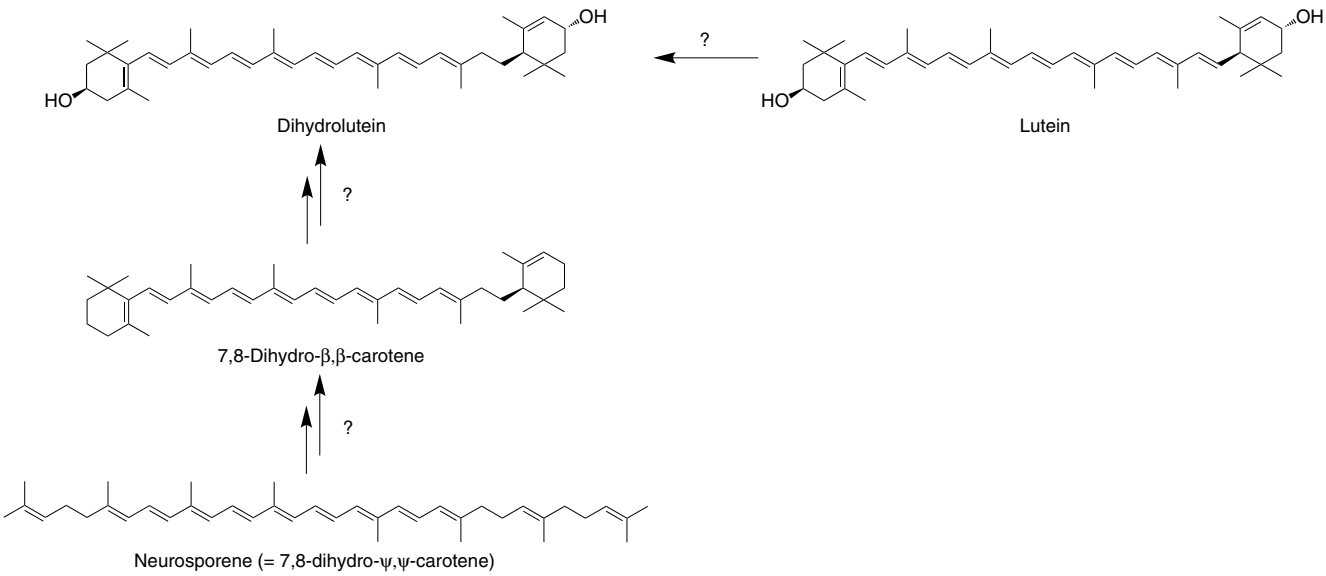


Fig. 33 Suggested biosynthesis of dihydrolutein

lisation itself may degrade some of the carotenoids, especially when the freeze-dried material is stored over some weeks or months before analysis.

4.3 Extraction from Algal Cells

Several solvents and solvent mixtures have been used for carotenoid extraction. The solvent to be used must be able to extract all pigments completely from the cells, as soon as possible, to prevent degradation of the carotenoids. In some cases, as for example when extracting red *Haematococcus pluvialis* cells, the cell wall must be disrupted (e.g. by enzymatic degradation), so the solvent can enter the cells and extract the carotenoids (e.g. Grung et al. 1992).

If the extract is to be evaporated later as for example when the pigments are to be dissolved in a different solvent, the solvents to be used should not contain any water and have boiling points less than 100 °C. When the extract will only to be injected directly into the HPLC, and does not need any evaporation, heavier solvents or water-containing solvent mixtures may be used. Be aware that water-containing solvent mixtures may cause degradation of carotenoids.

My personal favourite is propan-2-one (= acetone), whenever the cells are frozen, and 30 % methanol in propan-2-one, whenever extracting lyophilized cells. When prasinophytes are extracted, small amounts of buffer pH 7.2 buffer should be added prior to extraction, to prevent degradation of the epoxidic carotenoids. When extracting lyophilised algae, the cells may be soaked in a few drops of water prior to extraction, whenever the extraction is difficult by using solvent alone. In some cases, decanting the solvent and adding fresh solvent may be needed to extract all the pigments from the cell material. Make sure to extract the cells long enough to have all the pigments extracted, the material will usually obtain a pale brown colour, or a pale green colour, if degraded chlorophylls are left in the cells.

Any minor amounts of water may be evaporated away with benzene using a rotary evaporator (do not heat the water bath to more than 30 °C). Use some absolute ethanol to mix water and benzene prior to evaporation. Be aware, benzene is a known carcinogenic, so handle in a hood and keep the bottles closed. Although benzene has its strong disadvantage, it is still the preferred choice, as water can evaporate away with benzene without heating the sample too much.

Watch the evaporation carefully, as any water left in the flask may degrade the carotenoids, if not enough benzene has been added. If so, add more ethanol and benzene, and reapply the suction in the rotary evaporator.

For extraction, more information is given by Schiedt and Liaaen-Jensen (1995) and Pinckney et al. (2011).

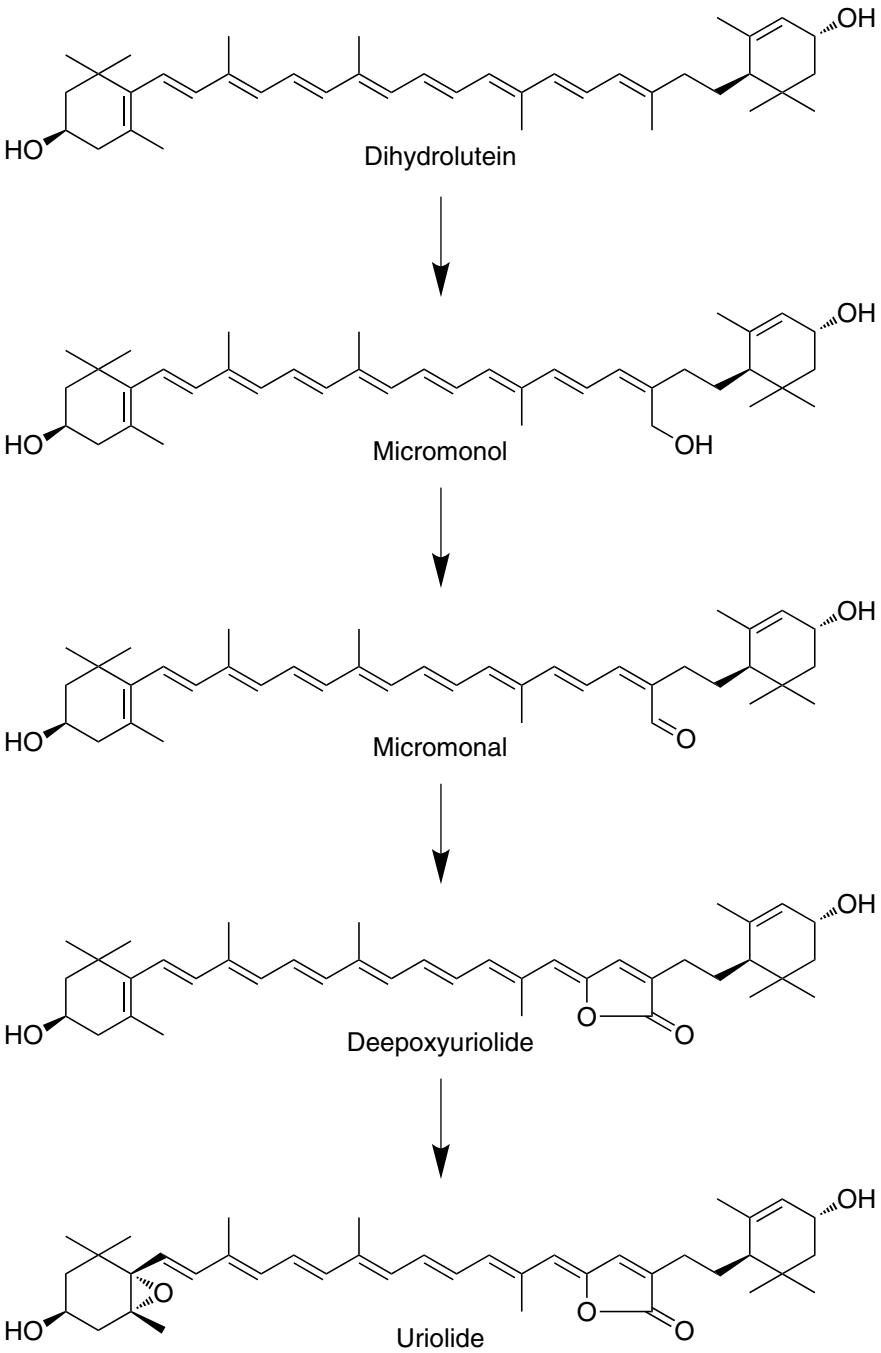
4.4 Chromatography

The extract may be used for chromatography, e.g. HPLC or thin layer chromatography (TLC). This will separate the carotenoids for identification by e.g. mass spectrometry, or for other experiments.

4.4.1 Thin Layer Chromatography (TLC)

TLC is a simple method, both concerning expenses and need of training. Watching someone with some experience is the easiest way to learn the technique. In general, a beaker is

Fig. 34 Suggested biosynthesis of uriolide

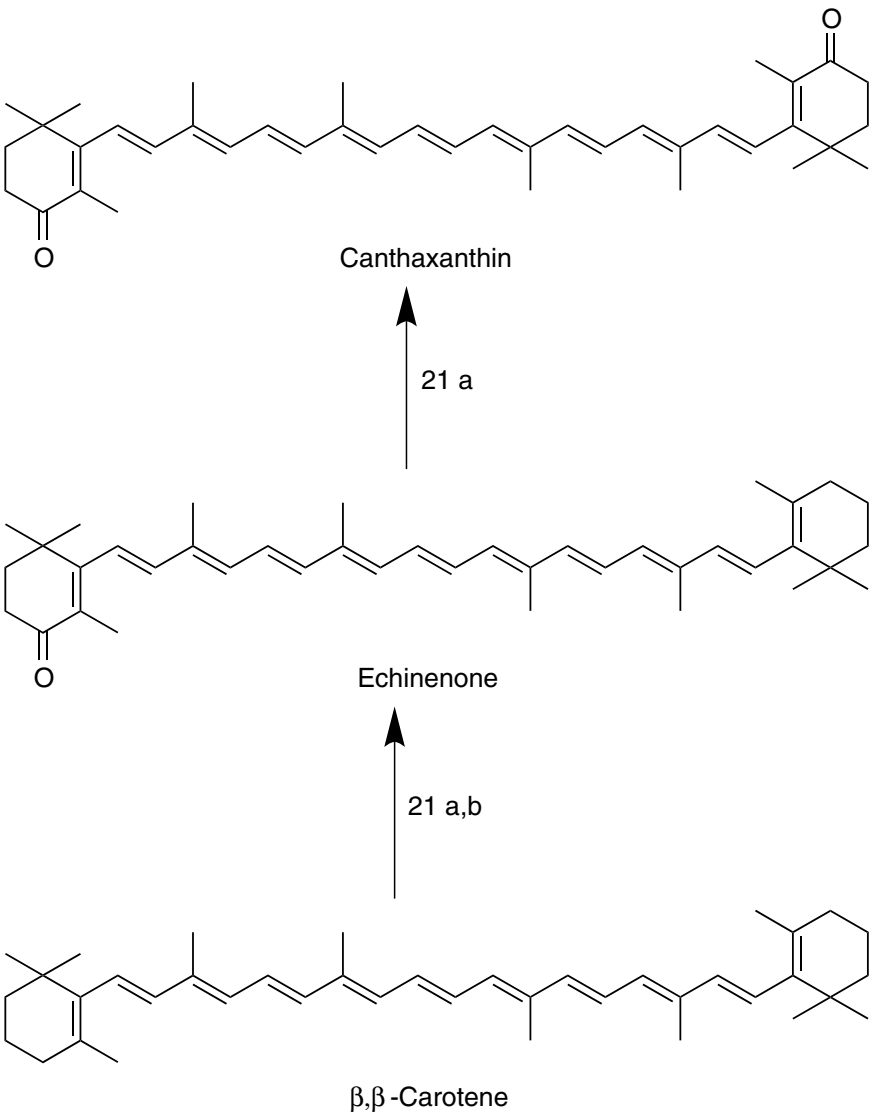


added half a centimetre with the solvent to be used, e.g. 20–30 % acetone in hexane for crude extracts, and closed by a lid. A sample of the extract is applied through a narrow glass tube near the shorter end of a TLC plate, a stream of nitrogen gas evaporates the solvent, and the plate is lowered into the solvent chamber, close the lid immediately. After a few minutes, separation may be seen.

The colours of the spots indicate what kind of pigments are present. Yellow spots are violaxanthin or ε,ε-carotene, while zeaxanthin or β,β-carotene is more orange-yellow. Fucoxanthin and other ketocarotenoids are brownish orange, and astaxanthin and ψ,ψ-carotene looks red on the plate. Chlorophylls vary in colour from green to grey, but also carotenoid furanoxides may look green on a silica TLC plate, due to partially presence of blue oxonium ions adding to the yellow colour (Haugan and Liaaen-Jensen 1994b).

Scraping off the solid layer band/spot, dissolving it in an appropriate solvent, e.g. acetone and filtrate away the solids, may collect each of the separated pigments.

Fig. 35 Biosynthesis of canthaxanthin



TLC of carotenoids is described by Schiedt (1995); general information about TLC can be found in e.g. Stahl (1962), Poole (2003), and Spangenberg et al. (2011).

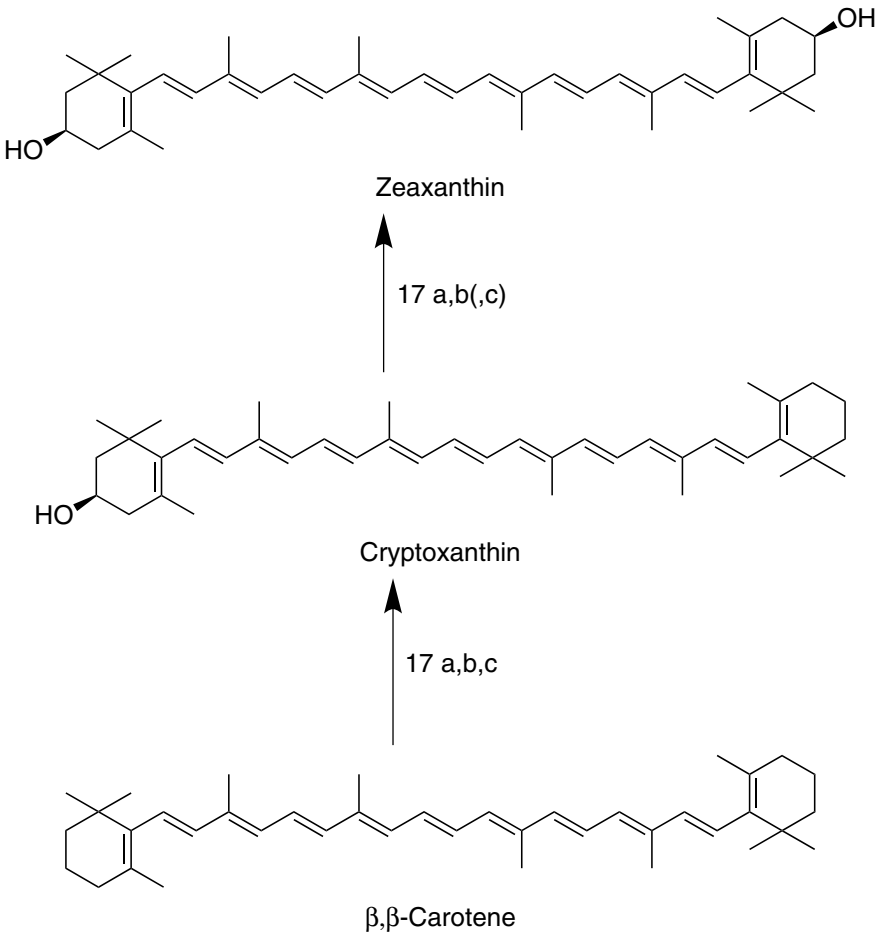
4.4.2 High Performance Liquid Chromatography (HPLC)

Whenever the purpose of the analysis is to quantify the amounts of each of the pigments present in the cells, high performance liquid chromatography (HPLC) is the preferred choice. This technique gives first a good separation of most, hopefully all pigments, quantification based on prior injection of carotenoid standards, assumed identity given based on the retention time of the same standards, and automatically summarised and printed results. HPLC, used correctly and carefully, can give the best possible results within a short period of time; usually the analysis of a sample takes 20–60 min.

The disadvantage of HPLC is first, the price. A good equipped HPLC with quaternary pump, a diode array detector and an cooled automated injector is expensive, and can cost up to about US$ 100,000. Second, the separation is performed on special columns, also expensive – easily US$1000 for one. Compared to the simple equipment needed for TLC, this is a lot of money.

A second disadvantage of HPLC is the automated analysis. When the computer nicely prints out calculated amounts of each carotenoid present with five decimals, it is easy to believe in the results. First, the accuracy is not five decimals. As the absorption coefficients, which the calibrations are founded on, have an uncertainty of ±5 %, and for some pigments probably ±20 %, five decimals are not trustworthy. Secondly, the identification of the pigments is not really any identification at all. It is just a probability, and may be wrong, either by misidentification of the peak by the instrument, or

Fig. 36 Biosynthesis of zeaxanthin



by the peak in the chromatogram accidently contains more than one pigment. The results of an automated HPLC analysis should always be checked manually for every analysis. If any mass spectrometer detector is coupled to the instrument, the certainty of the analysis is much, much higher, but the expenses of such an instrument may be ten times higher.

Third, the HPLC needs only tiny amounts of pigments to be analysed, so whenever the samples separated must be collected for further studies, or to keep as a reference standard for later analyses of other samples, it takes a lot of repeated analyses to achieve the needed amount of pigment.

Despite the disadvantages given here, the instrument is highly appreciated by carotenoid chemists or phytoplankton researchers.

For more information about HPLC on carotenoids, see e.g. Pfander and Riesen (1995) and several chapters in Roy et al. (2011). For general information about HPLC, see e.g. Poole (2003).

4.4.3 Other Kinds of Chromatography

Whenever large amounts of carotenoids (>1 mg) are needed, column chromatography may be used. This technique is poor in separation, but takes the largest amounts of pigment; see Bernhard (1995) for use on carotenoids, or Poole (2003) for general information about the technique.

Paper chromatography was used in older days, but is now only used when teaching pupils, else being replaced by TLC.

4.5 Spectroscopy

When working with carotenoids, a light absorption spectrophotometer is needed. This may be part of an HPLC instrument, as a detector, but a common spectrophotometer is needed for quantification of pigment standards for HPLC or isolated carotenoids from TLC. Other kinds of spectroscopy are very valuable, whenever available, making the identification of carotenoids go from 'probable' (best guess) to definite 'proven', when the pigment has underwent mass spectrometry and nuclear magnetic resonance analysis.

4.5.1 Visible Light Absorption Spectroscopy

A normal light absorption spectrophotometer is needed for any work with carotenoids. Various models are offered, having one-beam or double-beam setup, computer control and quantification, etc. For carotenoids, the spectrophotometer

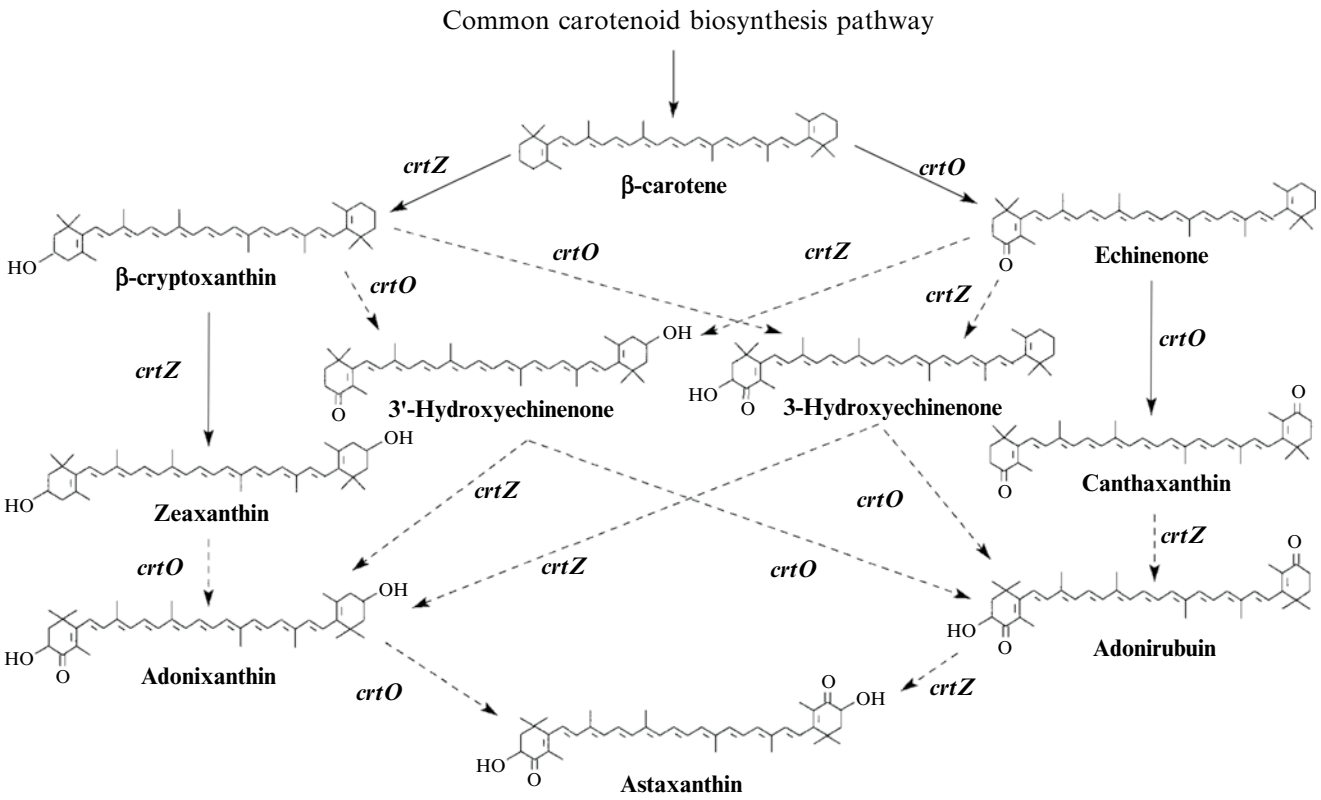


Fig. 37 Suggested biosynthesis of astaxanthin in *Synechococcus* cells. *Solid arrows* represent documented reactions and *dotted arrows* indicate speculated reactions in the biosynthesis pathway (From Harker and Hirschberg 1997; with permission)

should be able to scan and record between 350 and 700 nm. For quantification, the spectrophotometer should be calibrated/checked from time to time by analysing a standard, and be adjusted in case of any drift of baseline or changed light absorption recorded.

My personal experience is that such an instrument can last for many, many years, only needing a new lamp from time to time, and they offers stable and trustworthy results. The problem is often the computer device coupled with the instrument, as it may not last as long as the spectrophotometer, and it might be impossible to get a computer programme for a modern computer to command an old, but still good spectrophotometer.

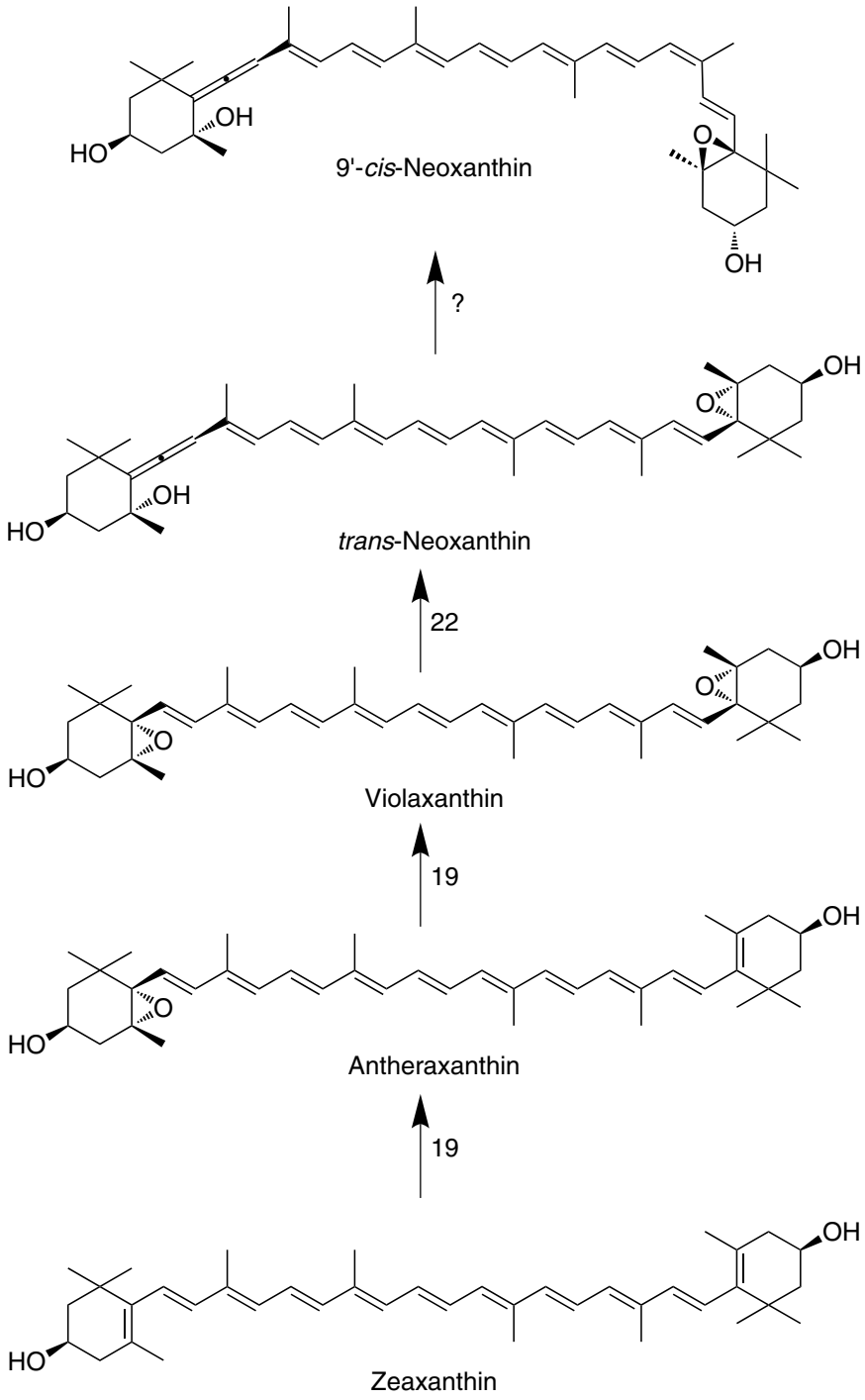
The main purpose is to see at what wavelength the pigment has its maximum absorption; this gives information about the length of the conjugated double bond chain (double and single bonds alternating). For β,β-carotene, having nine double bonds in the chain plus two ring double bonds, its maximal absorption is about 450 nm when dissolved in common solvents like propan-2-one (= acetone). Longer wavelength indicates more conjugated double bonds, shorter wavelengths fewer; see Fig. 47.

Next, the shape of the spectrum indicates what kind of conjugated chain that is present. If the spectrum is recognised as a 'hand' having distinct fingers, as in the spectrum of ψ,ψ-carotene ((31) in Fig. 48), the conjugated double bond chain is not part of any rings. If the right 'finger' is about half of the middle 'finger', the spectrum is typical for e.g. β,ψ-carotene ((12) in Fig. 48) or lutein, having conjugated double bond system reaching into one of the rings. Whenever the conjugated double bond system enters both end rings of the carotenoid, the spectrum looses some of its distinct shape, and the right 'finger' reduces to a distinct 'bump'. A typical example here is the spectrum of β,β-carotene, see (3) in Fig. 48. The rate of handedness is denoted as %III/II, see Fig. 49.

A conjugated carbonyl group (C=O) reduces the right 'finger' even more, removing the finger-shape at all, and the spectrum only looks like a round peak a bit flattened on one side, see e.g. the spectrum of echinenone ((283) in Fig. 50). Two conjugated carbonyl groups give a broad, round spectrum, as the spectrum obtained by canthaxanthin ((380) in Fig. 50; compared to β,β-carotene (3)). Here it is difficult to determine the maximum wavelength by only looking at the spectrum, as the peak is so round and broad.

Visible light absorption spectroscopy is commonly used for quantification of carotenoid pigments. To determine the amount of carotenoid, dissolve the carotenoid in a pure solvent (which one depends on the absorption coefficient used)

Fig. 38 Biosynthesis of 9′-*cis*-neoxanthin



and dilute to a known volume. The maximum absorbance is measured by the spectrophotometer using a one cm long cuvette, and the amount of carotenoid can be calculated from the formula:

$$\text{mg carotenoid} = \frac{\text{maximum absorbance} * \text{total volume of solution in mL}}{d}$$

The *d*-value, given in L g^{-1} cm^{-1}, can be found in Egeland (2011a, c). Alternatively, the following formula can be used:

$$\text{mg carotenoid} = \frac{10 * \text{maximum absorbance} * \text{total volume of solution in mL}}{A^{1\%}_{1cm}}$$

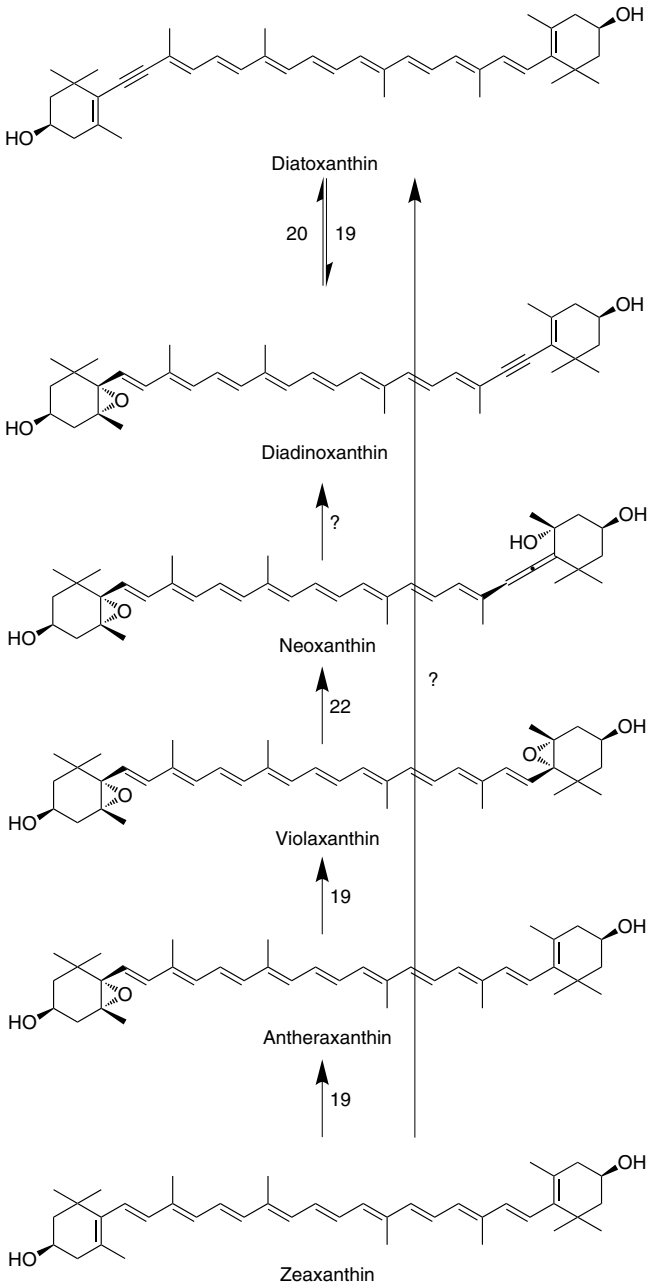


Fig. 39 Suggested biosynthesis of diatoxanthin and diadinoxanthin

The $A^{1\%}_{1cm}$ value, given in 100 mL g^{-1} cm^{-1}, can be found in Britton (1995) and Britton et al. (2004).

4.5.2 Mass Spectrometry

Mass spectrometry is the second most important spectroscopic technique to be used on carotenoids. Self-standing mass spectrometers are expensive and difficult to maintain, but nowadays, mass spectrometer detectors coupled to an HPLC instrument is gaining popularity.

The mass spectrum gives information about the molecular formula of the carotenoid, by giving its mass, and hence, the number of carbon, hydrogen and oxygen atoms. Fragment ions and the fragmentation pattern give important information about the molecule, and can e.g. indicate the number of hydroxyl groups (OH groups), adding more information about a carotenoid.

For details about the technique, see e.g. Enzell and Back (1995) and Airs and Garrido (2011).

A carotenoid cannot be denoted as 'identified' without a mass spectrum. The minimum requirement for the identification of a carotenoid is identical behaviour as a reference standard (e.g. bought from a company) in two chromatographic systems, identical visible light absorption spectrum as the standard, and a mass spectrum giving the molecular ion (Schiedt and Liaaen-Jensen 1995; Egeland 2011b).

4.5.3 Other Spectroscopic Techniques

Other important spectroscopic tecniques for carotenoid analysis is nuclear magnetic resonance (NMR) and circular dichroism (CD).

NMR instruments are very expensive to buy and laborious to maintain, as they need refill of liquid nitrogen and liquid helium on regular basis. Despite the disadvantages mentioned here, they are very popular, as they give information about every type of hydrogen and every type of carbon in the carotenoid molecule. It might be difficult to distinguish similar carotenoids using light absorption or mass spectrum, but any difference in the molecule, despite being minor, will be seen using NMR. Advanced techniques can further add information, e.g. by coupling hydrogen atoms to carbon atoms, or by elucidating the spatial arrangement in the molecule, indicating which atoms to be near each other in space. For an advanced description of NMR used on carotenoids, see Englert (1995).

CD is a rare technique, but it is needed whenever the chirality of a molecule should be determined, e.g. the difference of the two mirror-image β,ε-carotenes found in different cyanobacteria. The technique uses light absorption, like the common visible light absorption, but measures rotation of light instead. A compound cannot be assigned any chirality unless a reference standard is available, limiting the use of the technique on novel carotenoids, although some comparison can be done with carotenoids having similar structural elements. The technique used on carotenoids is described by Buchecker and Noack (1995).

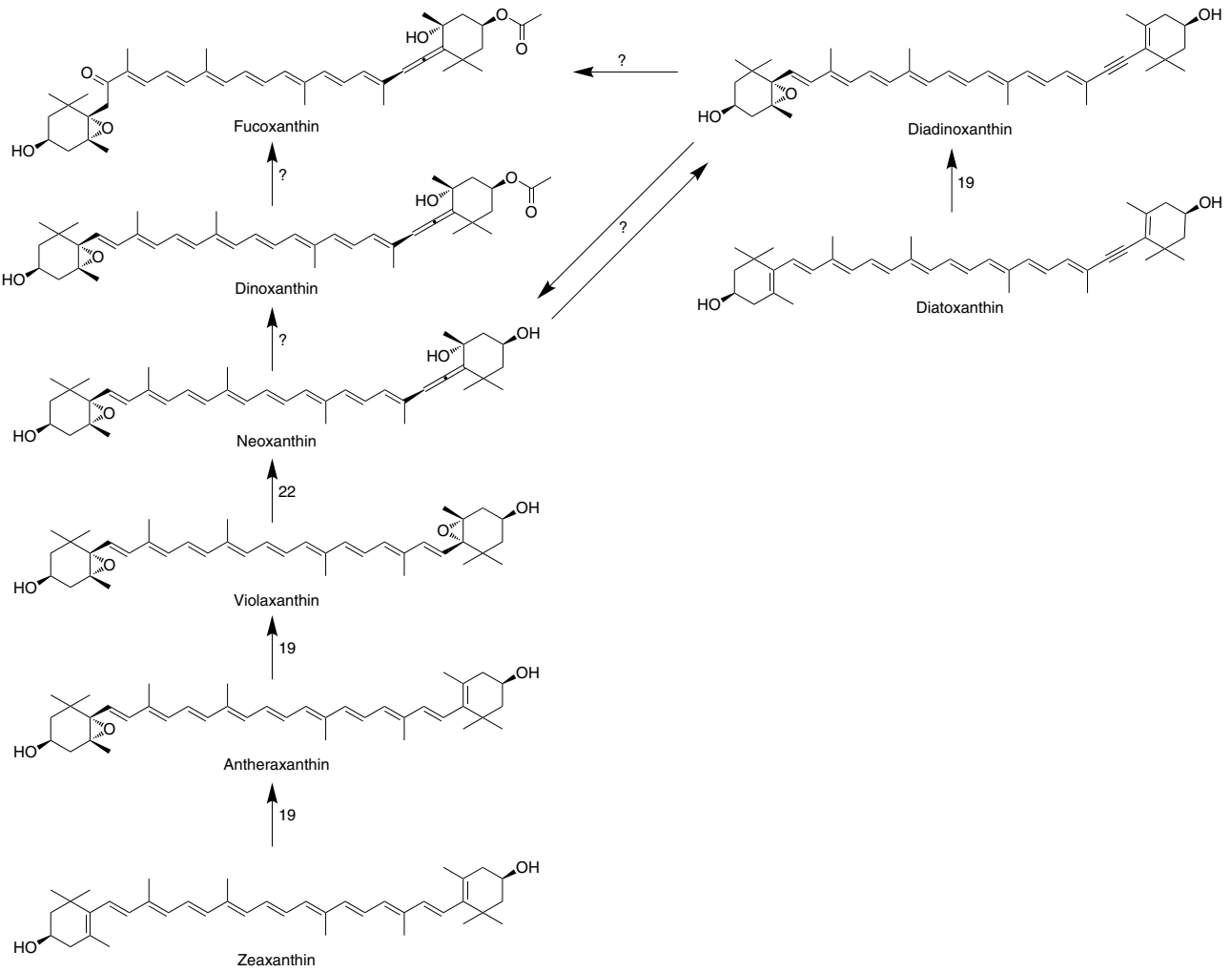


Fig. 40 Suggested biosynthesis of fucoxanthin

Since the CD instruments are rare, they may partially be replaced by HPLC using chiral columns, see Pfander and Riesen (1995).

A modern technique which seems to gain a lot of interest lately, is confocal Raman microspectroscopy, see e.g. Horath et al. (2006), Huang et al. (2009), Abbas et al. (2011), Collins et al. (2011), and Grosser et al. (2012).

4.6 Other Analytical Techniques

Other valuable techniques include derivatisation of the carotenoid molecule and chemical test reactions, e.g. to indicate epoxy groups or ester groups, see Eugster (1995) and Schiedt and Liaaen-Jensen (1995). Despite considered somewhat old-fashioned, it still offers easy and good indications of e.g. the number of hydroxyl groups in the carotenoid molecule, and the test reactions and derivatisation reactions described should not be forgotten.

4.7 Degradation Products

As carotenoids are unstable compounds, they may easily be altered by presence of light, acid, enzymes released from the algal material when filtered or frozen, etc. If the carotenoids only are modified into another carotenoid, it is usually possible to extrapolate the carotenoid distribution in the cells back to what was in the intact, natural cell. It is far worse if the carotenoids are so severely destroyed that they looses their colour, and therefore avoid detection, as the colour can neither be seen nor detected as for example by. the HPLC detector detecting at common carotenoid wavelengths.

As soon as the cell is disrupted by mechanical stress, freezing or by solvents penetrating the cell, the carotenoids

Fig. 41 Suggested biosynthesis of peridinin

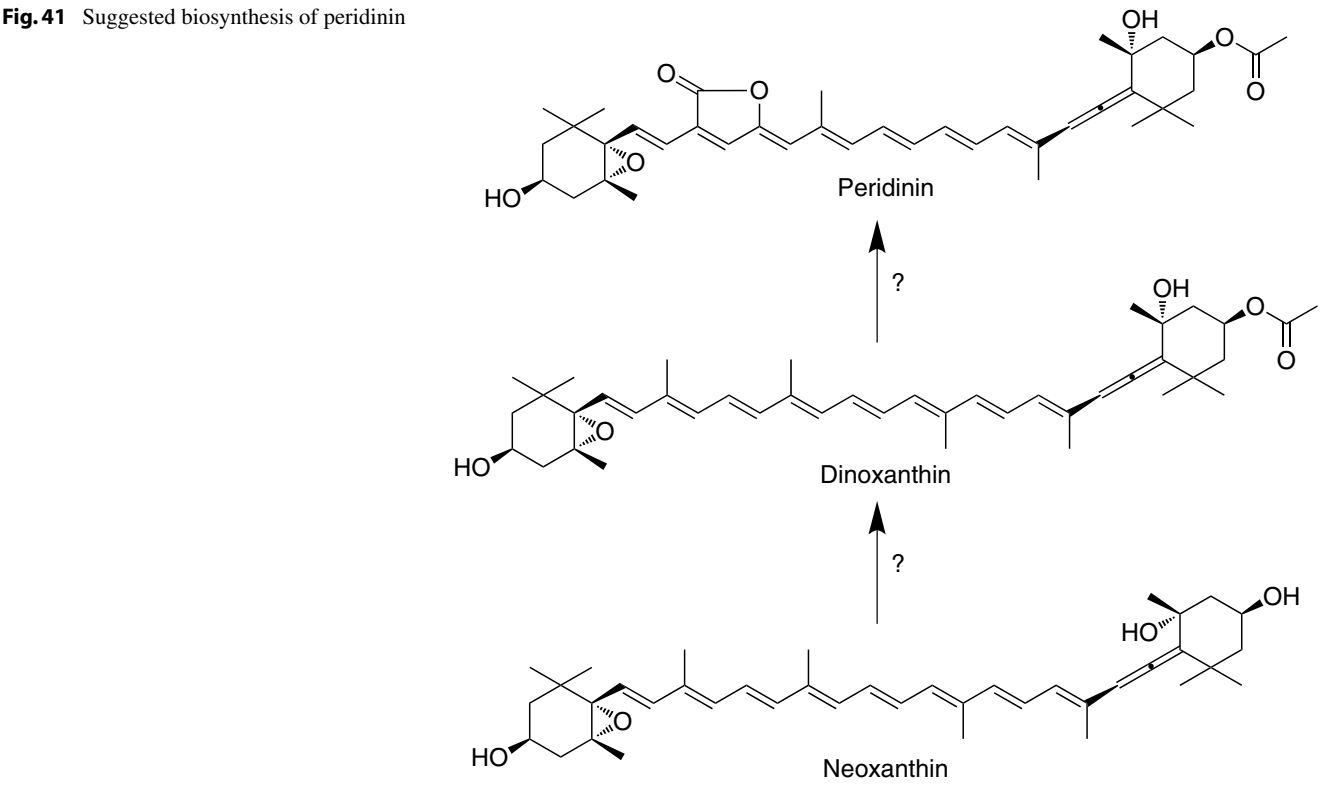


Fig. 42 Suggested biosynthesis of gyroxanthin

are freed from their natural, stable environment and exposed to air, solvent impurities, etc. Some common derivatives are described here. For further details, see Eugster (1995) and Schiedt and Liaaen-Jensen (1995).

4.7.1 Cis/Trans Isomers

Carotenoids have double bonds along the main backbone structure, almost exclusively in the *trans* form. Exceptions are the carotenals P-457 and micromonal. When a carotenoid is exposed to room temperature or above, to light, to traces of acids, to active surfaces, etc., one or more double bonds may isomerise from *trans* to *cis* form, commonly in the 9, 13, 15, 9′ or the 13′ position. *Cis*-isomers will usually be separated from the *trans* form on a good HPLC system, and their existence can be observed by an additional '*cis*-peak' appearing in the light absorption spectrum, (Fig. 51). The closer the *cis* bond is to the center of the chain, the higher *cis* peak. The main peaks of the spectrum will get slightly shifted to shorter

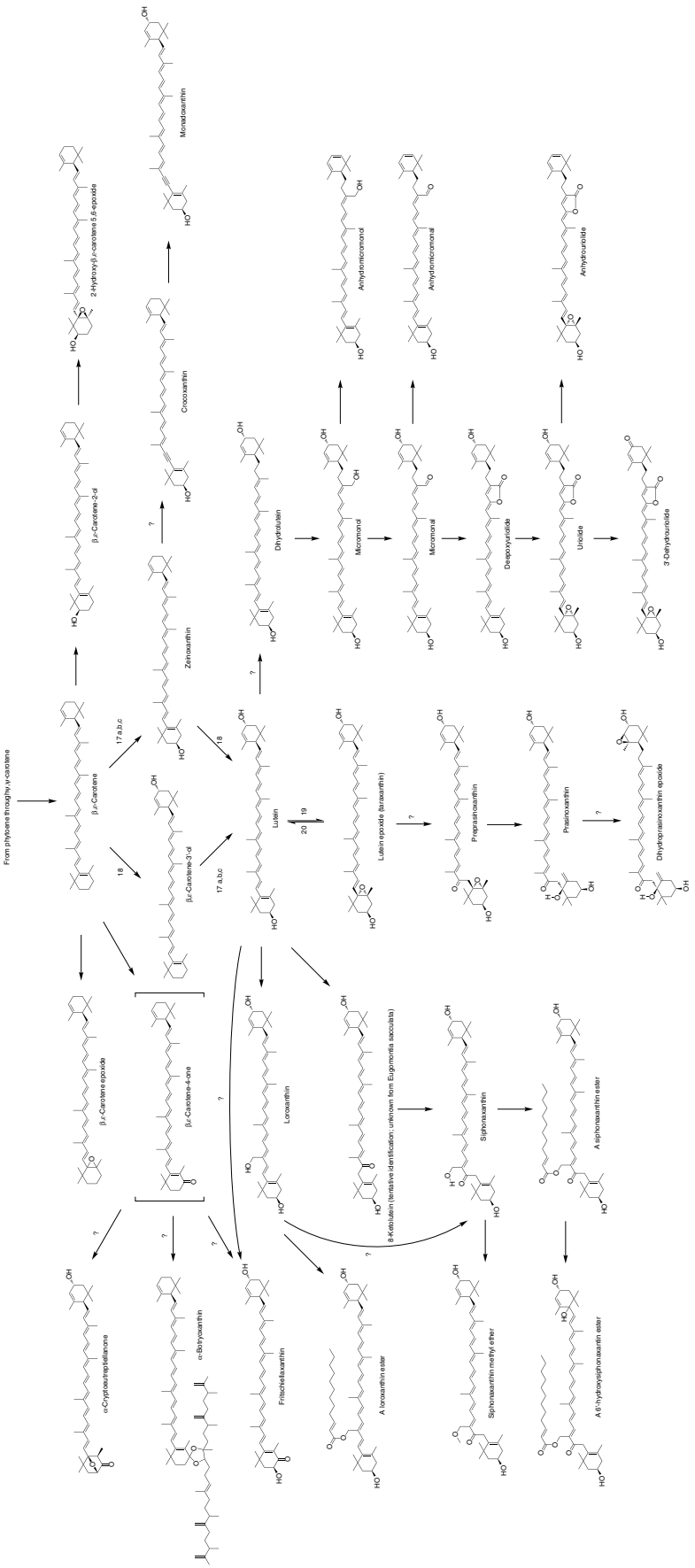


Fig. 43 Biosynthesis of derivates of β,ε-carotene

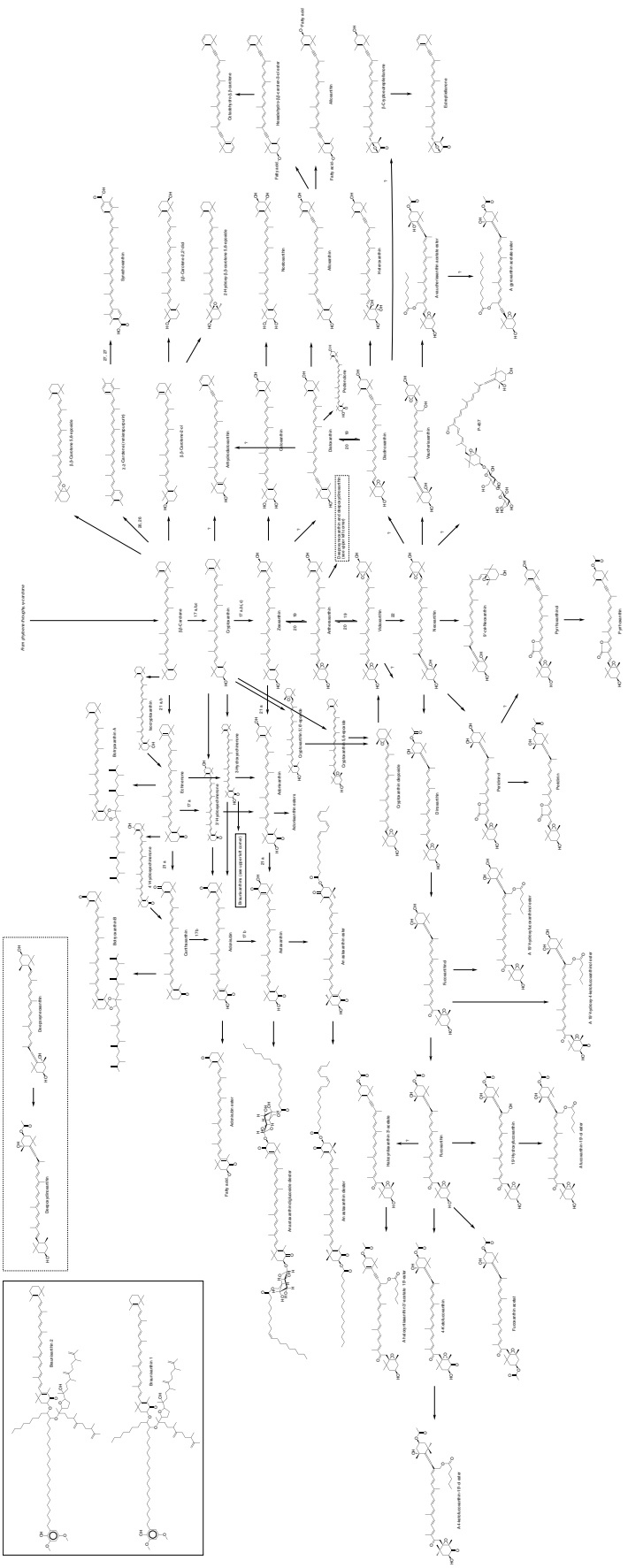


Fig. 44 Biosynthesis of derivates of β,β-carotene

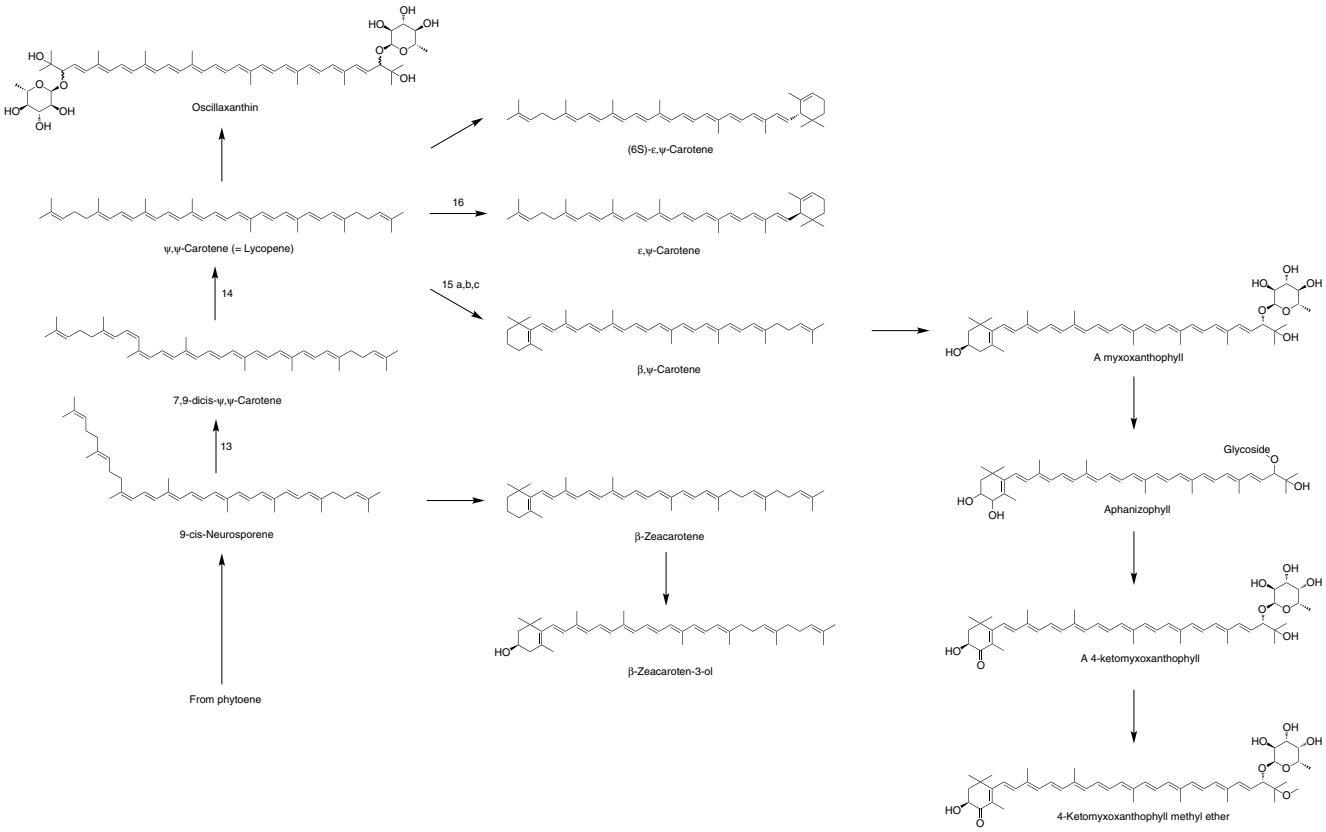


Fig. 45 Biosynthesis of derivates of β,ψ- and ψ,ψ-carotene

wavelengths, and the 'fine structure' (the distinctness of the 'fingers' in the spectrum) of the spectrum will be reduced, see Britton (1995).

4.7.2 Furanoxides

Furanoxides occur when carotenoid epoxides are exposed to traces of acids, as the three-membered oxygen ring will be opened to a five-membered ring. It is characteristic that two different diastereomers are formed, both an (8*R*)- and (8*S*)-furanoxide, usually in a 4:6 ratio via the intermediate cation. Examples can be found in Bjørnland et al. (1984) and Suzuki et al. (2003).

4.7.3 Alkali-Catalysed Derivatives

Astaxanthin, when exposed to alkali where traces of oxygen is present, will be oxidised to astacene, (Fig. 52). On silica or silica based stationary chromatography phases, the astacene binds efficiently to the silica, and will commonly not leave the HPLC column or move upwards on the TLC plate. Despite the additional two double bonds, compared to astaxanthin, the spectrum observes light at about the same wavelength, as the double bonds are 'cross-conjugated' in the two rings at the ends of the molecule.

Fucoxanthin and peridinin are, as astaxanthin, sentitive to alkali. A cation is formed, similar to the blue oxonium ion described above, and further a furanoxide (Haugan and Liaaen-Jensen 1994c). Also the carotenoids prasinoxanthin and 4-ketofucoxanthin (including 19′-alkanoyloxy-deriviatives) are cleaved β to the carbonyl group in the presence of alkali; leading to a new, less polar product; see Foss et al. (1986) and Egeland et al. (2000) for details. Carotenoid aldehydes react in the presence of alkali with propan-2-one (= acetone) to an aldol product, where the propanone molecule is added to the aldehyde group of the carotenoid (Schiedt and Liaaen-Jensen 1995).

Carotenoid esters (either esters of carotenoid carboxylic acids with alcohols, or carotenols esterified with acetate or fatty acids) may be hydrolysed by alkali to provide the parent carotenoid (given the carotenoid is not alkali-labile by itself), aiding the identification of the carotenoid structure (Eugster 1995; Schiedt and Liaaen-Jensen 1995).

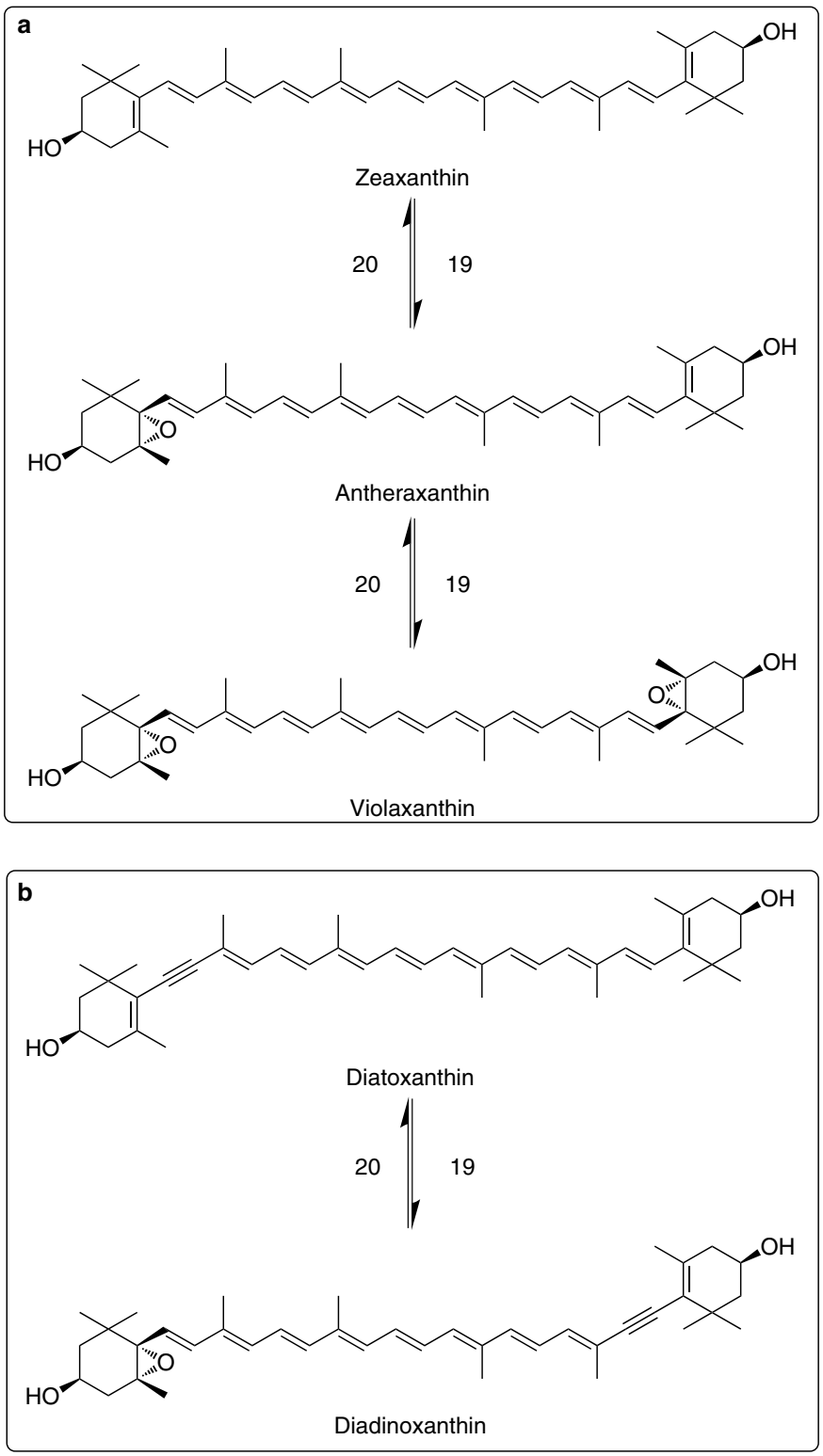


Fig. 46 Xanthophyll cycles: (**a**) zeaxanthin – antheraxanthin – violaxanthin; (**b**) diatoxanthin – diadinoxanthin

5 Examples of Uses of Algal Carotenoids

Algal carotenoids are used for a wide variety of purposes, from monitoring the algal community in the ocean to fish feed and colour agent in soft drinks. Some examples involving recent research studies will be given here.

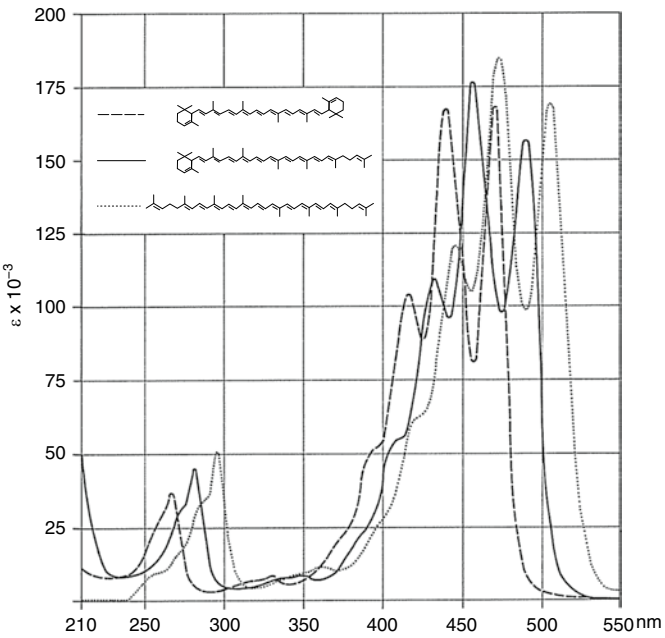


Fig. 47 Visible light absorption spectra of carotenoids with different number of conjugated double bonds (From Vetter et al. 1971)

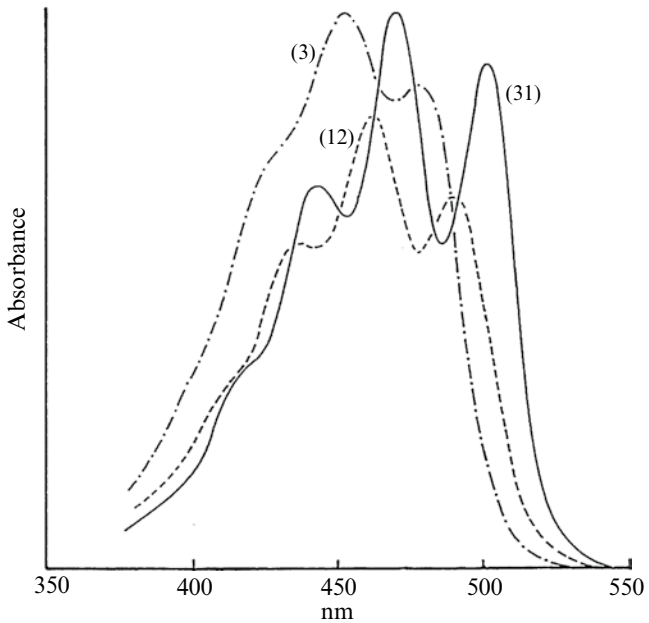


Fig. 48 Visible light absorption spectra of β,β-carotene with two rings (*3*), β,ψ-carotene with one ring (*12*) and ψ,ψ-carotene without rings (*31*) (From Britton 1995)

5.1 Oceanographic and Freshwater Surveys

Carotenoids are regularly used as tracers to phytoplankton community studies, mostly in oceanographic, but also freshwater analysis, see overviews given by Jeffrey et al. (1997) and Roy et al. (2011). Some recent articles, illustrating the use of carotenoids in oceanographic and freshwater surveys, are given here.

Descy et al. (2005, 2010) described the algal community composition of Lake Tanganyika, Africa, using the CHEMTAX software (Mackey et al. 1996) to calculate the biomass of the phytoplankton groups. Higgins et al. (2006) used the same programme for monitoring the phytoplankton distribution in the Bismark Sea, Papua New Guinea. Pereira et al. (2013) has reported the phytoplankton community in the Ria Formosa Lagoon, Portugal, Gin et al. (2003) along the coast of Singapore, Suzuki et al. (2011) in a phytoplankton spring bloom northeast of Japan and Dobrzyn et al. (2009) in Adventfjorden at Svalbard, an arctic group of islands north of Norway.

A study of the Nervion River estuary, Spain by Laza-Martinez et al. (2007) gives insights in the technique, and also to some of the problems of using carotenoids for phytoplankton community determination. Louda (2008) discusses difficulties with resuspension of sediments in coastal waters.

As the oceanographic surveys, also the benthic flora and sediments can be analysed by tracing the carotenoid pattern; see e.g. Reuss et al. (2005), Yakobi and Ostrovsky (2008), and McGowan et al. (2012).

5.2 Enriched Foods

Algal carotenoids are widely used as food additives; the isolated pigments can be added to food directly or offered as food supplement. Today, capsules with *Chlorella*, *Spirulina*, *Haematococcus*, *Pelvetia* and mixed seaweeds are for sale, in addition to purified β,β-carotene capsules (often from *Dunaliella salina*). Microorganisms and microalgae pigments, incl. carotenoids, as food pigment source has been

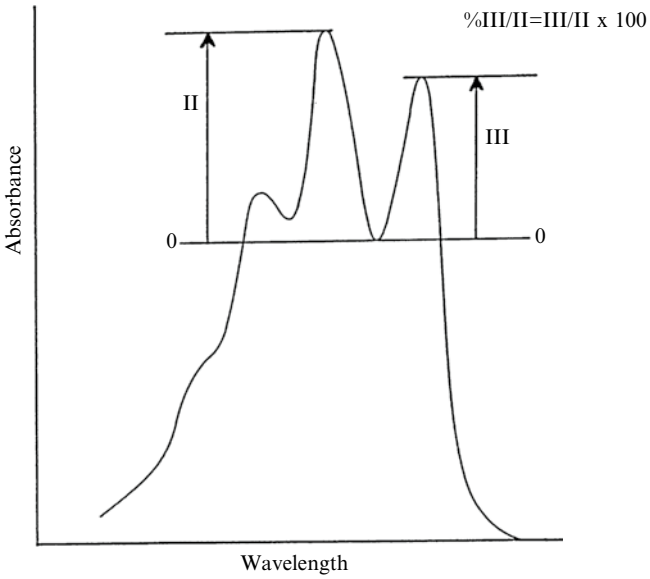


Fig. 49 Visible light absorption spectra: measurement of %III/II ratio (From Britton 1995)

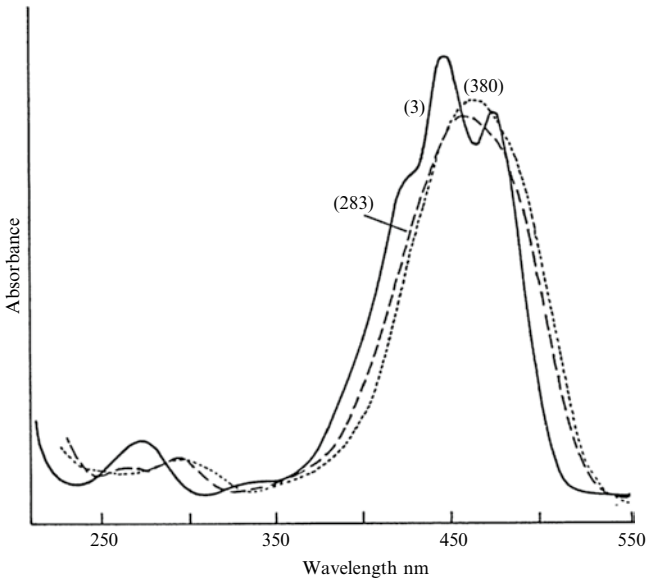


Fig. 50 Visible light absorption spectra for carotenoids with or without keto-groups. (*3*) β,β-Carotene – (*283*) Echinenone – (*380*) Canthaxanhtin (From Britton 1995)

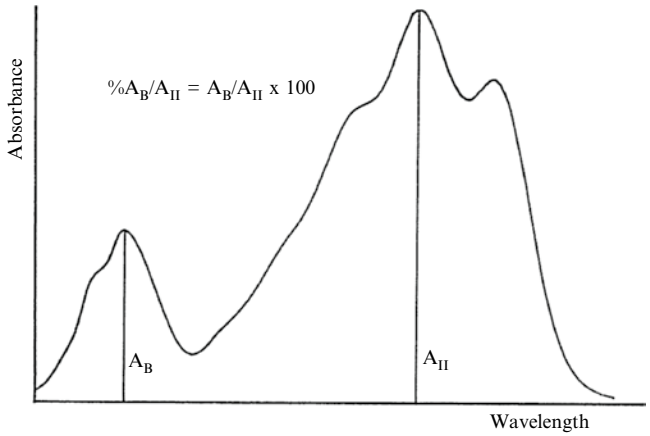


Fig. 51 Visible light absorption spectra for carotenoids with *cis* bonds (From Britton 1995)

discussed e.g. by Dufossé et al. (2005), Mortensen (2009), Dufossé (2009) and Borowitzka (2010). Sugimura et al. (2012) also describes scones baked with seaweed extract, and Ohi et al. (2009) have described astaxanthin bread.

5.3 Action Against Diseases

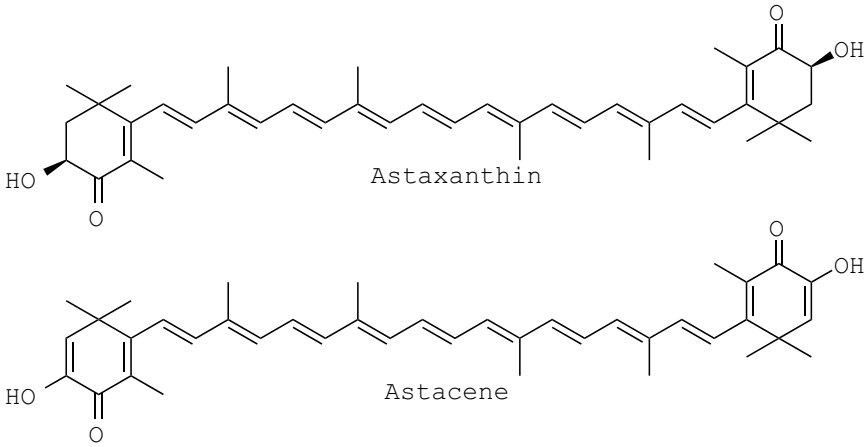
Algal carotenoids have been suggested to act against numerous diseases, although the use in present medical treatments are limited, the literature gives several examples on possible future uses. Well-known is β,β-carotene and some other carotenoids as vitamin A precursor, see Tang and Russell (2009). For a general overview on carotenoids and human health, see e.g. Britton et al. (2009) and Vílchez et al. (2011). Only a few recent examples are given here, to give a glimpse into this large and popular field of science.

Dunaliella salina capsules, rich in 9-*cis*-β,β-carotene (Fig. 53), reduced the severity of psoriasis, as reported by Greenberger et al. (2012), and 9-*cis*-β,β-carotene improved peripheral visual field in patients with fundus albipunctatus (Rotenstreich et al. 2010).

Fucoxanthin from seaweeds, diatoms, etc., as a 0.001 % solution, is reported to protect the skin of hairless mice against UVB-induced photoaging (Urikura et al. 2011).

Siphonaxanthin from green algae is reported to induce apoptosis in human leukemia (HL-60) cells (Ganesan et al. 2011), and siphonaxanthin, fucoxanthin and neoxanthin damaged human skin melanoma (A375) cells through carot-

Fig. 52 Astaxanthin and astacene



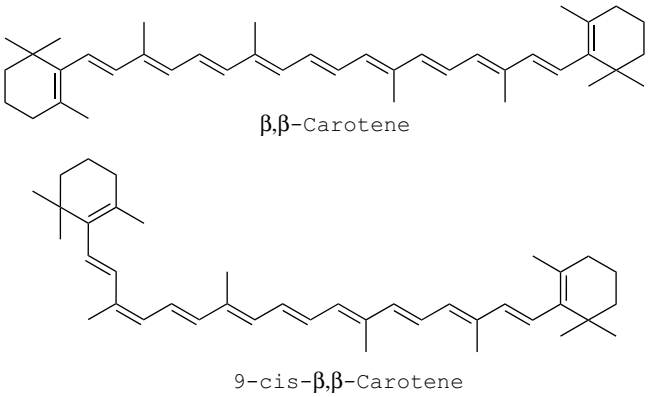


Fig. 53 β,β-Carotene and 9-*cis*-β,β-carotene

enoid photo-exitation, although other carotenoids as β,β-carotene, lutein, loroxanthin and violaxanthin did not enhance cell death (Yoshii et al. 2012).

5.4 Biological Feeds

Carotenoids are used in human food, sweets and soft drinks, but also widely used in feed. A few examples from recent literature are given here, using either algal carotenoids or algae as feed ingredients or as natural feed.

Chickens are fed xanthophylls to colour egg yolks or meat (Breithaupt 2008). These feed carotenoids may or may not be of algal origin. Kotrbáček et al. (2013) have described coloration of egg yolks of laying hens given feed supplemented with *Chlorella*, and Strand et al. (1998) for eggs from hens fed seaweed meal.

Farmed salmon are fed astaxanthin-enriched feed; commonly synthetic astaxanthin is used, although several natural sources have been described (Bjerkeng 2008). Astaxanthin-containing biomass of *Haematococcus pluvialis* can be used to colour the flesh of trout (Sommer et al. 1992), and the effects of *Spirulina* in feed to rainbow trout has recently been described by Teimouri et al. (2013).

Marine invertebrates get pigmented from natural algal feed, many of these pigments gets metabolised by the invertebrate; see e.g. Maoka et al. (2011a), but also, farmed shrimps can be fed feed with astaxanthin from *Haematococcus pluvialis* (Ju et al. 2011) or β,β-carotene from Dunaliella salina (Boonyaratpalin et al. 2001). Sacoglossans absorb not only carotenoids, but algal chloroplasts, from their feed (Evertsen and Johnsen 2009).

6 Large-Scale Production of Natural Carotenoids

Only a few carotenoids are offered for sale in large quantities. The carotenoids may either be made from chemical synthesis, or harvested from organisms naturally producing them.

The chemical syntheses of some commercially available carotenoids have been summarised by Britton et al. (1996). Here, only carotenoids harvested from natural sources will be taken into consideration. General information about algae as source of commercial carotenoids can be found (Walker et al. 2005; Campo et al. 2007; Borowitzka 2010; Guedes et al. 2011; Milledge 2011).

To obtain a pure carotenoid in large amounts from a natural source depends on mainly four variables: the choice of the right algal species; the cultivation method; the extraction of the carotenoid from the alga; and finally, the purification of the extract to obtain a carotenoid of acceptable purity.

Only a few species are being used for large-scale production (Guedes et al. 2011), the common species being *Dunaliella salina* and *Haematococcus pluvialis*, although also others have been suggested (Walker et al. 2005; Campo et al. 2007). Ideally, the species chosen should be able to produce large amounts of cells, and have a high content of

the carotenoid of interest, that can easily be extracted and purified.

6.1 β,β-Carotene

The best known example of large-scale cultivation for carotenoid isolation is β,β-carotene from *Dunaliella salina*, an alga able to produce large amounts of β,β-carotene. A disadvantage might be that the β,β-carotene is partly in the 9-*cis* form (see Fig. 53), while β,β-carotene from most other courses (including synthetic β,β-carotene) is in the *trans* form, although for most aspects, this difference is not of major importance. Various aspects of *Dunaliella*, e.g. cultivation, have been extensively described in a monograph (Ben-Amotz et al. 2009) and by Borowitzka (2013b). *Dunaliella* cultivation and β,β-carotene yield has even been described as a practical student laboratory exercise (Bosma and Wijffels 2003). Another source for large amounts of β,β-carotene is *Eustigmatos* cf. *polyphem* (Li et al. 2012).

6.2 Astaxanthin

Haematococcus pluvialis produces large amounts of astaxanthin, and several companies produce astaxanthin using this algal species. The disadvantage is that the cells richest of astaxanthin are cysts that have a rigid cell wall, making the extraction of the carotenoids difficult (Lee and Zhang 1999; Kobayashi 2003; Jeffrey and Egeland 2009). Another disadvantage is that astaxanthin from *Haematococcus* is present generally in esterified form (mono- and difatty acid esters of astaxanthin; Weesepoel et al. 2013), while synthetic astaxanthin is in the free form, so either the astaxanthin has to be hydrolysed without presense of oxygen (else, astacene is formed, see above) or by enzymatic hydrolysis (Zhao et al. 2011), or it may be necessary to use higher amounts of astaxanthin esters compared to free astaxanthin if used in e.g. salmon feed (see e.g. Hynes et al. 2009).

Other potential natural sources of astaxanthin are e.g. *Chromochloris zofingiensis* grown in the dark, crustaceans, e.g. *Calanus*, and flowers of *Adonis aestivalis*, also here, astaxanthin is mainly present in the esteried forms (Hynes et al. 2009; Maoka et al 2011b; Wang et al. 2013).

6.3 Lutein and Zeaxanthin

Lutein has traditionally been isolated from marigold flowers (*Tagetes* spp.), although some green algal species as sources for lutein have also been suggested (Campo et al. 2007; Guil-Guerrero and Rebolloso-Fuentes 2008; Cordero et al. 2011). Zeaxanthin may also be produced by green algae, e.g. under high irradiances (see above).

6.4 Future Aspects

The choice of algal isolate, the large-scale cultivation techniques, the harvesting of algae, the extraction of pigments and finally, the pigment purification are all important steps in the production of large amount of carotenoids, and all steps are the subject for improvements. Recent articles e.g. describe the possibility of combined cultivation and in situ extraction (Kleinegris et al. 2011).

Genetic engineering may be the best solution to obtain large amounts of carotenoids from algal sources (Jin et al. 2003; Walker et al. 2005), although genetic engineering is subject of resistance by the consumers.

Acknowledgements The library at University of Nordland gets my sincere thanks for once again have provided several hundreds article copies and also some books loaned from other libraries. Always promptly, always with a smile, never questioning why I need it or why I need all that much. Luckily for you, this time, most of the literature needed was available through electronic access, and not so many had to be ordered manually.

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Exocellular Polysaccharides in Microalgae and Cyanobacteria: Chemical Features, Role and Enzymes and Genes Involved in Their Biosynthesis

Federico Rossi and Roberto De Philippis

1 Introduction

Microalgae and cyanobacteria have complex carbohydrate metabolic pathways encompassing the ability to synthesize intracellular monosaccharides, polymeric reserve glucans and structurally complex extracellular polysaccharides (EPSs). The latter complex carbohydrates are synthesized following tightly regulated and energy-consuming processes (Coesel and Wardenaar 1994) and can be incorporated in the cell-wall, excreted as definite structures (sheaths, capsules or stalks), or released as mucilage.

EPSs are molecules with a great ecological significance, related to both organism survival and ecological fitness. These polysaccharidic excretions represent a physical barrier protecting cells from harmful agents and/or environmental constraints (De Philippis et al. 2001; Kumar et al. 2007) and serve in a wide array of physiological processes including cell adhesion, cell-to-cell interactions and biofilm formation (Mann and Wozniak 2012).

Depending on the characteristics of the secreted polymers, i.e. molecular weight, degree of branching and ultrastructure, they can have unique properties and different biological activities. During the last decades, there has been a growing interest for EPSs produced by microalgae and cyanobacteria and the possibility to use these value-added compounds in industrial, pharmaceutical and medical applications. The increased interest in using these phototrophic organisms as a source of polysaccharides instead of the formerly exploited land plants and seaweeds owes to the higher ease in controlling their growth conditions, combined with a lower variability in costs. Microalgae and cyanobacteria have simple growth requirements, consisting in a source of nitrogen (apart from the nitrogen-fixing organisms), phosphate, iron, magnesium, calcium and other minor salts. Harvesting is independent from climate and season and polymers with similar and reproducible properties can be more easily obtained (de Jesus Raposo et al. 2013).

Up to 2009, nearly 150 cyanobacterial strains had been screened in regard of their EPSs (Pereira et al. 2009). Currently, commercial applications of seaweed hydrocolloids are numerous, in products such as stabilizers, thickeners, emulsifiers, food and beverages (Bixler and Porse 2010), and there are some patents also concerning microalgal strains (Laurienzo 2010). EPSs secreted by cyanobacteria could find industrial application as gums, bioflocculants, soil conditioners and biosorbents (Li et al. 2001a; De Philippis et al. 2011).

Even in medical industry, these polymers are considered promising due to their proven effects on human immune system, exerting antitumor activity (Pugh et al. 2001) or acting as antivirals (Talyshinsky et al. 2002), as in the case of EPSs obtained from *Arthrospira platensis*[1] and *Arthrospira maxima* (Hayashi et al. 1996; Hernández-Corona et al. 2002).

In this chapter, the most important chemical features of the EPSs synthesized by microalgae and cyanobacteria will be reviewed along with the information available on the factors affecting their synthesis and on the metabolic pathways leading to the synthesis of these complex polymeric carbohydrates.

2 Morphological and Chemical Features of Microalgal and Cyanobacterial Extracellular Polysaccharides

In microalgae and cyanobacteria, EPSs are visible as a mucous mass surrounding cells or group of cells. They can tightly adhere to the cells and/or be more or less abundantly

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[1]Wherever possible the currently accepted names for species are used. The name used in the paper cited is also indicated. For details of names see chapter "Systematics, Taxonomy and Species Names: Do They Matter?" of this book (Borowitzka 2016).

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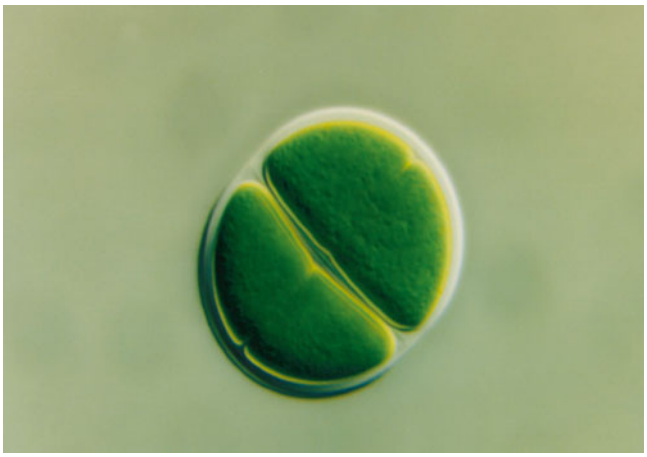

Fig. 1 Sheath of the unicellular cyanobacterium *Chroococcus* sp. observed using Normarski differential interference contrast microscopy, after staining with India ink (from De Philippis and Vincenzini 1998)

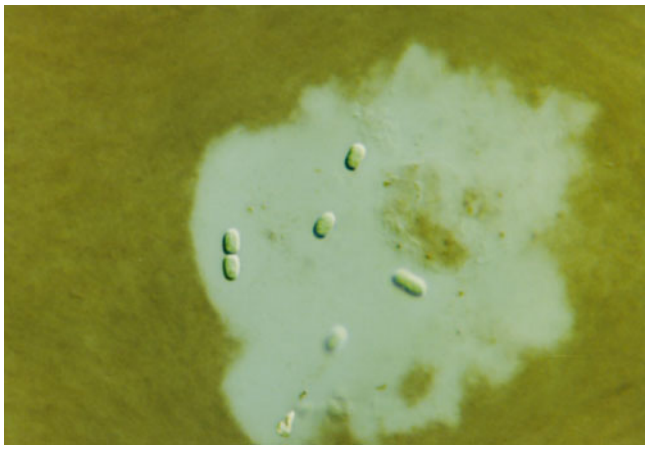

Fig. 3 Dispersed mucilage (slime) produced by the cyanobacterium *Cyanothece* PCC9224 observed using Normarski differential interference contrast microscopy, after staining with India ink (from De Philippis and Vincenzini 1998)

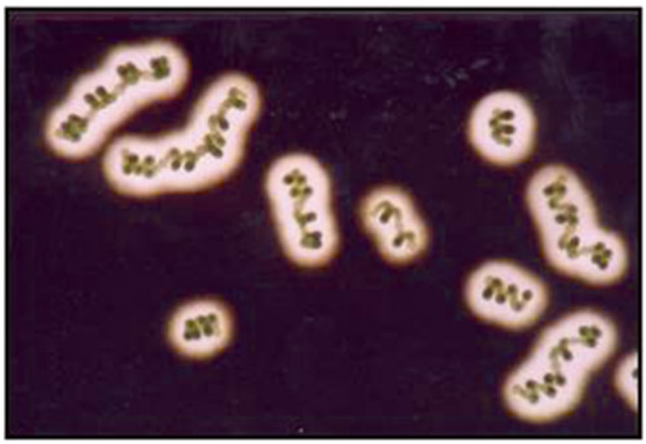

Fig. 2 Photomicrograph showing the polysaccharidic capsule of the cyanobacterium *Cyanospira capsulata* observed using Normarski differential interference contrast microscopy, after staining with India ink (Courtesy Dr Claudio Sili, Institute for Ecosystem Study, CNR, Italy)

released in the surrounding medium (Sutherland 1982; Decho 1990; De Philippis and Vincenzini 1998; Wotton 2004). They can be structured as a sheath (Fig. 1), which is a thin layer immediately next to the outer cell membrane containing concentric or radial fibres, or as a capsule (Fig. 2), which is intimately associated with the cell surface and may be covalently-bound to the cell wall.

When EPSs are loosely associated with the cell surface and do not constitute envelopes with definite limits, they are usually encompassed under the term slime (Fig. 3).

In diatoms, extracellular compounds are associated with the frustule, which is composed by two overlapping halves and girdle bands made of hydrated amorphous silica. Polymers are extruded through an apical pore field (Hoagland et al. 1993) or from the raphe (a longitudinal slit in the valve). EPSs provide cell adhesion by assembling as a stalk, aside from the possible structuring as capsule or sheath. The stalk (Fig. 4) is a permanent attachment structure constituted by (i) a surface adhered pad, (ii) a collar associated with the frustule at a terminal nodule or apical pore field, and (iii) an intervening shaft that separates the cells from the substrate (Daniel et al. 1987; Wang et al. 1997; Aboal et al. 2012).

In cyanobacteria, capsular polysaccharides (CPSs) and/or slime can represent a large share of the cell dry weight, while sheaths usually represent a small amount (De Philippis et al. 2001). In red microalgae, external portion of CPSs dissolves more or less partially in the growth medium and a fraction is released, thus increasing medium viscosity (Ramus 1972). This fraction, sometimes referred to as soluble fraction or as released polysaccharide (RPS) when referring to the dispersed material, in red microalgae represents up to 30 % of the total EPSs, while the bulk fraction, more condensed and adherent to the cells, was demonstrated to be the most abundant (Arad and Levy-Ontman 2010). It was proposed (Ramus 1972; Arad 1988), and later demonstrated (Keidan et al. 2009), that soluble EPSs are accumulated by red microalgae either following the release from the bulk fraction and following the direct transfer from the cell to the growth medium.

In general, due to the higher ease in its recovery, the most studied EPS fraction in red microalgae is the soluble one (Arad and Levy-Ontman 2013). Also in the case of cyanobacteria, RPSs represent the fraction that can be more easily recovered, simply precipitating the cell-free supernatants with cold ethanol (Lupi et al. 1991; Li et al. 2001a; Di Pippo et al. 2013). The selection of the optimal extraction method to recover the bulk EPS fractions from microalgae and cyanobacteria may not be immediate. Sheath extraction from *Chroococcus minutus* SAGB.41.79 for example was triggered through differential sucrose gradient centrifugation

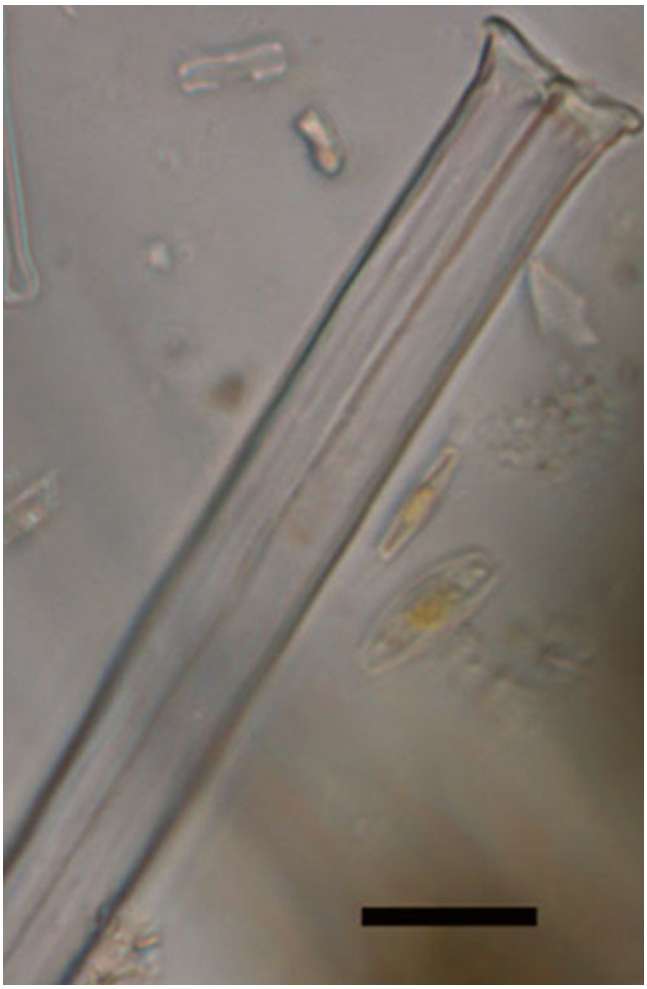

Fig. 4 Photomicrograph showing the stalk of *Didymosphenia geminata* (from Aboal et al. 2012). Bar = 20 µm

of the homogenized cells (Adhikary et al. 1986), while different approaches have been used to solubilize CPSs. Some authors performed hot water treatment of the pelleted cells (Bertocchi et al. 1990), while others performed deionized water extractions (Nakagawa et al. 1987; Plude et al. 1991). Filali-Mouhim et al. (1993) used low ionic strength buffer at 100 °C. In other cases, cyanobacterial CPSs were extracted by treating pelleted cells with 1.5 % sodium chloride at 60 °C (Vincenzini et al. 1990; De Philippis et al. 1993). Recently, Di Pippo et al. (2013) recovered cyanobacterial CPSs by extracting with 0.1 M sulfuric acid for 1 h at 95 °C. Freire-Nordi et al. (2006) extracted CPSs from *Staurastrum inversenii* by fixing medium-deprived cells with 0.5 % formaline followed by progressive 4 % Dakin liquid washings and by 30 min stirring at 40 °C.

Abdullahi et al. (2006) used alternatively H_2O at 90 °C, 0.5 M $NaHCO_3$ at 95 °C and 1 M NaOH containing 0.2 M $NaBH_4$ at 95 °C, in order to extract the bulk mucilage from the diatom *Phaeodactylum tricornutum*. The treatment with sodium hydroxide and bicarbonate was demonstrated to dissolve the mucilage coating, recovering the more tightly bound fractions.

Wustman et al. (1997) used mechanical and chemical methods to recover the most tightly bound fractions of the extracellular matrix from diatoms. In the mechanical approach, they recovered capsular polymers by the passage through a 23-gauge followed by an 18 gauge needle, the resulting suspension being filtered through an 8 µm bicarbonate filter and then dialyzed for 3 days against distilled water. In the chemical approach, they used EDTA and $NaHCO_3$ as extracting agents.

2.1 Characteristics and Composition of EPSs from Microalgae and Cyanobacteria

Microalgae, diatoms and cyanobacteria produce polymers that can be highly complex in terms of monosaccharidic composition. Some red microalgal strains were shown to produce polymers constituted by up to eight to ten monomers (Table 1) and some diatoms up to ten (Abdullahi et al. 2006 and references therein). In most cases, glucose, galactose and fucose are detected as major constituents, although sometimes rhamnose and arabinose were found to be dominant as well. Fructose is not usually found in microalgal and diatom EPS, although it has been often detected in EPS produced by cyanobacteria. One exception is represented by *Dunaliella salina* which represents a peculiar case (Mishar and Jha 2009). Fructose was also detected in the culture medium of *Chlorella pyrenoidosa* S-39 (Maksimova et al. 2004).

The presence of methyl, sulphate groups and uronic acids have been often reported, although their detection highly depends on the analytical technique utilized. In the EPS produced by *Botryococcus braunii* UC58, apart from the presence of glucose (29 %), fucose and galactose in a ratio 0.6:1 and uronic acid residues were detected (Allard et al. 1987; Fernandes et al. 1989). Other more recent investigations on *B. braunii* reported also the presence of arabinose and 3-*O*-methyl-fucose, 3-*O*-methyl-rhamnose and 6-*O*-methyhexose (Banerjee et al. 2002). The differences found in the composition of the EPS produced by the same microorganism confirm the previously reported considerations on the influence of the use of different hydrolytic and analytical procedures on the results obtained in the characterization of very complex polysaccharides (De Philippis and Vincenzini 1998). Extracellular polymers can present various fractions with different gelification properties depending, among the factors, on the monosaccharidic composition. The desmid *Penium margaritaceum* synthesizes a polymer constituted by different gelification fractions, but with similar sugar com-

Table 1 Chemical composition of exopolysaccharides produced by: **a** cyanobacteria; **b** diatoms; **c** green microalgae, red microalgae and dinoflagellates

a. Cyanobacteria												
Detected sugars												
Species	Fuc	Rha	Ara	Gal	Glu	Man	Fru	Xyl	Rib	UA	Other	Reference(s)
Anabaena augstumalis VRUC162[c]	+	+	+	+	d	–	–	+	+	+	*galN, glucN*	Di Pippo et al. (2013)
Anabaena augstumalis VRUC162[r]	+	+	–	+	d	+	–	+	–	d	*galN,*	Di Pippo et al. (2013)
Anabaena ATCC33047	–	–	–	+	+	+	–	d	–	+	*pr*	Moreno et al. (2000)
Anabaena C5	+	–	–	+	+	+	–	–	–	d	*pr*	Gantar et al. (1995)
Anabaena cylindrica 10C	+	+	–	+	+	+	–	d	–	+	*s*	Lama et al. (1996)
Anabaena flos-aquae A-37	–	–	–	–	d	–	–	+	+	+		Moore and Tischer (1965); Wang and Tischer (1973)
Anabaena spherica	–	–	+	d	+	+	–	–	–	–		Nicolaus et al. (1999)
Anabaena spiroides (= Dolichospermum spiroides)	+	d	–	+	d	d	–	+	–	d	*NAcGlu, NAcGal*	Gouvea et al. (2005)
Anabaena torulosa	+	+	+	+	+	–	–	d	–	–	*o*	Nicolaus et al. (1999)
Anacystis nidulans (= Asterocapsa nidulans)	–	–	–	+	d	+	–	–	–	–		Sangar and Dugan (1972)
Anabaena sp.	+	+	–	+	–	–	–	+	–	+		Huang et al. (2007)
Aphanocapsa halophytia MN-11	d	+	–	+	+	+	–	+	–	–	*s*	Sudo et al. (1995)
Aphanothece halophytia GR02	+	+	+	+	d	+	–	–	–	+		Li et al. (2001a)
Calothrix sp. VRUC166[c]	+	–	+	+	d	+	+	+	–	+	*galN, glucN*	Di Pippo et al. (2013)
Calothrix sp. VRUC166[r]	+	–	+	+	d	+	–	+	–	+	*glucN*	Di Pippo et al. (2013)
Chlorogloeopsis sp. 6912	+	+	+	+	d	+	–	–	–	+		Nicolaus et al. (1999)
Chroococcus minutus	+	+	+	+	d	+	–	+	–	+	*m, o*	Fischer et al. (1997)
Chroococcus submarinus c	d	+	+	+	d	+	–	d	–	+	*glcN*	Richert et al. (2005)
Chroococcus submarinus r	d	+	–	+	d	+	–	d	–	+	*glcN*	Richert et al. (2005)
Cyanospira capsulata	+	–	+	–	+	+	–	–	–	d	*p, pr, o*	Garozzo et al. (1995)
Cyanothece 16SOM2	+	–	–	+	d	+	–	+	–	d	*s*	De Philippis et al. (1993, 1998)
Cyanothece CA3	+	+	d	–	+	+	–	–	–	d	*a, p, s*	De Philippis et al. (1998)
Cyanothece CE4	+	+	+	+	+	–	–	–	–	d	*a, p, s*	De Philippis et al. (1998)
Cyanothece CE9	+	+	–	+	d	+	–	–	–	d	*p, s*	De Philippis et al. (1998)
Cyanothece CH1	+	+	–	+	–	+	–	+	–	d	*a, p, s*	De Philippis et al. (1998)
Cyanothece ET2	+	+	+	+	+	+	–	–	–	d	*a, p*	De Philippis et al. (1998)
Cyanothece ET5	+	+	–	+	d	+	–	d	–	+	*a, p*	De Philippis et al. (1998)
Cyanothece IR20	+	d	–	+	+	+	–	–	+	+	*a, p, s*	De Philippis et al. (1998)
Cyanothece PE13	+	+	–	+	d	+	–	+	+	+	*p, s*	De Philippis et al. (1998)
Cyanothece PE14	+	+	d	–	+	+	–	–	–	+	*a, p, s*	De Philippis et al. (1998)
Cyanothece TI4	+	+	–	+	+	+	–	+	–	d	*a, p, s*	De Philippis et al. (1998)
Cyanothece TP5	+	+	+	–	+	–	–	+	–	d	*p, s*	De Philippis et al. (1998)
Cyanothece TP10	+	+	d	–	+	+	–	+	–	d	*p, s*	De Philippis et al. (1998)
Cyanothece VI13	+	+	–	+	d	+	–	+	–	d	*p, s*	De Philippis et al. (1998)
Cyanothece VI22	+	+	–	+	+	+	–	+	–	d	*a, p, s*	De Philippis et al. (1998)
Fischerella maior Nav 10bis*c*	+	+	+	+	+	+	–	–	–	d		Bellezza et al. (2006)
Fischerella maior Nav 10bis*r*	+	+	+	+	d	+	–	–	–	+		Bellezza et al. (2006)
Fischerella muscicola	+	–	–	+	d	+	–	+	–	–		Nicolaus et al. (1999)
Geitlerinema sp.[c]	+	+	–	–	d	–	–	+	–	–		Richert et al. (2005)
Geitlerinema sp.[r]	+	+	–	–	d	–	–	+	–	–		Richert et al. (2005)

(continued)

Table 1 (continued)

a. Cyanobacteria												
Detected sugars												
Species	Fuc	Rha	Ara	Gal	Glu	Man	Fru	Xyl	Rib	UA	Other	Reference(s)
Gloeocapsa sp.	+	+	+	+	d	+	+	d	+	+		Rossi et al. (2012)
Gloeocapsa gelatinosa	–	+	+	–	+	+	+	+	+	+		Raungsomboon et al. (2006)
Gloeocapsosis sp.	d	+	+	+	+	+	+	+	+	+		Rossi et al. (2012)
Gloeocapsosis sp.	+	+	+	+	d	+	+	+	+	+		Rossi et al. (2012)
Gloeothece PCC6909^{r}	+	+	+	+	d	+	+	+	+	+	*s*	Micheletti et al. (2008)
Gloeothece PCC6909^{s}	–	+	–	+	d	+	–	–	+	+	*glucN, galN*	Micheletti et al. (2008)
Johannesbaptistia pellucidac	+	d	+	+	+	+	–	–	–	+		Richert et al. (2005)
Johannesbaptistia pellucidar	+	+	d	d	d	d	–	–	–	+		Richert et al. (2005)
Leptolyngbya sp. VRUC 135	+	+	–	+	+	+	–	+	–	+		Piro et al. (2005)
Leptolyngbya sp.	+	+	+	+	+	+	+	+	+	+		Rossi et al. (2012)
Lyngbya confervoides S9g	+	+	+	+	d	+	–	+	–	+	*u*	Gloaguen et al. (1995)
Microcoleus vaginatus	+	+	+	d	d	d	–	+	–	+	*m, NAcGlu*	Hu et al. (2003)
Microcystis sp.	+	+	–	+	–	–	–	+	–	+		Huang et al. (2007)
Microcystis aeruginosa	+	d	–	d	+	+	–	d	–	+	*NAcGlu*	Gouvea et al. (2005)
Microcystis aeruginosa f. aeruginosa	–	d	–	+	+	+	–	–	–	–		Forni et al. (1997)
Microcystis aeruginosa f. flos-aquae	–	–	–	–	d	+	–	–	–	+		Forni et al. (1997)
Microcystis PCC7005	+	–	–	–	d	+	–	–	–	+		Forni et al. (1997)
Microcystis viridis	–	+	–	–	d	+	–	+	–	+		Forni et al. (1997)
Nostoc calcicola 79WA01	+	+	+	+	d	+	–	+	–	+	*pr*	Flaibani et al. (1989)
Nostoc carneum	–	–	–	–	–	+	–	d	–	–	*pr*	Parikh and Madamwar (2006)
Nostoc commune DRH-1	–	–	–	+	d	–	–	+	+	+		Helm et al. (2000)
Nostoc insulare 54.79	–	–	+	–	+	–	–	–	–	–	*m*	Volk et al. (2007)
Nostoc PCC 6302	+	+	+	+	d	+	–	+	–	+		De Philippis et al. (2000)
Nostoc PCC 6310	+	+	–	+	d	+	–	+	+	+		De Philippis et al. (2000)
Nostoc PCC 6705	+	+	+	+	d	+	–	+	+	+		De Philippis et al. (2000)
Nostoc PCC 6719	+	+	+	+	d	+	–	+	–	+		De Philippis et al. (2000)
Nostoc PCC 6720	+	+	–	+	d	+	–	+	–	+		De Philippis et al. (2000)
Nostoc PCC 7107	+	+	+	+	d	+	–	–	+	+		De Philippis et al. (2000)
Nostoc PCC 7119	+	+	+	+	d	–	–	+	–	+		De Philippis et al. (2000)
Nostoc PCC 7413	+	+	+	+	d	+	–	+	+	+		De Philippis et al. (2000)
Nostoc PCC 7416	+	+	+	+	d	+	–	+	–	+		De Philippis et al. (2000)
Nostoc PCC 7422	+	+	+	+	d	+	–	+	–	+		De Philippis et al. (2000)
Nostoc PCC 7423	+	+	–	+	d	+	–	+	–	+		De Philippis et al. (2000)
Nostoc PCC 7706	+	+	–	+	d	+	–	+	–	+		De Philippis et al. (2000)
Nostoc PCC 7803	+	+	–	+	d	+	–	+	+	+		De Philippis et al. (2000)
Nostoc PCC 7807	+	+	–	–	d	+	–	–	+	+		De Philippis et al. (2000)
Nostoc PCC 7906	+	+	+	+	d	+	–	+	–	+		De Philippis et al. (2000)
Nostoc PCC 7933	+	+	+	+	d	+	–	+	–	+		De Philippis et al. (2000)
Nostoc PCC 7936	+	–	–	+	d	+	–	–	–	+		De Philippis et al. (2000)
Nostoc PCC 7937	+	+	+	+	d	+	–	+	–	+		De Philippis et al. (2000)
Nostoc PCC 8009/1	+	+	+	+	d	+	–	+	–	+		De Philippis et al. (2000)
Nostoc PCC 8109	+	+	–	+	d	+	–	+	–	+		De Philippis et al. (2000)
Nostoc PCC 8112	+	+	+	+	d	+	–	+	–	+		De Philippis et al. (2000)
Nostoc PCC 8113	+	+	–	+	d	+	–	+	+	+		De Philippis et al. (2000)

(continued)

Table 1 (continued)

a. Cyanobacteria												
Detected sugars												
Species	Fuc	Rha	Ara	Gal	Glu	Man	Fru	Xyl	Rib	UA	Other	Reference(s)
Nostoc PCC 8306	+	+	–	+	d	+	–	+	–	+		De Philippis et al. (2000)
Nostoc PCC 9202	+	+	+	+	d	+	–	+	–	+		De Philippis et al. (2000)
Nostoc PCC 9305	+	+	+	+	d	+	–	+	–	+		De Philippis et al. (2000)
Nostoc sp.	–	+	–	+	d	+	–	+	–	–	*m*	Hu et al. (2003)
Nostoc sp.	–	–	–	–	–	d	–	+	–	–	*pr*	Parikh and Madamwar (2006)
Nostoc sp.	+	–	+	–	+	–	–	–	–	+		Moore and Tischer (1964)
Nostoc PCC7423	+	+	–	+	d	+	–	+	–	+		De Philippis et al. (1998)
Nostoc PCC7936	+	–	–	+	+	+	–	–	–	d		De Philippis et al. (1998)
Oscillatoria amphibia PCC7105 (= *Geitlerinema amphibium*)	+	+	+	+	d	+	–	+	–	+		Gloaguen et al. (1995)
Oscillatoria corallinae CJ1	+	+	+	+	d	+	–	+	–	+		Gloaguen et al. (1995)
Oscillatoria sp.	+	+	–	+	–	–	–	+	–	+		Huang et al. (2007)
Oscillatoria sp.	–	–	–	–	d	–	–	+	+	–	*pr*	Parikh and Madamwar (2006)
Oscillatoria sp.	+	+	–	+	d	+	–	+	–	+	*u,o*	Bender et al. (1994)
Phormidium cf.joveolarum C52	+	+	+	+	d	+	–	+	–	+	*o,u*	Gloaguen et al. (1995)
Phormidium ectocarpi C86 (= *Leptolyngbya ectocarpi*)	–	+	–	+	d	+	–	+	–	+	*u*	Gloaguen et al. (1995)
Phormidium cf. *joveolarum* MEU	+	+	+	+	d	+	–	+	–	+	*o,u*	Gloaguen et al. (1995)
Phormidium autumnale VRUC164* (= *Microcoleus autumnalis*)	+	+	+	+	d	+	+	–	+	+		Di Pippo et al. (2013)
Phormidium autumnale VRUC164 ^{r} (= *Microcoleus autumnalis*)	+	d	–	+	+	+	+	+	+	+		Di Pippo et al. (2013)
Phormidium ectocarpi K5 (= *Leptolyngbya ectocarpi*)	+	+	+	+	d	+	–	+	–	+	*o,u*	Gloaguen et al. (1995)
Phormidium ectocarpi ME3 (= *Leptolyngbya ectocarpi*)	+	+	+	+	d	+	–	+	–	+		Gloaguen et al. (1995)
Phormidium ectocarpi N182 (= *Leptolyngbya ectocarpi*)	+	–	–	+	d	+	–	+	–	+		Gloaguen et al. (1995)
Phormidium ectocarpi PCC7375 (= *Leptolyngbya ectocarpi*)	+	+	+	+	d	+	–	+	–	+	*u*	Gloaguen et al. (1995)
Phormidium minutum D5	+	+	–	d	d	+	–	+	–	+	*u,o*	Gloaguen et al. (1995)
Phormidium minutum NB5	–	+	+	+	d	+	–	+	–	+	*u,o*	Gloaguen et al. (1995)
Phormidium minutum RT6	+	+	d	+	d	+	–	+	–	+	*o*	Gloaguen et al. (1995)
Phormidium tenue (= Leptolyngbya tenuis)	+	+	d	+	d	+	–	+	–	+	*NAcGlu*	Hu et al. (2003)
Phormidium tenue (= Leptolyngbya tenuis)	+	+	+	+	d	+	+	+	+	+	*GlcN*	Xu et al. (2013)
Phormidium sp.	+	+	+	+	d	–	–	d	+	–		Nicolaus et al. (1999)
Phormidium sp. 90-14/1	+	+	+	+	d	+	–	+	–	+	*o,u*	Gloaguen et al. (1995)
Phormidium sp. CCAP1463/4	+	+	+	+	d	+	–	+	–	+	*u*	Gloaguen et al. (1995)
Phormidium sp. CCAP1463/3	–	+	+	+	d	+	–	+	–	+	*o*	Gloaguen et al. (1995)
Phormidium sp. PNG91	+	+	+	+	d	+	–	+	+	+	*o,u,r*	Gloaguen et al. (1995)

(continued)

Table 1 (continued)

a. Cyanobacteria												
Detected sugars												
Species	Fuc	Rha	Ara	Gal	Glu	Man	Fru	Xyl	Rib	UA	Other	Reference(s)
Phormidium sp. J-1	–	+	–	+	–	d	–	–	–	+	*pr, s*	Bar-Or and Shilo (1987)
Plectonema golenkinianum^{c} (= *Pseudophormidium golenkinianum)*	+	+	–	+	d	+	–	+	–	+		Richert et al. (2005)
Plectonema golenkinianum^{r} (= *Pseudophormidium golenkinianum)*	+	+	–	+	d	+	–	d	–	+		Richert et al. (2005)
Plectonema sp.	+	+	+	+	d	+	+	+	+	+		Rossi et al. (2012)
Plectonema battersii^{c} (= *Pseudophormidium battersii)*	+	–	+	+	d	+	–	+	–	+		Richert et al. (2005)
Plectonema battersii^{r} (= *Pseudophormidium battersii)*	+	–	+	+	d	+	–	+	–	+		Richert et al. (2005)
Scytonema javanicum	+	+	+	d	d	d	–	+	–	+	*m*	Hu et al. (2003)
Scytonema hofmani	–	–	–	+	+	d	–	–	–	–		Nicolaus et al. (1999)
Scytonema ocellatum CP8-2^{c}	+	+	+	+	d	+	–	–	–	+		Bellezza et al. (2006)
Scytonema ocellatum CP8-2^{r}	+	+	d	+	d	+	–	–	–	+		Bellezza et al. (2006)
Scytonema sp.	+	+	+	+	+	+	–	+	–	–	*m*	Sassaki et al. (2005)
Spirulina maxima (= Arthrospira maxima)	+	+	–	+	d	+	–	+	–	+		Nie et al. (2002)
Synechocystis aquatilis VRUC165^{c}	+	+	–	–	d	–	–	+	–	+	*glcN*	Di Pippo et al. (2013)
Synechocystis aquatilis VRUC165^{r}	d	+	–	+	d	+	–	+	–	+	*glcN*	Di Pippo et al. (2013)
Synechocystis PCC6803	+	+	+	+	d	+	–	+	–	+	*pr, s, o, m*	Panoff et al. (1988)
Synechocystis PCC6714	+	+	–	+	d	+	–	+	–	+	*pr, s, o, m*	Panoff et al. (1988)
Tolypothrix tenuis	+	+	+	+	d	+	–	–	–	–		Nicolaus et al. (1999)
Trichormus variabilis VRUC162^{c}	+	+	+	+	d	–	–	+	+	–	*glcN, galN*	Di Pippo et al. (2013)
Trichormus variabilis VRUC162^{r}	+	+	+	+	d	+	–	+	+	–	*glcN, galN*	Di Pippo et al. (2013)
Trichormus variabilis VRUC168^{c}	+	–	+	+	d	+	–	–	+	+	*glucN*	Di Pippo et al. (2013)
Trichormus variabilis VRUC168^{r}	+	+	+	+	d	+	–	+	–	+	*glucN*	Di Pippo et al. (2013)

+ detected, – absent/not detected, *Fuc* fucose, *Rha* rhamnose, *Ara* arabinose, *Gal* galactose, *Glu* glucose, *Man* mannose, *Fru* fructose, *Xyl* xylose, *Rib* ribose, *UA* uronic acids, *a* acetate, *d* dominant sugar, *galN* galactosamine, *glucN* glucosamine, *m* methylated sugars, *NAcGal* N-acetyl galactosamine, *NAcGlu* N-acetyl glucosamine, *o* osamine, *p* pyruvate, *pr* proteins, *s* sulphate groups, *u* unidentified component(s), *c* capsular exopolysaccharides, *r* released polysaccharides

(continued)

Table 1 (continued)

b. Diatoms												
Detected sugars												
Species	Fuc	Rha	Ara	Gal	Glu	Man	Fru	Xyl	Rib	UA	Other	Reference(s)
Achnantes longipes	d	+	+	d	+	+	–	+	–	+	gul	Wustman et al. (1997)
Aulacoseira granulata	d	d	–	+	d	+	–	d	–	+	NAcGlu	Gouvea et al. (2005)
Amphora coffeaeformis (= Halamphora coffeaeformis)	d	+	+	d	+	+	–	+	–	–		Patil and Anil (2005)
Amphora coffeaeformis (= Halamphora coffeaeformis)	+	+	+	+	+	d	–	+	–	+		Wustman et al. (1997)
Amphora rostrata (= Halamphora costata)	d	+	+	+	+	+	–	+	–	–		Patil and Anil (2005)
Amphora salina CCMP1119 (= Halamphora coffeaeformis)	–	+	–	+	d	d	–	+	–	+	NAcGlu	Tesson and Hildebrand (2013)
Chaetoceros affinis	+	d	–	+	+	+	–	+	–	–		Haug and Myklestad (1976)
Chaetoceros curvisetus	d	+	–	d	+	+	–	+	+	–		Haug and Myklestad (1976)
Chaetoceros decipiens	d	d	–	+	+	+	–	+	–	–		Urbani et al. (2012)
Chaetoceros decipiens	+	+	–	d	+	+	–	+	+	–		Haug and Myklestad (1976)
Coscinodiscus radiatus CCMP310	–	+	–	+	+	d	–	+	–	+		Tesson and Hildebrand (2013)
Cylindrotheca closterium (= Ceratoneis closterium)	–	+	+	+	d	+	–	+	–	–		Staats et al. (1999)
Cylindrotheca closterium LG20 (= Ceratoneis closterium)	+	+	–	+	d	+	–	+	–	+		Bellinger et al. (2005)
Cylindrotheca closterium G (= Ceratoneis closterium)	+	+	–	–	d	–	–	+	–	–		Bellinger et al. (2005)
Cylindrotheca fusiformis	+	+	+	d	+	+	–	+	–	+	s	Magaletti et al. (2014)
Cymbella cistula	+	+	+	d	+	+	–	+	+	–		Wustman et al. (1997)
Cymbella mexicana	+	+	+	d	+	+	–	+	–	+		Wustman et al. (1997)
Chaetoceros debilis	+	+	–	+	+	+	–	+	d	–		Haug and Myklestad (1976)
Chaetoceros socialis	+	+	–	+	+	+	–	+	+	–		Haug and Myklestad (1976)
Corethron hystrix	d	+	–	+	+	+	–	+	+	–		Haug and Myklestad (1976)
Navicula curvilineata CCMP555	–	+	–	+	d	d	–	+	–	+		Tesson and Hildebrand (2013)
Navicula phyllepta	+	+	+	d	+	+	–	+	+	–		Bellinger et al. (2005)
Navicula salinarum	+	+	+	d	+	+	–	+	+	–		Staats et al. (1999)
Navicula subinflata (= Dickieia subinflata)	+	+	+	+	+	+	–	+	–	–		Patil and Anil (2005)
Nitzschia closterium (=Ceratoneis closterium)	–	+	–	+	d	+	–	+	–	–		Brown (1991)
Nitzschia epithemoides	+	+	+	+	d	d	–	+	+	–		Bellinger et al. (2005)
Phaeodactylum tricornutum	–	+	–	+	+	d	–	–	–	–		Brown (1991)
Pinnularia viridis	+	+	+	+	+	+	–	+	+	+	m	Chiovitti et al. (2003)
Thalassiosira gravida	d	–	+	+	+	+	–	+	+	–		Haug and Myklestad (1976)
Triceratium dubium CCMP147	–	+	–	+	+	d	–	+	–	+	NAcGlu	Tesson and Hildebrand (2013)

+ detected, – absent/not detected, *Fuc* fucose, *Rha* rhamnose, *Ara* arabinose, *Gal* galactose, *Glu* glucose, *Man* mannose, *Fru* fructose, *Xyl* xylose, *Rib* ribose, *d* dominant sugar, *gul* gulose, *m* methylated sugars, *NAcGlu* N-acetyl glucosamine, *s* sulphate groups

(continued)

Table 1 (continued)

c. Green and Red Microalgae and Dinoflagellates												
Detected sugars												
Species	Fuc	Rha	Ara	Gal	Glu	Man	Fru	Xyl	Rib	UA	Other	Reference(s)
Botryococcus braunii	+	+	+	d	+	–	–	–	–	+	m	Banerjee et al. (2002)
Chlamydomonas mexicana[cw] *(= Chlamydomonas oblonga)*	d	+	d	+	d	+	–	+	+	–		Barclay and Lewin (1985)
Chlamydomonas mexicana (= Chlamydomonas oblonga)	d	+	+	+	d	+	–	+	+	+		Barclay and Lewin (1985)
Chlamydomonas reinhardtii	–	+	–	+	d	+	–	–	–	–		Choi et al. (2010)
Chlamydomonas sajao[cw] *(= Lobochlamys segnis)*	–	+	d	d	d	+	–	+	+	–		Barclay and Lewin (1985)
Chlamydomonas sajao (= Lobochlamys segnis)	–	+	+	d	+	+	–	+	–	+		Barklay and Lewin (1985)
Chlorella pyrenoidosa Chick S-39	+	+	+	+	–	+	+	+	+	–		Maksimova et al. (2004)
Chloroccum sp.	+	–	–	+	d	+	–	+	+	–		Harun and Danquah (2011)
Chroococcus minutus	–	–	–	–	d	–	–	–	–	+	m, glucN	Adhikary et al. (1986)
Cosmarium sp. 1	d	+	+	+	+	+	–	+	+	+	s	Kiemle et al. (2007)
Cosmarium sp. 2	+	+	+	+	d	+	–	+	+	–		Kiemle et al. (2007)
Desmococcus olivaceus	+	+	+	d	d	+	–	+	–	+	m	Hu et al. (2003)
Dixoniella grisea	–	–	+	+	+	+	–	+	+	+	m,s	Arad (Malis) and Levy-Ontman (2010)
Dunaliella salina	–	–	–	+	+	–	+	+	–	–		Mishra and Jha (2008)
Dunaliella tertiolecta	–	+	–	+	d	+	–	+	–	–		Brown (1991)
Heterosigma akashiwo	d	d	+	d	+	d	–	–	–	+	ma	Lopes et al. (2012)
Heterosigma akashiwo[c]	+	d	+	d	+	d	–	+	–	+	ma	Lopes et al. (2012)
Koliella antarctica[cw]	–	+	+	+	+	+	–	+	–	+		Piro et al. (2013)
Netrium digitus	d	+	+	+	+	+	–	+	+	+		Kiemle et al. (2007)
Netrium interruptum (= Planotaenium interruptum)	+	+	+	+	+	+	–	d	+	+	s	Kiemle et al. (2007)
Netrium interruptum UTEX2509 *(= Planotaenium interruptum)*	+	+	+	d	+	+	–	+	+	–		Kiemle et al. (2007)
Netrium oblongum	+	+	+	d	+	+	–	+	+	+	s	Kiemle et al. (2007)
Palmella mucosa	+	–	+	–	+	–	–	–	–	+		Moore and Tischer (1964)
Penium cylindrus	d	+	+	+	+	+	–	+	+	+		Kiemle et al. (2007)
Penium margaritaceum	+	–	+	+	+	+	–	d	–	+	m	Domozych et al. (2005)
Penium spirostriolatum	+	+	d	+	+	+	–	+	+	+	s	Kiemle et al. (2007)
Pleurotaenium trabecula	d	+	+	+	+	+	–	d	+	+	s	Kiemle et al. (2007)
Porphyridium aerugineum	–	–	+	+	+	+	–	+	+	+	m,s	Arad (Malis) and Levy-Ontman (2010)
Porphyridium cruentum	–	–	+	+	+	+	–	+	+	+	m,s	Arad (Malis) and Levy-Ontman (2010)
Porphyridium sp.	–	–	+	+	+	+	–	+	+	+	m,s	Arad (Malis) and Levy-Ontman (2010)
Staurastrum iversenii var. *americanum*	+	+	–	+	–	–	–	+	–	–		Freire-Nordi et al. (2006)
Tetmemorus brebissonii	d	+	+	+	+	+	–	+	+	+	s	Kiemle et al. (2007)

+ detected, – absent/not detected, *Fuc* fucose, *Rha* rhamnose, *Ara* arabinose, *Gal* galactose, *Glu* glucose, *Man* mannose, *Fru* fructose, *Xyl* xylose, *Rib* ribose, *UA* uronic acids. *d* dominant sugar, *glucN* glucosamine, *m* methylated sugars, *s* sulphate groups, *ma* mannuronic acid, *c* capsular exopolysaccharides, *cw* cell wall polysaccharides, *r* released exopolysaccharides

ponents, namely xylose, fucose, glucuronic acid, arabinose, galactose, glucose and methyl – sugars (Domozych et al. 2005).

CPS composition in microalgae and diatoms has been rarely investigated. Freire-Nordi et al. (2006) determined the main components of CPSs synthesized by the microalga *Staurastrum iversenii var. americanum*, which resulted composed by galactose, fucose, rhamnose and xylose, while Abdullahi et al. (2006) reported the composition of different fractions of EPSs excreted by *Phaeodactylum tricornutum*.

Besides using a limited repertoire of assembly and secretion procedures typical of bacteria (Pereira et al. 2009), cyanobacterial EPSs are often complex molecules composed by a higher number of constituents compared to microalgae, although for some cyanobacterial species only four constituents were detected (Forni et al. 1997). Xylose, arabinose, fucose, rhamnose and galactose are generally present, along with uronic acids, in cyanobacterial EPSs. In the notable case of *Microcystis wesenbergii*, uronic acids were the only constituents (Forni et al. 1997). Another peculiar case is represented by Cyanothece sp. 113, which produces an α-D-1,6-homoglucan exopolysaccharide, thus constituted entirely by D-glucose (Chi et al. 2007). Glucose is often the dominant sugar although in other cases rhamnose, xylose, arabinose, fucose, mannose and uronic acids resulted dominant instead (Li et al. 2001a).

In most cases, no qualitative differences were detected between the composition of CPSs and soluble EPSs (Vincenzini et al. 1990; Freire-Nordi et al. 2006), with few exceptions observed in cyanobacteria (Di Pippo et al. 2013) and diatoms (Abdullahi et al. 2006). The latter authors reported significant differences in the relative abundance of the sugar constituents between soluble and bulk EPSs produced by *Phaeodactylum tricornutum*.

The complexity of EPSs and the marked amphiphilic character observed in a large number of cyanobacterial polysaccharides is thought to increase the fitness of the producing strains, as it confers physiological plasticity towards environmental constraints. While the hydrophobic character owes to uronic acids, sulphated sugars and ketal-linked pyruvyl groups, the hydrophobic character is provided by the presence of ester-linked acetyl groups (up to 12 % of polysaccharide dry weight), by the frequent marked presence of deoxysugars, and by the presence of peptidic moieties (Pereira et al. 2009). Sulphated moieties contribute, together with uronic acids, in determining the negative charge of these polymers, thus increasing their exploitability for a wide array of applications (Colica and De Philippis 2013). While the hydrophobic fractions enhance the cell capacity to adhere to solid surfaces (Nielsen et al. 1997), hydrophilic fractions are more involved in binding minerals, nutrients, and water molecules (Rossi et al. 2012).

The high number of different constituents is often associated to the presence of complex repeating units and a huge variety of structures and architectures. For example, EPSs produced by *Nostoc commune* DRH-1, *Nostoc insulare* and *Cyanothece* ATCC 51142 are composed by repeating units of respectively 6, 4 and 3 monosaccharides, while other strains such as *Mastigocladus laminosus* and *Cyanospira capsulata* were shown to produce polymers containing repeating units of 15 monosaccharides (Pereira et al. 2009).

EPSs extracted from diatoms were also demonstrated to be branched and very complex, with glucose occurring as 4-, 3-, 2,3-, and 2,3,6- linked glucosyl residues, galactose occurring as 3-, 3,6-, and 4 galactosyl residues, and rhamose occurring as 2- and 2,3-linked rhamnosyl residues (Abdullahi et al. 2006).

In red microalgae, cell wall lacks the rigid microfibrillar layer found in red seaweeds, displaying solely a sulphate polysaccharidic envelope with a MW of $2–7 \times 10^6$ Da constituted by galactans heteropolymers, negatively charged due to the presence of uronic acids and half-ester sulphate groups (Arad and Levy-Ontman 2010). The most represented sugars are glucose, galactose and xylose, in a ratio of 1:1.9:3.2, while mannose, fucose, ribose, arabinose, rhamnose and other methylated sugars were detected in very small molar percentages or in traces (Lupescu et al. 1991). A molecule representing a block of the polysaccharide structure is the aldobiouronic acid O-(α-d-glucopyranosyluronic acid)–I-galactopyranose disaccharide, detected in *Porphyridium* sp., *Dixoniella grisea* (as *Rhodella reticulata*) and *Porphyridium aerugineum*. This backbone unit is part of a linear and larger block containing (1→2)- or (1→4)-linked xylopyranosyl, (1→3)-linked glucopyranosyl (1→3)-linked galactopyranosyl and (1→3)-linked glucopyranosyl or glucopyranosyluronic acid residues (Geresh et al. 2009). Although the sugar composition of the red microalgal polysaccharide has been clarified, the exact sequence of the constituents remains unknown.

Due to the lack of proper enzymes to degrade and recover the bulk polysaccharidic fraction of the cell wall, there are limited information on its chemical composition. From an anionic polymer extracted from the bound fraction of the polysaccharide synthesized by *Porphyridium* sp., two oligosaccharides different from the aldobiuronic acid were isolated. This bulk polymer resulted composed by xylose, glucose, galactose and galacturonic acid (Gloaguen et al. 2004).

Sulphated sugars are present in the bulk phase of the cell wall of algae. They have been found in red algae, green algae (El-Sheekh et al. 2012) and diatoms (Hoagland et al. 1993). They are also constituents of cyanobacterial EPSs (Bar-Or and Shilo 1987; De Philippis et al. 2001), representing a unique case among the EPSs produced by eubacteria

(Micheletti et al. 2008). Sulphated sugars in red microalgae constitute from 0.5 to 10 % (w/w) of the EPSs, with sulphate groups attached to glucose and galactose in position 3 and 6 (Lupescu et al. 1991; Arad and Levy-Ontman 2013). The characterization of sulphated moieties of *Porphyridium* sp, carried out by acetyl pyridinium chloride precipitation, calcium chloride solubilization and ethanol precipitation, showed that they contain 8.5 % sulphate and 1–2 % proteins (Arad et al. 1985).

In cyanobacteria, sulphated sugars are involved in important processes such as cell recognition and cell adhesion (Guzman-Murillo and Ascencio 2000) while in microalgae they are involved in the protection of the cells against harmful environmental factors, due to their stability over a wide range of temperatures (30–160 °C), pH and salinity values (Arad and Levy-Ontman 2013). It has been reported that sulphated EPSs inhibits the development of some human pathogens. For example, they were proved to inhibit the cytoadhesion process of *Helicobacter pilori* on HeLa S3 cells (Guzman-Murillo and Ascencio 2000; Ascencio et al. 2004). On these basis, a treatment using sulphated EPSs to block the adhesion of these pathogens would be possible.

Proteins have been often found as constituents of outer envelopes in microalgae and cyanobacteria. Analysis through SDS-PAGE showed the presence of a 66 kDa glycoprotein in the cell wall of *Porphyridium*, consisting of a polypeptide of 58 kDa and a glycan moiety of approx 8 kDa. The sequencing of cDNA demonstrated that it is a novel protein sharing some similarities with the protein superfamilies of the SCOP databases, i.e. glycosyltransferases, pectin lyase-like, sialidases, and CoA-like lectins/glucanases. This information hints at a possible role of the 66 kDa glycoprotein in the synthesis or modification of the polysaccharidic cell wall (Arad and Levy-Ontman 2010). In cyanobacteria, the presence of polypeptides in outer envelopes was observed by many authors. For example, exudates enriched with glycine, alanine, valine and leucine were observed in secretions by *Cyanospira capsulata* and *Nostoc calcicola* (Flaibani et al. 1989; Marra et al. 1990), while proteins bound to aspartic and glutamic acid were observed in secretions by *Schizothrix* sp. (Kawaguchi and Decho 2002). Proteins were also observed in EPSs produced by diatoms (Hoagland et al. 1993). In general, proteins form hydrogen bonds with carbohydrate polymers, helping the stabilization of the macromolecular constituents of the cells (Potts 1994).

Interestingly, some amino acid-derived compounds known to act as UV-screens (i.e. mycosporine-like aminoacids (MAAs) and scytonemin) have been found stored in the sheath of several cyanobacterial strains (Garcia-Pichel and Castenholz 1991; Garcia-Pichel et al. 1993; Rossi et al. 2012).

3 Factors Affecting EPS Synthesis in Microalgae and Cyanobacteria

It was observed that the optimal growth conditions for metabolite accumulation are often species-specific and that intraspecific differences can also be observed. Environmental conditions were proven to affect EPS production and characteristics, including the chemical composition and the solubility of the polymers (Arad et al. 1988, 1992; Ucko et al. 1994).

Nutrient availability is a paramount factor but other parameters, such as salinity, illumination (light period and wavelength) and temperature, are also to be considered.

3.1 Light Intensity and Temperature

Light intensity which growing cells are subjected to is a key parameter affecting EPS production (Matsunaga et al. 1996). The quantity and the quality of light affect biomass composition. Generally speaking, light intensity is positively correlated to carbohydrate synthesis in both microalgae (Friedman et al. 1991) and cyanobacteria (De Philippis et al. 1992). Arad and collaborators (1985) demonstrated that *Porphyridium* grown under two different light intensities (15 and 3.8 µmol photons m^{-2} s^{-1}) synthesized different amount of EPS, being both bulk and soluble EPS fractions 7 % higher under high light conditions.

Light intensity and temperature have a synergistic effect (Carvalho et al. 2009; Jensen and Knutsen 1993) as temperature affects light inhibition.

Temperature affects nutrient uptake, cell membranes and oxygen-evolving activity of PSII (Vonshak 2002) and thus supposedly also biosynthetic processes. The green alga *Botryococcus braunii* UC58 has been demonstrated to produce soluble EPSs critically depending on growth temperature, with an optimal range for production (25–30 °C) coinciding with the optimal range for growth (Lupi et al. 1991). At the lower temperature tested by the authors (20 °C), *B. braunii* maintained a relatively high growth rate, but very low production of soluble EPSs. Interestingly, EPSs produced at optimal temperature, when dissolved in water produced also the highest viscosity. This hints at the fact that the temperature may have influenced the molecular weight of the secreted polymers.

3.2 Carbon Availability

Carbon dioxide concentration negatively affects EPS accumulation due to the Carbon Concentrating Mechanism (CCM) by which microalgae concentrate inorganic carbon from the environment under low carbon dioxide conditions. For example, microalga *Parachlorella* (*Chlorella) kessleri* was demonstrated to increase carbohydrate accumulation when carbon dioxide concentration decreased from 3.0 to 0.04 % (Izumo et al. 2007). In general, carbon metabolic mode (photoautotrophic, heterotrophic, photoheterotrophic and mixotrophic) is an important factor to consider. Heterotrophic and mixotrophic modes improve the growth rate and lead to a higher carbohydrate accumulation, as it was observed for *Chlorella* (Abreu et al. 2012; Choix et al. 2012). Cell-wall EPS metabolism in *Porphyridium* also is affected by CO_2 concentration. The ratio of soluble to bound EPSs and the ratio of galactose to xylose was also affected, suggesting an effect of carbon dioxide on carbon partitioning. ^{14}C-NMR and GC analysis showed that the main low molecular weight product in *Phorphyridium* is floridoside, a disaccharide composed by galactose and glycerol, which acts like a carbon precursor for macromolecules, for example cell wall components (Li et al. 2001b).

3.3 Nitrogen Availability

Nitrogen availability, and in particular the variation of C:N ratio, affects carbohydrate accumulation. Nitrogen is an essential constituent of vital compounds, e.g. DNA, proteins and pigments. *Chroomonas* sp. was shown to increase carbohydrate productivity from 3.52 to 7.56 mg L^{-1} day^{-1} with $NaNO_3$ increasing 4–12 mM (Bermùdez et al. 2004).

The presence of nitrogen in combined form elicits carbohydrate synthesis (Kumar et al. 2007). Higher EPS production and cell growth in the presence of combined nitrogen source was observed for different *Anabaena* strains (Lama et al. 1996). The quantity and the composition of produced polymer can also vary according to the provided N-source (De Philippis and Vincenzini 1998), although some exceptions can be found (Tischer and Davis 1971).

N-starvation also elicits EPS synthesis, likely owing to the increase in C:N ratio (Kumar et al. 2007), although in some cases no effect was reported (Piorreck et al. 1984). Although the reaction to nitrogen starvation is specie-specific, in this condition generally photosynthetically fixed carbon flux is directed towards lipid or carbohydrate synthesis pathway (Hu 2004; Olivier 2011; Brányiková et al. 2011), although in some cases any of these products was accumulated (Markou et al. 2012). Different studies confirm carbohydrate accumulation (De Philippis et al. 1992; Keidan et al. 2009; Sassano et al. 2010) or report variation in lipid contents (Tedesco and Duerr 1989; Kataray and Dönmez 2011) under N-starvation. In particular, the highest intracellular accumulation of carbohydrates was shown for *Arthrospira maxima* (De Philippis et al. 1992) and *Arthrospira platensis* (Sassano et al. 2010) which accumulated up to 60–70 % and 55–65 %, respectively. N-starved cells of *Chlorella vulgaris* accumulated up to 38–41 % (Branyikova et al. 2011), while *Tetraselmis subcordiformis* accumulated up to 35 % (Ji et al. 2011). A strain-specific response to N starvation was observed in a study carried out with 15 *Cyanothece* strains isolated from various environments (De Philippis et al. 1998). Under nitrogen starvation, eight strains showed an increase in the amount of soluble polymeric carbohydrates released in the culture medium; two strains showed an increase in both the intracellular and the extracellular carbohydrates while other five strains showed a significant accumulation of intracellular carbohydrate reserves.

A very interesting behaviour was observed in the case of the filamentous heterocystous cyanobacterium *Cyanospira capsulata*. Under conditions that caused a mere diversion of carbon flux from protein synthesis owing to the addition of various inhibitors of nitrogen assimilation, the accumulation of intracellular carbohydrate reserves (namely glycogen) was observed. Under conditions that effectively enhanced the amount of carbon available to the cells, due to the addition of glyoxylate, an increase in the amount of EPS synthesized and released by the cells was observed (De Philippis et al. 1996).

Reddy et al. (1996), observing the results obtained with *Cyanothece* ATCC 51142 grown on solid media, suggested the possibility that EPS excretion represents a way to provide cells with essential nutrients, such as calcium and iron, to sustain nitrogen fixation under N-starving conditions. The same authors also hinted at the possible role of EPSs in excluding oxygen, enabling nitrogen fixation. Indeed, EPS deposits over heterocyst-differentiating cyanobacterial cells is one of the required step to create a micro-oxic intracellular milieu in order to protect nitrogenase from O_2 inactivation (Huang et al. 2005).

3.4 Phosphorus Availability

Phosphorus is another essential element present in compounds of fundamental importance for cell metabolism, notably DNA and ATP. The lack of a P-source seems to elicit EPS synthesis. Cade-Menun and Paytan (2010) hypothesized the presence of a lower threshold of phosphorus concentration at which carbohydrates start to accumulate. Later, Markou et al. (2012) confirmed this hypothesis. Studying *Arthrospira platensis*, they quantified the concentration at which carbohydrates and biomass production is maximum, which corresponds to 1.82 ± 0.16 mg P g^{-1} dry biomass.

The most frequently observed effect of P-starvation is the switch from protein synthesis to carbohydrate and lipid accu-

mulation (Markou et al. 2012), although no carbohydrate accumulation was observed in some cases (De Philippis et al. 1991; Ji et al. 2011) and a decrease in carbohydrate content was observed in others (Nicolaus et al. 1999). Rodolfi et al. (2009) and Xin et al. (2010) reported only lipid accumulation for *Nannochloropsis* sp. F&M-M24 and *Scenedesmus* sp. LX1, respectively. *Cyanothece* 16 SOM 2 increases EPS release under P-starvation (De Philippis et al. 1993), while in the case of *Phormidium* J-1 and *Cyanospira capsulata* no significant effects were observed (De Philippis et al. 1991; Fattom and Shilo 1984). For *Anabaena cylindrica* 10 °C, a decrease in EPS release was observed under P-shortage (Lama et al. 1996).

Under P-limiting conditions, which may occur in thick and intertidal sandy biofilms (Thornton et al. 2002), carbohydrate synthesis resulted stimulated in different diatom strains (Miklestad and Haug 1972; Guerrini et al. 2000; Alcoverro et al. 2000; Magaletti et al. 2004). The pennate *Phaeodactylum tricornutum* produced roughly double the quantity of EPSs under P-starvation, compared to non starving conditions. Under P-limiting conditions substantial changes in the relative contents of uronic acids, sulphate, rhamnose, xylose, fucose, mannose, glucose, and arabinose were also observed (Abdullahi et al. 2006).

3.5 Sulfur Availability

S-starvation also affects carbohydrate synthesis. During the first days of S-starvation, carbohydrate accumulation occurs due to the need to exploit the excess of electrons coming from PSII and due to the activation of carboxylic acid cycle, where the degraded products of Rubisco are recycled (Melis 2007; Fouchard et al. 2005; Markou et al. 2012). *Chlamydomonas reinhardtii* was shown to accumulate carbohydrates from a 10-fold (Melis 2007) to a 15.5-fold (Ball et al. 1990) content under S-starvation. Even manganese and potassium starvation have been reported to elicit carbohydrate synthesis, but sulfur starvation is considered by some authors the most feasible method to produce carbohydrate-rich biomass, as the lag between the obtainment of the higher carbohydrate yield and cell death is longer compared to when cells are deprived of other nutrients (Markou et al. 2012).

3.6 Stress-Responsive EPS Synthesis

EPSs can be synthesized in response to a stress. This is not surprising considering their key role in providing protection to cells against biotic and abiotic factors (De Philippis and Vincenzini 1998). In *Acutodesmus* (*Scenedesmus) obliquus* and *Chlorella vulgaris*, for example, EPS and intracellular carbohydrate accumulation follow the exposure to different concentrations of microcystins (El-Sheekh et al. 2012). EPSs can act as antioxidants (Tannin-Spitz et al. 2005; Li et al. 2011) and they have been suggested to function as ROS-scavengers following oxidative stress. The scavenging action seems to start intracellularly during the sugar assembly, before polymers are extruded in the surrounding environment (De Philippis and Vincenzini 1998). Exposure to metals also has been reported to inhibit carbohydrate accumulation as in the case of copper exposure, which reduces carbohydrate accumulation in *Scenedesmus communis (*as *S. acutus)*, *Tetraselmis suecica* and *Dunaliella tertiolecta* (Morsi AbdEl-Monem et al. 1998; Lim et al. 2006).

Change in the moisture status can influence EPS production. Cyanobacteria can change their metabolic activity according to the moisture level which in turn influence EPS synthesis (Mazor et al. 1996). The importance of EPSs in conferring the capability to tolerate desiccation has been hypothesized for some EPS-producing cyanobacteria (Potts 2001; Billi and Potts 2002) and green algae (Demura et al. 2014). Hill et al. (1994) proposed for EPSs the role of water repository. Indeed, due to their amphiphilic nature, EPSs are able to trap water molecules and control their uptake and their loss (Pereira et al. 2009). The critical role of EPSs in drought tolerance was demonstrated by Tamaru et al. (2005) who observed a significantly damaged photosynthetic O_2 evolution and a decrease in viability in EPS-depleted cells of *Nostoc commune*. The main role attributed to EPSs by the authors is to maintain the integrity and the functions of the biological membranes, preventing damages occurring during the desiccation process. Indeed, in a previous study the in vitro addition of RPSs from *Nostoc commune* CHEN to artificial membrane vescicles prevented their fusion, which is the major outcome following dehydratation and rehydratation (Hill et al. 1997). Tamaru et al. (2005) also observed a decrease in the freeze-thaw tolerance of EPS-deprived cells, pointing out the importance of the polysaccharidic envelope under low temperatures.

The cryoprotective role of EPSs was demonstrated also in the case of green microalga *Coccomixa subellipsoidea* C-169, in which they represent a coat protecting against the low temperatures that this strain is adapted to (Blanc et al. 2012). The intracellular synthesis of osmotically active sugars such as threalose and sucrose following salt stress is common ground. Similarly, with some exceptions (Pereira et al. 2009), in the presence of increasing salt concentrations cyanobacteria were demonstrated to produce larger amounts of EPSs (Su et al. 2007). Similarly, EPS synthesis in microalgae can be elicited by salt stress (Mishra and Jha 2009). Diatoms *Phaeodactylum tricornutum* and *Nitzschia frustulum* synthesized EPS responsively to increasingly salinity levels (Allan et al. 1972; Abdullahi et al. 2006). Salinity alterations lead to changes in the relative contents of uronic acids, sulphate, rhamnose, xylose, fucose, mannose, arabinose and *O*-methylated moieties in diatom-secreted EPSs (Abdullahi et al. 2006; Yoshimura et al. 2012).

Co-culture systems of microalgae and bacteria represent potentially stressing environments in which usually EPS accumulation of every strain involved is enhanced (Angelis et al. 2012). This effect could possibly represent a way to overcome the constraint represented by nutrient competition, or to protect from possible toxins released in the environment by other microorganisms (Bell 1983). Other authors suggest also the possibility that the increase in EPS production in co-cultures is the result of the higher amounts of carbon dioxide, vitamins and growth factors released by one or more of the strains involved, or is the result of the influence that each of the strains can have on the pH of the growth medium (Fernandes et al. 1989). The diatom *Encyonopsis* (*Cymbella) microcephala* was shown to secrete a polysaccharidic capsule when grown in co-cultured with *Bacteroidetes* strain 32, while the capsule was not observed in *E. microcephala* axenic cultures (Bruckner et al. 2008).

4 Mechanisms of EPS Synthesis in Microalgae and Cyanobacteria

In microalgae and cyanobacteria, carbohydrate synthesis takes place in the chloroplasts and/or in the cytosol during the Calvin cycle, using NADPH and ATP, in turn generated by protons and electrons coming from the splitting of water during the photosynthetic reactions. In the Calvin cycle, carbon dioxide is assimilated through the intervention of ribulose-1,5-biphosphate carboxylase oxygenase or Rubisco. Rubisco is carboxylated to 2 molecules of 3-phosphoglycerate, one of which is used for carbohydrate formation (Markou et al. 2012).

4.1 EPS Synthesis in Microalgae

Following investigations carried out by using the antibiotic brefeldin A (BFA) and various polysaccharide staining techniques (including the silver hexamine method), it was demonstrated that in microalgae polysaccharide biosynthesis and polysaccharide sulfation take place through the Golgi apparatus (GA) (Keidan et al. 2009). Product-packed vesicles can be followed in their path from GA until their fusion with the plasma membrane. GA is constituted by stacked cisternae, named dictyosomes, and two multi-subunit organized protein complexes (membrane tetrads) which are thought to be multi-enzymatic complexes. In plants and animals, GA is involved in the synthesis of proteins, glycoproteins, proteoglycans and complex sugars. Polysaccharide-staining approaches used on the desmid *Micrasterias americana* showed that between all the vesicle populations, at least two "large vesicle" (LV) and one "dark vesicle" (DV) types, this latter involved in primary wall formation, contain polysaccharides (Brosch-Solomon et al. 1998). Studies on *Closterium acerosum* showed that when the cells are marked with the antibody Skd-Clos-1, specific for a carbohydrate moiety of the secreted slime, labeling resulted concentrated at GA (Domozych 1999). BFA is known to alter the microarchitecture of the GA stacks, blocking the transport of secretory proteins from the endoplasmic reticulum and causing the reabsorption and disintegration of GA. Treatment with BFA of *Closterium acerosum* resulted in a lack of slime excretion by the cells grown on solid medium (Fig. 5).

The key role of GA in EPS synthesis was confirmed by the results obtained treating with BFA N-starved *Phorphyridium* sp. cells during the stationary phase (no cell-division). An inhibition in both soluble and bulk EPS excretion was observed. While the synthesis of both fractions decreased, the synthesis of the soluble EPS fraction resulted more pronounced. Conversely, cells treated with BFA while

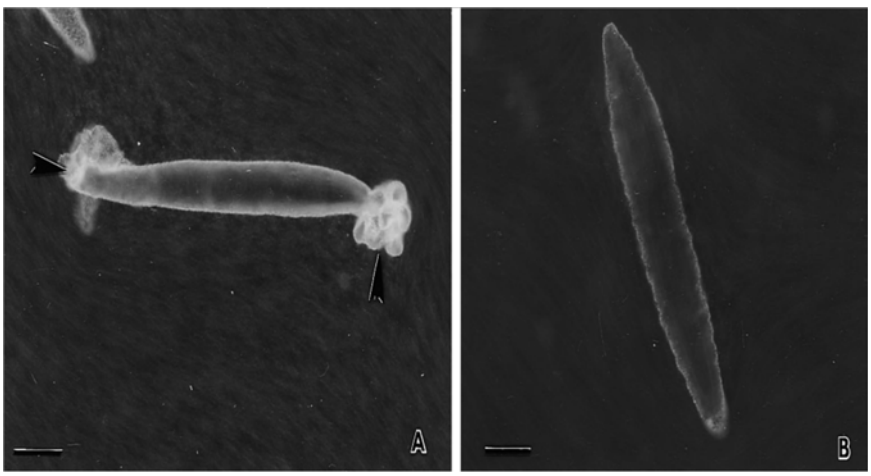

Fig. 5 Slime excretion (*arrowheads*) in untreated *Closterium acerosum* cells (**a**) and absence of slime excretion (**b**) in cells treated with 5 µg mL^{-1} breveldin A (*BFA*) for 5 min. Cells were cultured on fluoresbrite bead-WHM agar. Fluoresbrite beads bind to the acidic group of the excretion, allowing visualisation of slime trails; bar = 100 µm (from Domozych 1999)

growing in the normal growth medium showed decreased bulk-EPS production (Keidan et al. 2009).

The EPS synthesis pathways in microalgae also have been investigated using different approaches, including synchronized cultures, cell-wall modified mutants, inhibitors of polysaccharide formation, and addition of ^{14}C-compounds. Attempts to describe sulfation pathways and glycoprotein formation have also been pursued (Arad and Levy-Ontman 2010).

Synchronized cultures, also known as DLD (dark, light, dilution), were first carried out with the microalga *Chlorella* (Tamiya 1966). Following synchronized cultures of *Porphyridium* sp. and *Dixoniella grisea*, a 0.5×10^6 Da sulphated poly-xylose compound was detected in the growth medium at the beginning of the cell cycle (2nd hour). This molecule was supposed to polymerize further (from 4th to the 6th hour), incorporating other intermediate sugars to produce new higher molecular weight intermediate (MW from 0.5 to 2×10^6 Da). The polymer resulted constituted by galactose, glucuronic acid, mannose, methylated galactose and arabinose. The appearance of sugars was observed mainly during the light period. During the dark period, these sugars polymerized extracellularly to the final molecular weight, following membrane mediated exocytosis (Fig. 6; Simon-Bercovitch 1997; Simon-Bercovitch et al. 1999).

Treatment with the herbicide 2,6-dichlorobenzonitrile (DCB) represents another approach used to study cell wall formation and EPS synthesis in red microalgae, including *Porphyridium* and *Rhodella* (Simon-Bercovitch 1997; Arad et al. 1993, 1994), and also in diatoms such as *Achnantes longipes* (Wang et al. 1997). DCB is known to block cell growth and EPS excretion but minimally affecting DNA synthesis, protein synthesis, and frustule formation in diatoms. The treatment allows the isolation of DCB-resistant mutants which differ for cell-wall composition but with protein and sulphate contents similar to the wild type (Arad et al. 1993). In DCB-mutants of *Rhodella*, higher proportions of methyl galactosyl residues were observed, while higher proportions of xylosyl residues were observed for DCB-mutants of *Porphyridium*. The use of the herbicide allowed also observation of protoplast regeneration inhibition in *Porphyridium* (Arad et al. 1993).

A two-pronged approach, combining DLD with the use of DCB on *Dixoniella grisea* (as *Rhodella reticulata)* (Cohen and Arad 1998) enabled the description of a sequence of events in EPS biosynthesis during cell cycle, by progressively detecting different sugars dissolved in the growth medium during the cell cycle. EPS formation and polymerization was observed during the light period. A sulfur group binds to a xylose polymer during the 2nd hour of the cycle. Subsequently, other moieties bind to the xylose-sulfur polymer in the following order: glucose, galactose, mannose, and glucuronic acid (9th hour of the cycle), ribose and methyl sugars (11th hour of the cycle). Since two different MW fractions with a similar composition were detected, the authors speculate that the fraction at lower MW (0.5×10^6 Da) is first synthesized inside the cell. Furthermore, this polymer is extruded and further polymerized outside the cell to a higher MW (1.15×10^6 Da).

The study of polysaccharide sulfonation in red microalgae was carried out by supplying *Porphyridium* cells with $Na_2{}^{35}SO_4$ or [^{35}S]cysteine. Results suggested the role of cysteine as sulfur donor, with the intervention of the enzyme sulfotransferase that catalyzes the attachment of sulfur to the cell-wall polysaccharides (Keidan et al. 2006). The gene encoding for sulfotransferase was also identified in the ESTs (expressed sequence tag databases) (Plesser et al. 2007). By supplying cells of *Porphyridium* with ^{14}C-floridoside

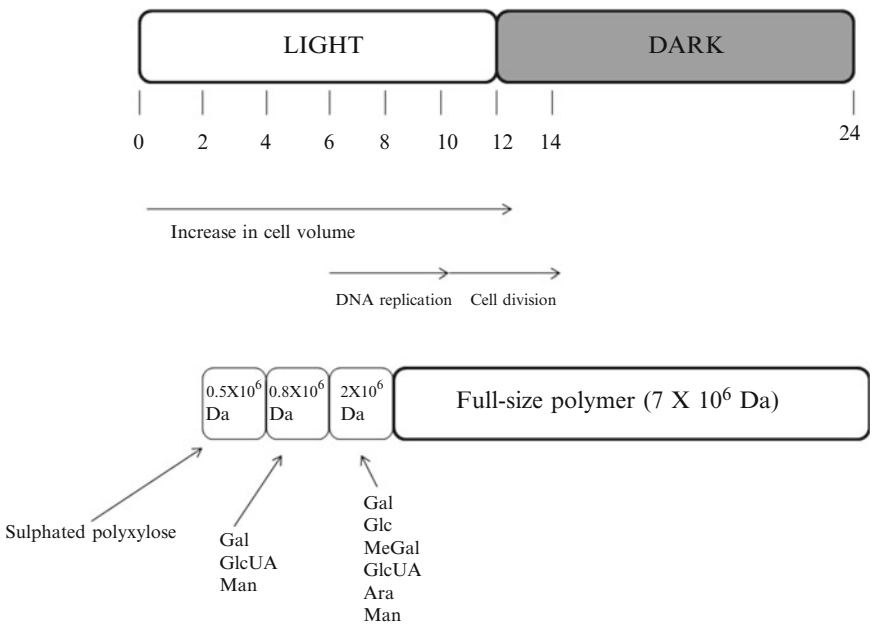

Fig. 6 Schematic of the synthetic process observed using DLD technique. *Ara* arabinose, *Gal* galactose, *Glc* glucose, *GlcUA* glucuronic acid, *Man* mannose, *MeGal* methylated galactose (Simon-Bercovitch et al. 1999)

(a disaccharide constituted by galactose and glycerol), it was shown that cells are able to metabolize this disaccharide. Hence, it was suggested that floridoside acts like a precursor which channels the fixed carbon towards the synthesis of cell-wall polysaccharides. It was also suggested that the polysaccharide, synthesized inside the cells, is transferred outside via the solubilization of the cell wall or directly (Keidan et al. 2009; Li et al. 2001b).

4.2 EPS Synthesis in Cyanobacteria

In cyanobacteria, EPS synthesis pathways are complex and still they to be fully elucidated. However, since the mechanisms are relatively conserved among bacteria, a typical process for EPS assembly and extrusion can be assumed. The process has to include (Pereira et al. 2009):

(i) the activation and conversion of monosaccharides in sugar nucleotides in the cytoplasm;
(ii) the assembly of the repeating units by sequential addition of sugars onto a lipid carrier;
(iii) the polymerization of the repeating units at the periplasmic face of the plasma membrane;
(iv) the extracellular export of the polymer.

In bacteria, exopolysaccharide synthesis was found to be organized in (i) a synthase-dependent or (ii) a Wzy- and ABC-dependent pathway (Whitfield 2010; Whitfield and Larue 2008).

The synthase-dependent pathway has not been fully investigated as yet and relies on a glycosyltransferase that acts both as polymerase and as exporter (Cuthbertson et al. 2010). These so-called "dual function glycotransferases" are present in bacteria, fungi, animals and plants and operate transporting, while synthesizing, the sugar polymer (Davis 2012).

Pathways (i) and (ii) are typical of the capsule assembly process in *Escherichia coli* (Cuthbertson et al. 2009; Steenbergen and Vimr 2008; Whitfield 2006; Whitfield and Paiment 2003; Whitfield and Roberts 1999). In the Wzy-dependent pathway (leading to the assembly of *E. coli* group 1 capsules), the sugar repeating units are transferred into a lipid carrier located at the plasma membrane. From there, the nucleotide sugars are transferred to the periplasmic face of the plasma membrane by another integral membrane protein called Wzx where they are polymerized by the intervention of another protein, called Wzy, in the presence of the polysaccharide copolymerase protein (PCP) Wcz, which in turn interact with the outer membrane exporting protein (OPX) Wza (Cuthbertson et al. 2009; Whitfield 2006).

In the ABC-dependent pathway, leading to the assembly of *E.coli* group 2 capsules, polysaccharides are entirely polymerized in the inner face of the cytoplasmic membrane by single sugar residue addition to the non-reducing end of the forming polymer. The complete polymer is then exported, by an ABC transporter, through the plasma membrane. ABC transporter is constituted by two nucleotide-binding domains (encoded by *kpsM* and *kpsT* genes) and two transmembrane domains. Other genes with unknown role have been detected, namely *kpsC*, *kpsS*, *kpsF* and *kpsU*. The export of the polymer needs the intervention of a PCP protein, named kpsE, and an OPX protein called kpsD (Cuthbertson et al. 2010).

Pereira et al. (2009) proposed a hypothetical working model for EPS synthesis in cyanobacteria (Fig. 7) based on the Wzy-dependent mechanism, which is the most common among Gram-negative bacteria (Whitfield and Larue 2008), combined with the information available on the cyanobacterial genome sequences and on the genes involved in EPS biosynthesis in *Anabaena* sp. PCC7120 (Yoshimura et al. 2007). In this model, simple sugars are assembled in the cytoplasm to form sugar nucleotides, which are then transferred to a lipid acceptor on the plasma membrane – most likely an undecaprenol diphosphate (Skorupska et al. 2006) – to assembly polysaccharide units. Through the intervention of the membrane integral Wzx protein, this lipid-linked polysaccharide is flipped across the membrane. The protein Wzy then polymerizes the repeating units in the periplasmic face of the membrane with the intervention of the protein Wzc, supposedly for the control of the chain length of the growing polymer. Another protein, called Wza, forms pores allowing the externalization of the formed strand. This latest process could require the intervention also of proteins Wzc and Wza.

Since the polymer has to be extruded extracellularly (Whitfield and Larue 2008), the presence of a specific translocation mechanism in the cell envelopes assigned to carbohydrate export must be supposed. Hoiczyk and Baumeister (1998) first described a translocation system in cyanobacteria. The system, named JPC (junctional pore complex), an example of multi-component export machinery (Hoiczyk and Hansel 2000), consists in rows or girdles encircling the bacterial filament on both sides of each cross wall. When isolated, JPC was found to be composed of two substructures, the first being a 13 mm diameter tube-like, and the second a pore with an overall length of 32 nm and a maximal width of 20 nm. Staining with India ink of the pores showed mucilage secretion, the slime bands emerging with a velocity of 3 μm s^{-1}. This mucilage excretion is involved in cell locomotion.

5 Enzymes and Gene-Encoding Enzymes Involved in Microalgae and Cyanobacteria EPS Synthesis

Although the composition and the structure of EPSs produced by living organisms can present huge differences, certain stages in their biosynthesis are unavoidable. These stages include the synthesis of precursor molecules and

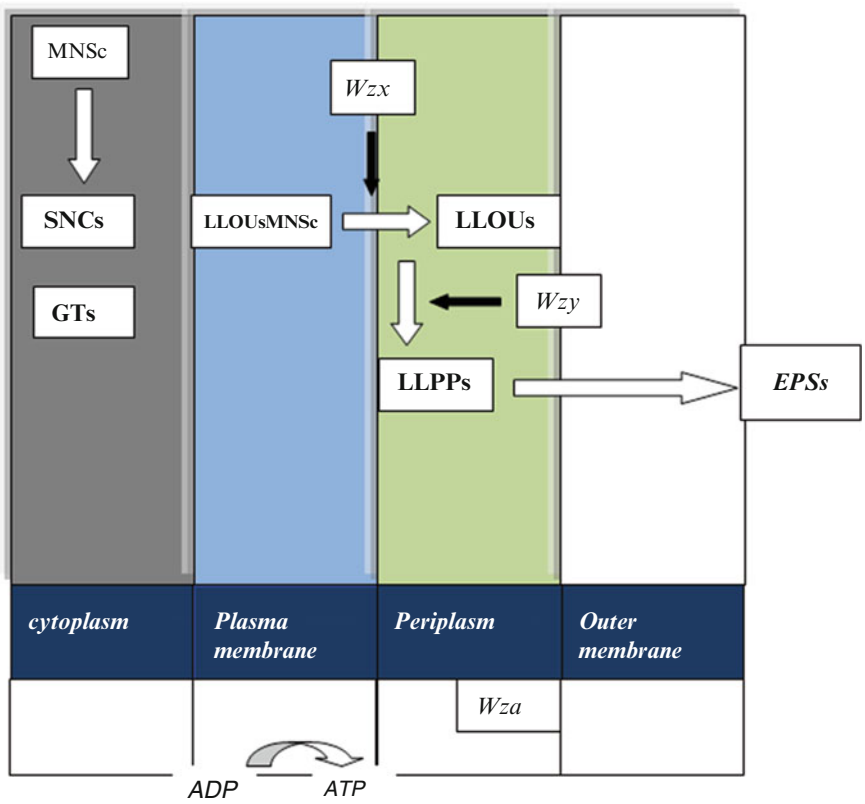

Fig. 7 Proposed pathway for EPS synthesis and compartimentation (Adapted from Pereira et al. (2009)). *MNS* monosaccharides, *SNCs* sugar nucleotides, *GTs* glycosyltransferases, *LLOUs* lipid-linked oligosaccharide units, *LLPPS* lipid-linked polymerized polysaccharide units

polymerization of the component sugars, as discussed above. Then, since the component sugars are synthesized inside the cell, the transport of the products, either extracellularly or through an endomembrane, is necessary (Davis 2012). An example of the transport of sugar molecules partially synthesized include the bacterial peptidoglycan precursor Lipid II (undecaprenyl–pyrophosphoryl–N–acetylmuramyl–(pentapeptide)–N–acetylglucosamine) and hyaluronan synthesized by "class II" hyaluronan synthase (Schultz et al. 2007; Mohammadi et al. 2011; Davis 2012).

A huge number of enzymes act on glycoconjugates, oligo- and polysaccharides which constitute some of the most structurally diverse substrates on Earth (Cantarel et al. 2009). These enzymes are collectively called Carbohydrate-Active Enzymes (CAZymes). CAZymes are classified in different families (Cantarel et al. 2009):

(i) Glycosyl hydrolases (GHs), including glycosidases and transglycosidases. These enzymes are responsible for the hydrolysis and transglycosilation of glycosidic bonds;
(ii) Glycosyltransferases (GTs), responsible for the synthesis of glycosidic bonds from phosphor-activated sugar donors;
(iii) Polysaccharide lyases (PLs), responsible of cleaving the glycosidic bonds of uronic-acid containing sugars, by a ß-elimination mechanism;
(iv) Carbohydrate esterases (CEs), which remove ester-based modifications and facilitate GH action on complex sugars;
(v) Carbohydrate-binding molecules (CBMs), which have no *per se* activity, but potentiate the activity of the aforementioned enzymes by promoting interaction with the substrates.

CAZymes are being collected in the Carbohydrate-Active Enzymes database (CAZy database; www.cazy.org), including sequence annotations from public sources, and data on taxonomy, sequence, family classification and known references (Cantarel et al. 2009). The gathering of genomic information on microalgae and cyanobacteria from genomic sequencing will allow more-in depth studies on carbohydrate synthesis, also known as glycome, in order to understand the mechanisms, observe possible evolutionary inferences between organisms, and use this knowledge for biotechnological applications.

Between CAZymes, GTs are key enzymes implied in the synthesis of EPS repeating units. Precisely, they are involved in the biosynthesis of disaccharides, oligosaccharides and polysaccharides by catalyzing the transfer of sugar moieties from an activated donor to specific acceptor molecules (Pereira et al. 2009). GTs can be subdivided as retaining or inverting GTs according to the stereochemistry of the final products (Sinnott 1990). The CAZy database assigns GTs to

different families using nucleotide diphospho-sugar, nucleotide monophospho-sugars and sugar phosphates (EC 2.4.1.x) and related proteins, following the method proposed by Campbell et al. (1997) and Coutinho et al. (2003).

5.1 Enzymes and Enzyme-Encoding Genes in Microalgae

The extracellular envelopes and the cell wall of microalgae and their mucilaginous exudates can be profoundly different, both biochemically and structurally. The complexity and the differences observed between the outermost polymeric structures of microalgae also is reflected in the high number of enzymes involved in polysaccharide synthesis and modification, e.g. in cell wall metabolism. Different enzymes participate in the synthesis and their identification requires the screening of the organisms' proteomes and the use of GT-encoding models. The identification of GTs involved in these complex processes requires a new systematic and efficient classification system, similar to the one currently used for plants (Banks et al. 2011; Vogel et al. 2010).

Microalgal genomes have started to be explored only relatively recently. Interesting results were obtained by Blanc et al. (2010, 2012) on the genome of *Chlorella variabilis* NC64 and *Coccomyxa subellipsoidea* C-169 NIES 2166. In *C. variabilis* NC64A, 233 predicted enzymes involved in carbohydrate metabolism were detected, while 92 and 168 predicted enzymes had been previously detected respectively in *C. reinhardtii* and *Ostreococcus tauri*, two other EPS-producing chlorophytes. Between the eukaryotic ortholog groups, one (324 proteins) is putatively involved in carbohydrate transport and metabolism, one (99 proteins) is involved in cell wall/membrane envelope biogenesis, one (210 proteins) is involved in extracellular structure building, while another (315 proteins) is involved in intracellular trafficking, secretion and vesicular transport (Blanc et al. 2010). In *C. variabilis*, whose cell wall includes chitin and chitosan, 2 paralogs for chitin synthase and 25 paralogs for chitin deacetylase, the latter converting chitin into chitosan, were detected. Two chitinase genes (plant and prokaryotic type GH19 and GH18) and four chitosinase genes, involved in cell wall degradation, were also detected (Blanc et al. 2010). The four chitosanases contain the three catalytic residues Glu-36, Asp-40 and Thr-45 of *Streptomyces* sp. N174 chitosanases (Lacombe-Harvey et al. 2009). These results represent an example of comparative genomics, which is necessary to determine unsuspected functions and to understand evolutionary pathways. Furthermore, in *C. subellipsoidea*, five expanded protein families are putatively involved in polysaccharide and cell wall metabolism, and 7 proteins with putative 5 UDP-glucoronosyl and UDP-glucosyl transferase have been identified (Blanc et al. 2012).

Due to their large size, microalgal genomes have been investigated using transcriptome sequencing. Transcriptome data have been used for phylogenomics, although post-genomic approaches to study physiology and metabolism are possible (Cadoret et al. 2012). For example, through the "Marine Microbial Eukaryotic Transcriptome project" (www.marinemicroeukaryotes.org), 39 transcripomes have been sequenced, allowing a for a better understanding of synthetic pathways and regulation networks of microalgal-derived compounds.

Following an investigation on proteome screening of different species conducted by Ulvskov et al. (2013) utilizing the CAZy database as bait, the number of GT sequences and the % of their codifying genes in the genome were reported (Table 2). Moreover, for these organisms the number of genes coding for each detected GT family has been reported (Table 3).

5.2 Enzymes and Enzyme-Encoding Genes in Cyanobacteria

In bacteria, EPS biosynthetic pathways, recently reviewed by Pereira et al. (2013), are relatively well conserved, regardless of the variety of polysaccharides produced. The process requires the participation of:

(i) Enzymes involved in the biosynthesis of nucleotide sugars;
(ii) GTs catalyzing the transfer of nucleotide sugars to specific acceptors in the plasma membrane;
(iii) Proteins involved in the assembly and export of EPS (Reeves et al. 1996).

Table 2 Total number of GTs detected following proteome screening of green and red microalgal strains and percent of GT-codifying genome of different microalgal strains (From Ulvskov et al. 2013)

Species	Phylum (Class)	Total number of GTs	Percent of genome being GTs
Chlamydomonas reinhardtii	Chlorophyta (Chlorophyceae)	76	0.50
Micromonas sp.	Chlorophyta (Prasinophyceae)	77	0.77
Ostreococcus tauri	Chlorophyta (Prasinophyceae)	52	0.66
Ostreococcus lucimarinus	Chlorophyta (Prasinophyceae)	57	0.75
Cyanidioschyzon merolae	Rhodophyta (Cyanidiophyceae)	32	0.64
Galdieria sulphuraria	Rhodophyta (Cyanidiophyceae)	54	0.75

Table 3 Detected families of GTs and number of detected genes in the genomes of different investigated microalgal strains (from Ulvskov et al. 2013)

Strains	Detected families of GTs (*number of genes codifying for each GT family*)
Chlamydomonas reinhardtii	GT2(5), GT4(10), GT5(9), GT8(1), GT10(1), GT20(4), GT21(1), GT24(1), GT28(3), GT32(1), GT33(1), GT34(2), GT35(2), GT47(17), GT48(6), GT49(2), GT50(1), GT57(2), GT64(1), GT66(2), GT71(1), GT75(1), GT77(6)
Micromonas sp.	GT2(6), GT4(11), GT5(9), GT7(3), GT13(3), GT20(3), GT21(1), GT22(4), GT24(1), GT25(2), GT28(2), GT32(2), GT33(1), GT34(1), GT35(3), GT41(1), GT47(3), GT48(2), GT50(1), GT57(2), GT58(1), GT60(2), GT64(1), GT66(2), GT71(1), GT76(1), GT77(11)
Ostreococcus tauri	GT2(6), GT4(8), GT5(6), GT7(1), GT20(3), GT22(4), GT24(1), GT31(1), GT32(1), GT33(1), GT34(1), GT35(3), GT41(2), GT47(3), GT48(2), GT50(1), GT58(1), GT60(1), GT64(1), GT66(1), GT71(1), GT76(1), GT77(1)
Ostreococcus lucimarinus	GT2(6), GT4(9), GT5(6), GT7(1), GT8(1), GT20(2), GT22(4), GT24(1), GT28(1), GT32(1), GT33(1), GT34(2), GT35(3), GT41(2), GT47(1), GT48(2), GT50(1), GT58(1), GT60(1), GT64(2), GT66(1), GT71(1), GT76(1), GT77(6)
Cyanidioschyzon merolae	GT2(5), GT4(5), GT5(1), GT8(2), GT19(2), GT20(4), GT22(1), GT24(1), GT28(1), GT33(1), GT35(1), GT39(1), GT39(1), GT49(1), GT57(2), GT58(1), GT66(1), GT77(1), GT83(1)
Galdieria sulphuraria	GT2(5), GT4(10), GT5(1), GT8(3), GT14(3), GT17(1), GT19(4), GT20(6), GT22(2), GT24(1), GT28(2), GT30(1), GT32(1), GT33(1), GT35(3), GT39(2), GT57(2), GT58(1), GT64(2), GT66(1), GT77(1), GT83(1)

The processes commonly include the activation of monosaccharides and their incorporation in nucleotide sugars and these are similar to what should happen in microalgae in the cytosol.

In cyanobacteria, EPS-related genes occur scattered throughout the genome, either isolated or in small clusters, while in bacteria the latter is usual (Pereira et al. 2009). The identification in the genomes of EPS-producing cyanobacteria so far sequenced of genes codifying for proteins involved in Wzy-dependent pathway, suggests that EPS synthesis in cyanobacteria follows this mechanism (Pereira et al. 2009; Whitfield and Larue 2008). Studying the sequences of 24 cyanobacterial genomes, Pereira et al. (2013) found proteins possessing the Wzy characteristic domain in all the strains, although nine of them were devoid of Wzx homologues. On the other hand, the low number of detected *KpsC* and *KpsS* putative genes, which codify for proteins involved in the ABC-dependent pathway, strengthens the Wzy-dependent hypothesis.

PCP protein sequences of cyanobacteria grouped separately (phylogenetic distance) from other PCP proteins belonging to other bacteria, which cluster together. They have the typical additional C-terminal region containing the Walker A and B domains and the DxD motif commonly found in bacterial tyrosine kinases (Soulat et al. 2007).

In a recent investigation (Cuthbertson et al. 2009), major phylogenetic groups defined for the PCP proteins matched those established for the correspondent OPX proteins, suggesting their co-evolution. Four export components, named A,C, D and E intervene in the Wzy-dependent pathway whilst other two components, named B and F are involved in ABC-dependent pathway. In cyanobacteria, PCP-encoding proteins resulted abundant, especially in filamentous cyanobacteria, where they are involved in the synthesis of heterocyst EPSs.

Fox genes are specifically required for nitrogen fixation in the presence of oxygen. These genes include those involved in heterocyst-EPS synthesis: *devRA* (*alr*0442) and *hep*K *(all*4496*)* whose products interact as parts of a two-part regulatory system; *alr0117* and *alr1086*; *hep*B (*alr*3698) and *hep*C (*alr*2834), whose products resemble a glycosyltransferase and an UDP-galactose lipid carrier transferase, respectively; and *hep*A (*alr*2835), which codifies for an ATP-binding cassette transporter and is activated in response to nitrogen deprivation (Huang et al. 2005).

OPX proteins in cyanobacteria are characterized by a common domain (PES; IPR003715), a common motif in cyanobacteria, and most of the putative proteins contained a soluble ligand-binding beta-grasp domain (SLBB; IPR019554). Most of the genomes analyzed by Pereira et al. (2013) possess at least one homologue, with only three exceptions, the unicellular *Prochlorococcus marinus* MIT9301, *Synechococcus elongatus* PCC6301 and *Cyanobacterium* UCYN-A. Results also suggested the presence of two OPX protein paralogues in the last common cyanobacterial ancestor, *opx*A and *opx*B.

Selective forces favor, in general, the loss of *opx*B and the duplication of *opx*A. In general, with a correlation with the morphological groups, the *opx* genes resulted more abundant in the filamentous strains, whilst *pcp* genes resulted more abundant in heterocystous strains. No correlation has been found between *opx* and *pcp* copies with the habitat and/or diazotrophic capacity and with EPS-producing capacity.

Cyanobacterial outer polysaccharidic structures are of different types: capsular, sheath-like, slimy and some release almost their entire excretion as RPSs. At the moment it is

unknown whether all these types are secreted following the same pathways.

6 Conclusions

From the above information it is possible to draw some general conclusion with regard to the characteristics of the exocellular polysaccharides produced by microalgae and cyanobacteria and their biosynthesis:

(i) Generally speaking, microalgae and cyanobacteria are capable of producing the most complex EPSs, in terms of number of different types of monosaccharides in the repeating unit of the polymers, in comparison with other EPS-producing microorganisms. However, it has to be stressed that an additional factor of complexity in the evaluation of the differences in EPS composition reported by different research groups is related with the methods they utilized for the hydrolysis and analysis of the polymers. Indeed, as it was previously reported (De Philippis and Vincenzini 1998), qualitative and quantitative analyses of the monosaccharides present in the EPSs may be strongly affected by the conditions utilised for the hydrolysis of the polymers, in particular in the case of uronic acids. Also the use of different chromatographic methods for the analysis of the monosaccharides may affect the results obtained, both in terms of identification and quantification of the monomers. Finally, the harsh procedures generally utilized for removing and recovering the EPSs tightly bound to the cells, as in the case of sheaths and capsules, may modify the chemical characteristics of the polymers.

(ii) From the data here reviewed, it is evident that the composition of the EPSs is variable from species to species and, in some cases, also affected by the growth conditions. Thus, this feature makes unreliable the use of the monosaccharidic composition of the EPSs for chemotaxonomical uses but extend the chances to find polymers with properties suitable for industrial applications.

(iii) The presence of sulphate groups linked to the macromolecule has been reported for a large number of EPSs. However, in spite of the above reported potential of sulphated polysaccharides for biomedical applications, only in few cases the biosynthetic pathway of sulfation was studied. Owing to the direct correlation between the degree of sulfation of a polysaccharide and its antiviral activity (Witvrouw and De Clercq 1997), the knowledge of the pathway of EPS sulfation in other EPS-producing cyanobacteria and microalgae would be very important. In fact, the knowledge of this process could give the possibility to apply the metabolic engineering techniques to the producing strains for increasing the degree of sulfation of the polymers they produce.

(iv) A large number of studies have shown that EPS biosynthesis is affected by one or more environmental factors, but it was shown that the effects are species-specific. As a consequence, in spite of the general agreement that EPSs have a protective role for the producing microalga or cyanobacterium, in most cases it is hard to make a more general definition of this role in most cases. A deeper knowledge on the genes involved in the biosynthesis of EPSs in microalgae and cyanobacteria would allow expression profiling studies aimed at defining the influence of specific environmental factors on the production of the polysaccharides. Knowledge of the genes involved in the biosynthesis would also allow the development of metabolic engineering strategies for increasing the production of EPS or for obtaining modification of their chemical characteristics aimed at increasing their suitability for specific industrial applications.

(v) The recent establishment of the CAZymes database is a very good starting point and gives promising perspectives for increasing the knowledge of EPS biosynthetic pathways in microalgae and cyanobacteria.

(vi) It has to be stressed that the mechanism that directs the newly synthesized EPS to the formation of the specific outermost extracellular structure that characterizes the single strain (i.e. sheath, capsule, slime, RPS) is still unknown. Understanding this mechanism could provide the possibility to modify the type of structure typical of that strain in order to facilitate the recovery of polymers having an industrial interest.

From the above listed points, it is evident that, in spite of the long history of this topic and the huge number of studies available on microalgal and cyanobacterial EPSs, there are many fascinating and important aspects that still need to be investigated and clarified.

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Algae Genome-Scale Reconstruction, Modelling and Applications

Cristiana G.O. Dal'Molin and Lars K. Nielsen

1 Introduction

Microalgae are interesting organisms because of their ability to produce a wide range of compounds, such as carotenoids (Macias-Sanchez et al. 2008; Goh et al. 2009; Guedes et al. 2011), lipids (Chisti 2007; Chen et al. 2011; Chen and Walker 2011), hydrogen (Escobar et al. 2000; de la Rosa et al. 2001; Melis et al. 2007), protein (Potvin and Zhang 2010), and starch (Fernandes et al. 2010; Branyikova et al. 2011). These algal compounds have numerous applications, varying from fine chemicals to biofuels to food additives.

To make commercial bulk production of these compounds economically feasible, maximization of algal biomass production and optimization of biomass composition are necessary. Moreover, to fully exploit the potential of microalgae and their products, a deeper knowledge of algal cell metabolism is required. Therefore, the development of systems biological tools for microalgae is needed.

In modern, system-level microbial metabolic engineering, genome-scale metabolic reconstructions are used as a systems based framework to integrate and analyze large 'omics' datasets as well as to evaluate cell design in silico. In doing so, systems-level metabolic engineering is changing the way microbial cell factories are designed and it is becoming a major driver towards bio-based production of chemicals, materials and fuels from renewables and thus one of the core technologies of global green growth (Kim et al. 2008; Kohlstedt et al. 2010; Lee 2010a, b; Lee and Park 2010; Jang et al. 2012a). Similarly, genome-scale reconstructions can be applied to systems-level algal metabolic engineering to advance algae biotechnology.

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Chlamydomonas reinhardtii is a model organism for green microalgae and has been studied extensively in the past decades. The availability of complete genome sequence data for *C. reinhardtii* and its functional annotation have enabled the reconstruction of genome-scale metabolic netwoks (Chang et al. 2011; de Oliveira Dal'Molin et al. 2011). These models provide quantitative and qualitative hypotheses that can be used to help iteratively guide additional experiments to further the state of algae metabolic engineering and biotechnology. In this chapter, the first generation of algae genome-scale metabolic reconstructions based on *C. reinhardtii* genome is discussed and the basic concepts of metabolic model formulation are explained.

2 Genome-Scale Metabolic Reconstruction

Genome scale modelling considers the reconstruction, pathway analysis and application of genome-scale metabolic models to study metabolism. Ultimately, such models may be used for systems-level metabolic engineering, pathway design and in silico analyses for strain improvement. A reconstruction breaks down metabolic pathways into their respective reactions and enzymes, and analyzes them within the perspective of the entire network. In simplified terms, a reconstruction collects all of the relevant metabolic information (at genome-scale) of an organism and compiles it in a mathematical model. The genome-scale metabolic network reconstruction encompasses existing knowledge in a highly mathematical, structured framework to study an organism (Rudd 2000; Forster et al. 2003; Thiele and Palsson 2010b). Importantly, the network topology readily reveals gaps in the annotation, e.g., a missing gene in amino acid biosynthesis pathway when the organism is known to be able to synthesise the amino acid. Thus, genome-scale metabolic reconstructions are also advanced functional annotations that guide the search for missing genes or missed annotations.

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Genome-scale reconstructions vary in size, depending on the organism. Typical genome-scale recomstrutions of prokaryotes cover an average of 650 genes, 600 metabolites, and 800 reactions, whereas genome-scale reconstructions of eukaryotes include on average 1000 genes, 1200 metabolites and 1500 reactions (Oberhardt et al. 2009).

Applications of genome-scale metabolic models have greatly influenced the field of systems biology by providing a platform on which high-throughput computational analysis of metabolic networks can be performed. In silico reconstructions and subsequent analysis of genome-scale metabolic models through constraint-based modeling enables a global interrogation of metabolism and guides to identify gene manipulation targets; not possible with standard experiments (Lee et al. 2011; Jang et al. 2012a, b). These models are routinely applied in the context of a systems-level approach for metabolic engineering and strain improvement of industrial microorganisms (Blazeck and Alper 2010), as illustrated in Fig. 2. These strategies are particularly valuable for gaining insight into unexpected problems, such as slow growth and low product yield that arise after rational engineering through systems-level interrogation of cellular networks.

Most of the genome-scale reconstructions are available for prokaryotes. The process is far more challenge for eukaryotes particularly due to the cell complexity and compartmentation. Although these challenges, considerable progress has been in algae and plant genome-scale reconstruction and modeling in recent years (de Oliveira Dal'Molin et al. 2010a, b, 2011, 2014; Chang et al. 2011; de Oliveira Dal'Molin and Nielsen 2013). Advances on modeling multi-tissue interactions in plants were critical to describe C4 photosynthesis in grasses (de Oliveira Dal'Molin et al. 2010a) and more recently, a multi-tissue genome-scale model framework (named, MultiGEM) has been developed to perform whole plant fluxomics across the diurnal cycle (de Oliveira Dal'Molin et al. 2015).

2.1 The Reconstruction Process

The reconstruction is done using publically available annotation databases and biochemical evidences found in the literature for the particular organism. Table 1 presents the information source used to build AlgaGEM, a genome scale metabolic network for the unicellular green alga *Chlamydomonas reinhardtii.*

In summary, the process to build a reconstruction is as follows:

(i) Draft of the reconstruction from genome annotation of a target organism:

Even for eukaryotes like algae or plants, at this stage the cellular network model is not compartmentalized, and the model is generated in a flat file.

(ii) Refinement of the reconstruction (curation):

The metabolic network is compartmentalized and the organelle transporters are added based on genome annotation and biochemical evidences.

(iii) Convert model into a mathematical/computational representation:

The metabolic network is represented into a stoichiometric matrix (**S**) with coefficients of the substrates and products for the different reactions, which also includes the transport reactions over the membranes.

(iv) Test model functionality through experimentation and model validation:

Physico-chemical constraints are imposed in the network and the metabolic fluxes are calculated by flux balance analysis (FBA).

The process of developing genome-scale metabolic reconstructions includes functional annotation of gene products based on sequence homology. More importantly, however, this also addresses assigning functions to unknown genes and this includes assigning genes to metabolic functions, for which no gene has been indentified yet.

The reconstruction process has been extensively reviewed (DeJongh et al. 2007; Duarte et al. 2007; Quek and Nielsen 2008; Durot et al. 2009; Feist et al. 2009; Oberhardt et al. 2009) and a detailed protocol of how to build a genome-scale metabolic model is described by Thiele and Palsson (2010a). An automated pipeline covering a detailed protocol is available to generate genome-scale reconstructions for prokaryotes and literally thousands of reconstructions have been generated by this means (Henry et al. 2010). Although a detailed protocol is available, the process requires an extensive manual curation effort for eukaryotes, particularly due to the cell compartmentation and multicellular metabolism in higher organisms.

2.2 The Stoichiometric Matrix

It is necessary to convert the metabolic network into a mathematical/computational representation in order to calculate the metabolic fluxes. The metabolism of an organism can be described by a set of reaction equations defining the stoichiometric conversion of substrates into products. The stoichiometry matrix **S** contains the stoichiometric coefficients of the substrates and products for the different reactions in the metabolic network, which also includes the transport reactions over the membranes. This approach combines standard functional annotations of metabolic genes – gene-protein-reaction (GPR) mapping – with a network topology captured

Table 1 Online resources for the reconstruction of the metabolic network of *Chlamydomonas reinhardtii* (AlgaGEM)

Database	Link
Genome database	
DOE Joint Genome Institute (JGI); *Chlamydomonas reinhardtii* v4.0	http://genome.jgi-psf.org/Chlre4/Chlre4.home.html
An online Informatics Resource for *Chlamydomonas (Chlamy Center)*	http://www.chlamy.org/chlamydb.html
Pathway databases	
Kyoto encyclopedia of genes and genomes (KEGG)	http://www.genome.jp/kegg/pathway.html
ChlamyCyc	http://chlamyto.mpimp-golm.mpg.de/chlamycyc/index.jsp
Metacyc	http://metacyc.org/
ExPASy biochemical pathways	http://www.expasy.ch/cgi-bin/search-biochem-index
Enzymes databases	
ExPASy enzyme database	http://ca.expasy.org/enzyme/
	http://www.brenda-enzymes.info/
Enzyme/protein localization and others databases[a]	
AraPerox (Arabidopsis protein from plant peroxisomes)	http://www.araperox.uni-goettingen.de/
SUBA (Arabidopsis subcellular database)	http://www.plantenergy.uwa.edu.au/applications/suba2/index.php
PPDB (plant proteome database)	http://ppdb.tc.cornell.edu/default.aspx
UniproKB/SwissProt	http://ca.expasy.org/sprot/relnotes/relstat.html
Transport DB	http://www.membranetransport.org/

[a]Manual curation based on literature and homology sequence (de Oliveira Dal'Molin et al. 2011)

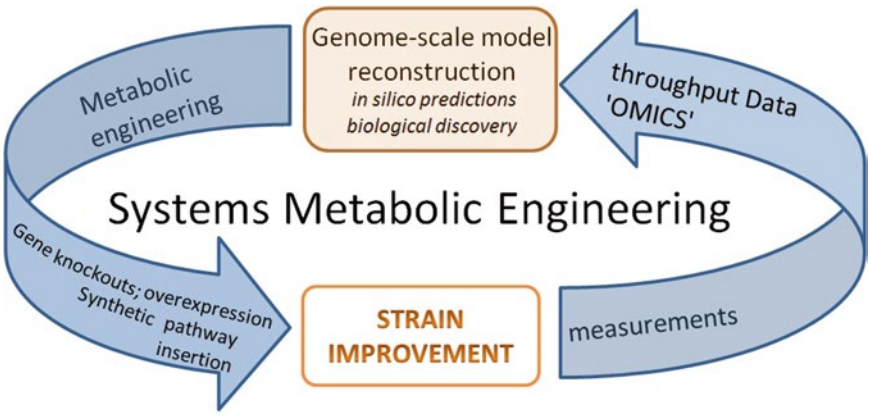


Fig. 1 Systems metabolic engineering incorporates the concepts and techniques of systems biology, synthetic biology and evolutionary engineering at the systems level. This iterative process offers a conceptual and technological framework; knowledge-based methods are incorporated into in silico metabolic models to design optimal metabolic pathways and identify genetic knockout candidates for the optimal production of desired products

in a stoichiometric matrix, **S**, with components (metabolites) in rows, connections (reactions) in columns, and stoichiometric coefficients in each cell (Fig. 1).

A list of reactions is collected that are either catalyzed by enzymes encoded in the genome or have been defined experimentally, and then expanded to define relationships between genes, enzymes, reactions, metabolites, and pathways in the network. For eukaryotes, the reactions are assigned to the correspondent organelle where the enzyme is located. Often many step reactions are not covered by the genome annotation and they are added to fill the gaps and to enable in silico cell functionality based on biochemical evidences.

3 From the Reconstruction to Model Formulations

Once the metabolic network is captured from the genome and converted into a mathematical/computational representation we can then test our hypotheses. This can be done by carefully implementing the model assumptions and constraints to solve the metabolic fluxes through the network. It is important to understand that metabolic reconstruction and model formulation are two distinct processes and should be treated separately. The reconstruction is never fully completed and it is always subjected to refinement. At genome-scale,

it is impossible to use dynamic models with parameterised kinetics; instead constraint-based modeling approaches are applied (Famili et al. 2003; Park et al. 2009; Schellenberger et al. 2011).

3.1 Principles That Underpin the Implementation of Flux Balance Analysis

Flux balance analysis (FBA) is a widely used approach for studying biochemical networks, in particular the genome-scale metabolic network reconstructions that have been built in the past decade (Edwards et al. 2001; Lee et al. 2006; Cakir et al. 2007; Mendoza 2007; Mo et al. 2007; Schwartz et al. 2007; Oberhardt et al. 2009; Orth et al. 2010). Like in metabolic flux analysis (MFA), FBA is based on the assumption that accumulation in any internal node of a metabolic network is trivial compared to the fluxes through the node; i.e.: the changes in concentration of any internal metabolite is too small compared to metabolite rates. If C is the cell-specific concentration of an internal metabolite (in mmol g^{-1} DW), X is the concentration of biomass (in g-DW g^{-1} cell), and $v \leftarrow$ and $v \rightarrow$ the specific fluxes (in mmol g-DW^{-1} h^{-1}) towards and away from the metabolite, respectively, the assumption can be formulated as:

$$\frac{1}{X}\frac{d(CX)}{dt} = C\frac{1}{X}\frac{dX}{dt} + \frac{dC}{dt} = C\mu + \frac{dC}{dt} << v\leftarrow, v\rightarrow \quad (1)$$

It is important to address two assumptions in the Eq. 1:

(i) The system is pseudo-steady state: $dC/dt \ll v\leftarrow, v\rightarrow$
(ii) The concentration of metabolites, C, is so small that the dilution due to growth term is trivial: $C\mu \ll v\leftarrow, v\rightarrow$

The pseudo-steady-state assumption has been found to hold under most conditions. It is well established in microbial systems but has been seriously misinterpreted when taken to higher eukaryotic systems like mammalian and plant cells. It should be stressed that the pseud-steady state assumption does not require a true steady-state to be established; the slow transients during the cell culture (batch) or plant growth also satisfy this assumption. Only where a dramatic transient such as induction occurs (i.e.: light set on/off, without a transient period), is this assumption likely to fail.

The assumption of trivial metabolite concentrations is generally valid throughout the network. For microalgae cells, an important exemption is the accumulation of lipids. In some microalgae species, lipids can reach very high concentrations and maintaining these concentrations places a non-trivial load on the metabolic network. In general, any highly abundant metabolite should be included in biomass or treated as 'storage components', i.e., should be considered a product rather than an internal metabolite. Other examples of components that should be treated as storage components and not internal metabolites are free amino acids and organic acids when accumulated in high levels in the vacuole.

Given the assumption in Eq. 1, we have that the sum of fluxes towards an internal metabolite, $v\leftarrow$, must equal the sum of fluxes away from a metabolite, $v\rightarrow$. For a large network, this is written in matrix form as:

$$S \cdot \mathrm{v} = 0 \quad (2)$$

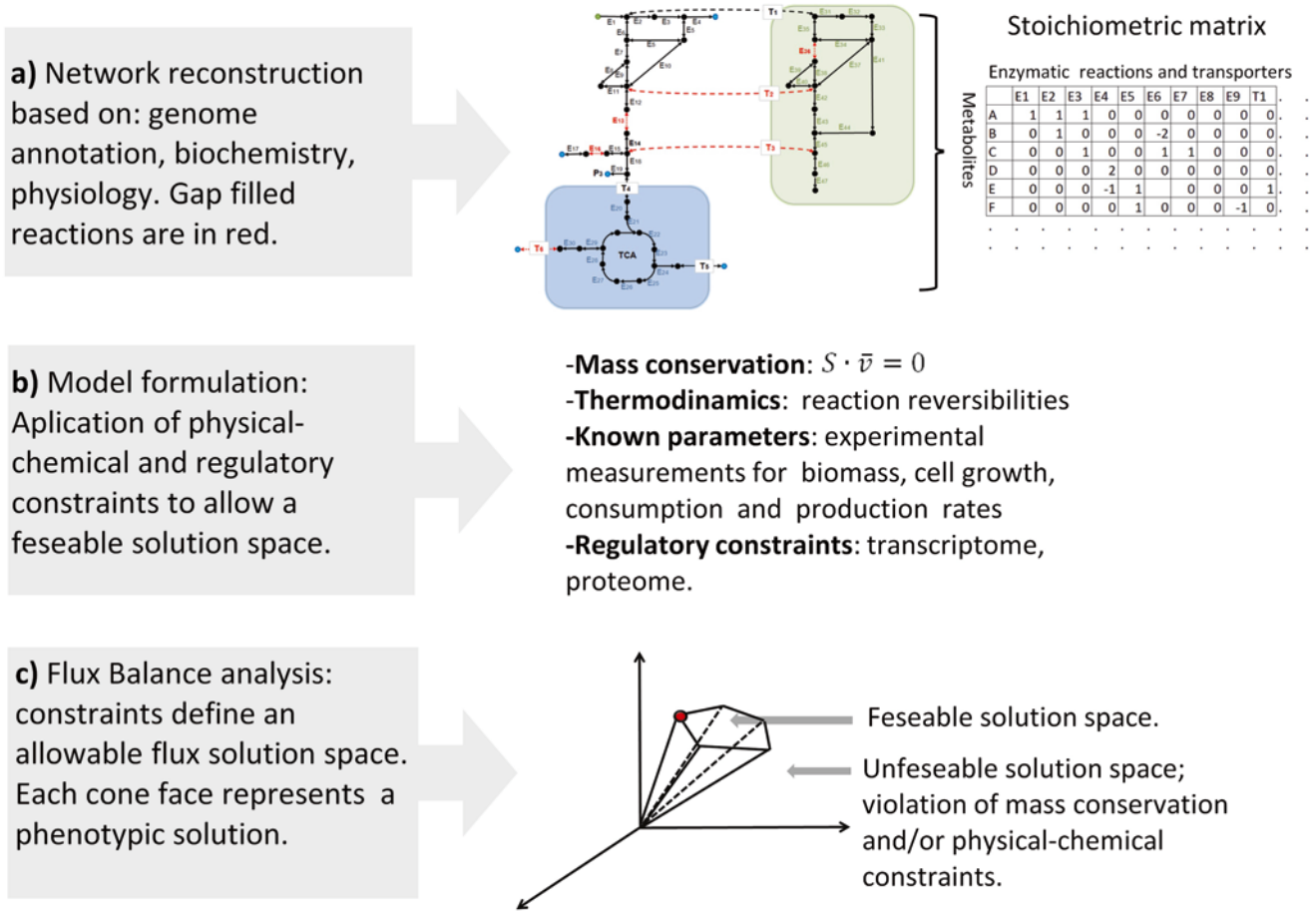
where $\boldsymbol{S}$ is a stoichiometric matrix with each row corresponding to a balanced internal metabolite and each column corresponding to a flux in the flux vector, **v**. Eq.2 defines a set of linear relationships between fluxes and thus reduces the degrees of freedom in the system. Given the assumption of biological pseudo steady-state, or homeostasis we have a direct application of linear programming to biological systems.

Thus, FBA can be defined as the use of linear programming to solve the equation $S.\bar{v} = 0$ given a set of upper and lower bounds on v and a linear combination of fluxes as an objective function. The model formulation combines hypotheses and information (known parameters) about the system such as the biomass composition, measurement rates and omics data; used as physical-chemical and regulatory constraints (Fig. 2b). In order to calculate the fluxes and identify particular network state, it is necessary to make assumptions about the cellular objectives (Fig. 2c) (Orth et al. 2010).

The constraints separate the feasible and unfeasible metabolic behaviours. From the flux distributions obtained, physiologic parameters such as growth yields, minimal medium components or minimum energy requirement for cell growth can be calculated by FBA. The output of FBA is a particular flux distribution, v, which maximizes or minimizes the objective function. Simulated and experimental data are compared and their distance provides a basis from where the model may need to be improved.

3.2 Cell Optimality: The Cell 'Goal'

The cell optimality function is part of the model's assumption and should be well formulated as the results and biological interpretations depend on such assumption. For bacteria for example, the objective function or the 'cell goal' is to maximize growth. A notable example of the success of flux balance analysis is the ability to accurately predict the growth rate of *Escherichia coli* when cultured in different conditions (Edwards et al. 2001). This method has been successfully used for systems-level engineering of industrial microorganisms to optimize the production of biotechnologically relevant metabolites (Kim et al. 2008; Kohlstedt et al. 2010; Lee 2010a, b; Lee and Park 2010; Jang et al. 2012a). The same approach is valuable in understanding metabolic capabilities of complex systems like algae, plant cells and



	E1	E2	E3	E4	E5	E6	E7	E8	E9	T1
A	1	1	1	0	0	0	0	0	0	0
B	0	1	0	0	0	-2	0	0	0	0
C	0	0	1	0	0	1	1	0	0	0
D	0	0	0	2	0	0	0	0	0	0
E	0	0	0	-1	1		0	0	0	1
F	0	0	0	0	1	0	0	0	-1	0

Fig. 2 Genome-scale metabolic reconstruction. (**a**) The network is reconstructed from a variety of biological data sources. Gap filling algorithms are combined with manual curation to complete missing reactions. Network topology is then captured in a stoichiometric matrix. (**b**) The capabilities of the model are assessed in the context of physical, chemical and regulatory constraints. (**c**) A feasible flux solution space is allowed, and the metabolic fluxes are calculated based on a cell optimality criteria imposed in the system

tissues. Interestingly, the agreement between experimental observations and in silico predictions based on optimization suggests that plant cell metabolism could be regulated to achieve resource efficiency (photons in photosynthetic tissue and sucrose in nonphotosynthetic tissue) in individual tissues (de Oliveira Dal'Molin et al. 2010a, b). Different optimaity criteria have been tested to study key features of algae metabolism using the reconstructions based on *Chlamydomonas reinhardtii* genome. The model applications and predictions are sumarized in the following session.

4 Algae Genome-Scale Metabolic Reconstructions

The first generation of algae genome-scale models have been reconstructed based on *Chlamydomonas reinhardtii* genome (AlgaGEM (de Oliveira Dal'Molin et al. 2011) and iRC1080 (Chang et al. 2011)). AlgaGEM accounts for the functions of 866 unique ORFs, 1862 metabolites, 2249 gene-enzyme-reaction-association entries, 1725 unique reactions and five cellular compartments (de Oliveira Dal'Molin et al. 2011). The active scope of AlgaGEM includes glycolysis (plastidic and cytosolic), the pentose phosphate pathway (PPP) (plastidic and cytosolic), tricarboxylic acid cycle (TCA cycle), light and dark reactions (Calvin cycle), fatty acid synthesis, beta-oxidation, glyoxylate cycle, photorespiratory cycle and fermentative reactions. iRC1080, a more comprehensive reconstruction, accounts for 1080 genes, associated with 2190 reactions and 1068 unique metabolites, and encompasses 83 subsystems distributed across 10 compartments (Chang et al. 2011). iRC1080provides a detailed account of metabolic photon absorption by light-driven reactions and lipid metabolism. These reconstructions made it possible to explore important metabolic phenotypes of *C. reinhardtii* through appropriate model formulation and optimization methods. While both metabolic recostructions vary in size and scope they well characterize essential features of algae

primary metabolism. AlgaGEM and iRC1080 reconstructions are important contributions of two independent research groups redressing the lack of microalgae metabolic reconstructions at genome-scale and are considered an important achievement in algae systems biology research.

4.1 AlgaGEM Applications

AlgaGEM captures key metabolic features of *Chlamydomonas reinhardtii*. This framework was used for example to test cell optimality and maximum bioproduct performance under different cell growth. The model was evaluated through the estimation of the flux distributions in three growth conditions: autotrophic, heterotrophic and mixotrophic. Two main optimality criteria were tested and the flux distributions were then determined using linear programming as follow:

1. Minimizing energy and carbon source

$$\begin{aligned} & \text{photon or acetate utilization} \\ \text{minimize} \quad & Sv = 0 \\ \text{subject to} \quad & v_{biomass} = b \\ & v_{\min} \le v \le v_{\max} \end{aligned}$$

i.e., the distributions that minimize the use of the key energy substrate (photons or acetate), while achieving a specified growth rate.

Using efficient resource utilisation as the optimality criterion, AlgaGEM predicted phosphoglycolate catabolism in algae. Photorespiration of *C. reinhardtii* deviates from the classical plant photorespiration in that instead of oxidizing glycolate to glyoxylate via glycolate oxidase in peroxisomes, *C. reinhardtii* and many other microalgae utilize glycolate in mitochondria. AlgaGEM accurately predicts that algae will catabolise rather than recycle phosphoglycolate, if sufficient oxygen is available and energy is needed, or alternatively secrete glycolate to the environment, as observed (Spencer and Togasaki 1981; Moroney et al. 1986; Stabenau and Winkler 2005).

2. Maximizing bio-product: H_2

Chlamydomonas is capable of producing H_2 but the strains need to be metabolicaly engineered in order to increase its production for biofuel applications. AlgaGEM has been used to test the network capacity to maximize H_2 in *Chlamydomonas* under different growth conditions. In silico flux predictions were then determined as follows:

$$\begin{aligned} & H_2 \text{ synthesis} \\ \text{maximize} \quad & Sv = 0 \\ \text{subject to} \quad & v_{biomass} = b \\ & v_{\min} \le v \le v_{\max} \end{aligned}$$

i.e., the flux distributions that maximize H_2 synthesis, while achieving a specified growth rate under autotrophic, mixotrophic or heterotrophic condition.

Under heterotrophic condition, the model predicted the physiological pathways used for H_2 production in *Chlamydomonas* under dark condition. AlgaGEM also predicted increased hydrogen production when cyclic electron flow is disrupted under mixotrophic conditions. The metabolic changes highlited by the simulations to increase H_2 yield show agreement with evidences observed in a stage transition mutant, Stm6 (Kruse et al. 2005). Moreover, the model suggests other potential metabolic targets that need further experimental validation.

4.2 iRC1080 Applications

iRC1080 is a comprehensive genome-scale metabolic reconstruction for *Chlamydomonas reinhardtii*, validated in content and function, and its application for detailed modeling of diverse light sources (Chang et al. 2011). In particular, iRC1080 model has been used to evaluate light source efficiency. By immplementing light-regulated constraints and basic environmental exchange constraints iRC1080 model simulates photoautotrophic, heterotrophic, and mixotrophic growth.

Given the importance of lipid metabolism in biofuel production, iRC1080 was reconstructed accounting for all lipid metabolic pathways supported by evidence in the literature and genome functional annotation. The capacity of iRC1080 as a knowledgebase was demonstrated through analysis of lipid metabolism to generate novel hypotheses about latent metabolic pathways resulting from algal evolution.

The models derived from iRC1080 were used as a platform for prediction of phenotypic outcomes of system perturbations, light source evaluation and design, and genetic engineering design for production of biofuels and other commodity chemicals. This platform was used, for example, to the design of an energetically efficient light source for algae growth. The results are of significant interest to the metabolic engineering and bioreactor-design communities as it demonstrates that the model is capable of accurately predicting light source efficiencies in terms of a metabolic objective.

4.3 In Silico Fluxomics Platform

Both algae genome-scale metabolic reconstructions discussed in this chapter have been explored using a well-established platform to interrogate the metabolic reconstruction is the constraint-based reconstruction and analysis (COBRA) methods (Schellenberger et al. 2011). COBRA methods have been used in many researches over the past decade, which characterize genome-scale properties of metabolic networks and their phenotypic states. COBRA toolbox (Schellenberger et al. 2011) provides access to a variety of common methods used in metabolic engineering including flux balance analysis (FBA), flux variability analysis (FVA), method of minimum metabolic adjustments (MOMA) and others constraint-based modelling approaches into algorithms in one mathematical package.

AlgaGEM and iRC1080 are accessible and readily adopted by the algae research community. Both reconstructions are available in the systems biology markup language (SBML) and are readable by COBRA Toolbox – a MATLAB package for implementing COBRA methods. AlgaGEM-COBRA (de Oliveira Dal'Molin et al. 2011) can be downloaded from http://web.aibn.uq.edu.au/cssb/resources/Genomes.html and iRC1080 model (Chang et al. 2011) can be downloaded from http://www.nature.com/msb/journal/v7/n1/suppinfo/msb201152_S1.html.

5 Concluding Remarks

The *Chlamydomonas* reconstructions represent an important achievment in algae systems biology.

The application of genome-scale models combined with omics technologies promise to be important on the development and hypothesis-driven research in algae biotechnology and metabolic engineering. The examples of in silico flux predictions illustrates the potential of using genome-scale models to explore complex, compartmentalized networks and develop non-trivial hypotheses.

Overall, algae genome-scale modeling is a powerful approach that is accessible and readily adopted.

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Part VI

Applications

Algal Physiology and Large-Scale Outdoor Cultures of Microalgae

Michael A. Borowitzka

1 Introduction

The large-scale culture of microalgae has been the subject of extensive research since the late 1940s (Burlew 1953a; Tamiya 1957) and commercial culture of microalgae for a variety of products has been undertaken for the last 50 years (Borowitzka 2013b, c). Most current commercial large-scale cultures use open air culture systems, especially paddle-wheel driven raceway systems, although there are also a few companies using closed photobioreactors. The number of microalgal species being cultured on a large-scale commercially is small, the major ones being *Arthrospira* (*Spirulina*) *platensis*[1] and *Chlorella* spp., produced mainly for use a nutritional supplements, and *Dunaliella salina and Haematococcus pluvialis* as sources of the carotenoids β-carotene and astaxanthin. On a significantly smaller, but still commercial, scale, species such as *Porphyridium cruentum* are being cultured for specialty products such as polysaccharides and phycobilin pigments (Arad (Malis) and van Moppers 2013), and there is also the very widespread production of microalgae for use in hatcheries for live aquaculture feeds including species such as *Nannochloropsis* spp., *Isochrysis galbana*, *Tisochrysis lutea* (previously known as Tahitian *Isochrysis* T-iso; (Bendif et al. 2013)), *Chaetoceros* spp., *Skeletonema costatum*, *Thalassiosira pseudonana*, *Tetraselmis* spp., *Pavlova lutheri* etc. (Borowitzka 1997; Muller-Fuega 2004). The 'green water' culture of algae for aquaculture is an often overlooked, but major method of algal production (Neori 2011). The culture of algae in high rate oxidation ponds for wastewater treatment is also well established (Craggs et al. 2012, 2013).

In the last decade the potential of the use of microalgae for the production of renewable fuels has reignited interest in the large-scale production of microalgae, including research and development on new species, culture systems and methods, and the search for products other than biofuels with commercialisation potential (Chisti 2007; Borowitzka and Moheimani 2013a; Da Silva et al. 2014). The extremely large scale that is required to produce a commercially meaningful quantity of biofuel as well as the low value of the product continues to present a significant challenge to the eventual commercialization of renewable biofuel from microalgae, despite decades of research and development (Sheehan et al. 1998; Stephens et al. 2010; Chisti 2013). In order to achieve successful exploitation of the potential of microalgae a good understanding of the physiology, biochemistry and ecology of microalgae in large-scale cultures is essential so as to achieve reliable cultures and to optimize both biomass productivity as well as the productivity of the desired final product.

The conditions algal cells experience in large-scale cultures are very different from those experienced in laboratory cultures. Most algal culture studies are done indoors in the laboratory under more-or-less constant conditions; especially constant temperature and irradiance. Furthermore, it is relatively easy to mix small laboratory-scale cultures well by shaking or aeration thus minimising any oxygen, pH, light, temperature, and nutrient gradients. Larger (>100 L culture volume) outdoor cultures, on the other hand, are subjected to diurnal temperature changes and a continually varying light environment. Long-term outdoor cultures are also exposed to seasonal changes in these parameters, especially irradiance and temperature. Furthermore, in very large commercial-scale cultures (≫100,000 L; Table 1) inadequate mixing, due to hydraulic limitations, can lead to significant localised variations in all physical and chemical parameters. As outdoor cultures are generally shallow, the irradiances the algae cells are exposed to can also be very much higher than those

[1]Wherever possible the currently accepted names for species are used. The name used in the paper cited is also indicated. For details of names see chapter "Systematics, Taxonomy and Species Names: Do They Matter?" of this book (Borowitzka 2016).

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Table 1 Main commercial microalgal culture systems currently in use, maximum volume, and algae species cultured (updated from Borowitzka 1999)

Culture system	Algae	Approximate maximum volume (L)[a]	Location
Tanks	Many species (for aquaculture)	1×10^4	World wide
Extensive open ponds	*Dunaliella salina*	1×10^9	Australia
Circular ponds with rotating arm	*Chlorella* spp.	1.5×10^4	Taiwan, Japan, Indonesia
Raceway ponds	*Chlorella* spp. *Arthrospira* spp. *Dunaliella salina*	3×10^4	Israel, Japan, Taiwan, USA, Thailand, China, India, Vietnam
Shallow cascade system	*Chlorella* spp.	3×10^4	Czech Republic, Bulgaria
Large bags	Many species (used for aquaculture)	1×10^3	World wide
Internally lit reactors	*Haematococcus pluvialis*	$>10^3$	Sweden
Fermenters (heterotrophic)	*Crypthecodinium cohnii*, *Chlorella* spp.	$>10^3$	USA, Japan, Taiwan
Tubular photobioreactors	*Chlorella* spp. *Haematococcus pluvialis*	$>10^4$	Germany, Israel, China
Two-stage system (closed reactor and paddle wheel ponds)	*Haematococcus pluvialis*	3×10^4	USA

[a]These are order of magnitude estimates only

experienced by the cells in the laboratory. Laboratory cultures also can be carried out axenically, but large-scale axenic culture is impossible for periods of time greater than a few days, even in closed photobioreactors. Only short-term axenic batch cultivation is possible on a large scale and this is economically feasible only for high value products as, for example, the production of long-chain polyunsaturated fatty acids EPA and DHA using the heterotrophic thraustrochytrid algae *Schizochytrium*, *Crypthecodinium* and *Ulkenia* (Barclay et al. 2010; Wynn et al. 2010).

This chapter considers the main factors which affect the growth, productivity and composition of microalgae with a focus on large commercial-scale culture, especially outdoor open pond cultures.

2 Light

The quantity and quality of light available to the algae are recognised as the single main factors limiting the productivity of algal mass cultures outdoors (Burlew 1953b; Richmond 1996; Borowitzka 1998). However the impact of irradiance on algal productivity is not straightforward, nor is the interaction of irradiance with other environmental factors such as temperature. Algal cultures growing outdoors in open ponds or closed photobioreactors are exposed to very high irradiances which vary throughout the day and with season. Apart from the diurnal variation in irradiance there is also shorter-term variation due to clouds and, in open ponds, due to reflection and focusing of the light by surface waves, both of which vary with sun angle.

The cell density of the cultures has a major impact on the light environment experienced by the algal cells. In the dense cultures generally used in shallow ponds and photobioreactors the irradiance decreases rapidly from the surface of the culture, so that cells deeper within the culture may actually be in the dark (Oswald 1988a; Janssen et al. 2003) (Fig. 1). Mixing circulates the algal cells from these dark/low light regions to the high-light surface layer so that the cells are exposed to an alternating light/dark pattern the frequency of which is determined by the type of reactor (pond, flat plate, tubular etc.), the mixing system used and the hydraulic conditions of the culture system. In large-scale raceway ponds vertical mixing is poor except in the vicinity of the paddle wheels (Prussi et al. 2014). Vertical mixing can, however, be improved by the addition of foils or 'wings' in the channels (Voleti 2012). Similarly, in tubular photobioreactors flow in the long straight tubes approaches laminar flow with little transfer of the cells from the center of the tubes to the surface unless turbulence generating devices are included (Muller-Feuga et al. 2003; Zhang et al. 2013). In the curved tubes of helical photobioreactors such as the Biocoil there is radial flow from the perimeter of the tubes to the center (Hall et al. 2003; personal observations).

The photosynthetic productivity of algal cultures at any given irradiance is sensitive to the concentration of cells, a phenomenon first demonstrated by Myers and Graham (1958) with *Chlorella ellipsoidea* (now known as *Chloroidium ellipsoideum*) and later again by Richmond and Vonshak (1978) and Hu et al. (1998) with *Arthrospira* (*Spirulina*) *platensis* and Hu and Richmond (1994) with *Isochrysis galbana*. In relatively dense cultures at constant irradiance there is an optimum cell density (OCD; cells mL^{-1}) where productivity reaches a maximum value; if the cell density is increased further the productivity starts to decline because of increased self-shading by the algae, which reduces the amount of light available to individual cells. Increased irradiance will increase the OCD. Mixing/circulation of the medium will expose the algal cells to alternatively higher and lower irradiances. However, the frequency of

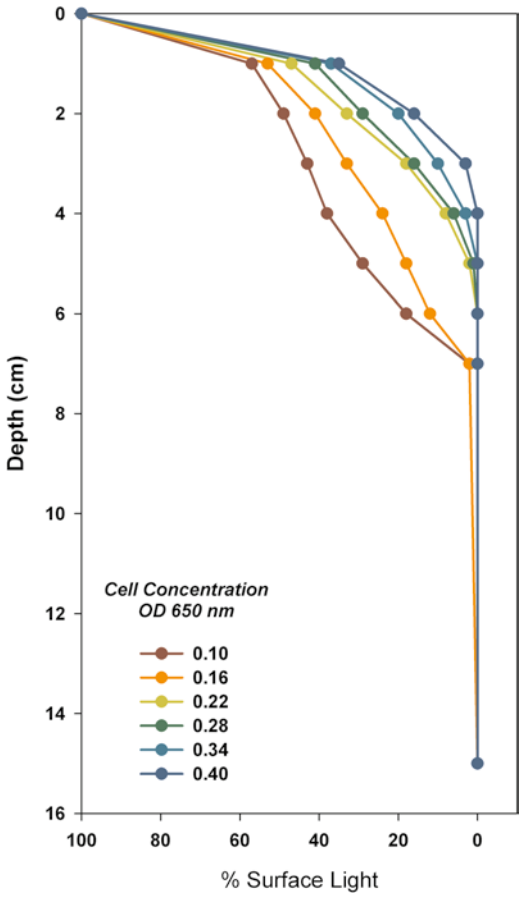

Fig. 1 Light distribution in a shallow raceway pond with respect to cell density as measured by OD_{650}. 100 % incident light = 2300 µmol photons m^{-2} s^{-1} (Based on data from Richmond et al. (1980))

these changes in irradiance in large-scale cultures is sufficiently slow so that, for practical purposes, it is the average irradiance that the cells are exposed to which affects their photosynthetic rate. The optimum average irradiance for photosynthesis can be estimated from the photosynthesis vs irradiance (P vs E) curve.

Culture management strategies also impact on the light environment. For example, if the algae are grown as semi-continuous cultures and the partial harvesting and subsequent topping-up of the culture is done during the day, the effective dilution of the culture exposes of the remaining cells in the pond/bioreactor to markedly increased irradiances. This increased irradiance received by the cells, in turn, can lead to photoinhibition and even photodamage (see later), resulting in a reduction in productivity. Only when the cell density subsequently has increased due to growth, so that photoinhibition/photodamage is reduced, will the culture productivity again increase (Isdepsky 2015).

The important role of the OCD in maximising the productivity of cultures outdoors has been discussed extensively by Richmond (Richmond and Zou 1999; Richmond 2000, 2013). Note that other researchers use alternative, and generally equivalent, parameters such as the optimum chlorophyll concentration (OCC; in g chlorophyll-*a* m^{-3}) (Sukenik et al. 1991) or the optimum areal density (OAD; in g dry weight m^{-2}) (Soeder 1980; Hartig et al. 1988).

This OCD phenomenon can be demonstrated by simple modelling. The light distribution in an algal pond can be described from the Beer-Lambert law by the following expression:

$$E_d = E_0 \exp(-kd) \tag{1}$$

where E_0 is the irradiance at the surface, E_d is the irradiance at depth d, and k is the extinction coefficient. This equation can then be used to calculate the average irradiance in the pond for any given E_0 and k, where the value of k is a function of the cell density in the culture.

In the model used here, the photosynthetic rate of the algal culture serves as a proxy for the rate of new biomass production and can be described by a standard photosynthesis vs irradiance (*P vs E*) model. Here I use the hyperbolic tangent model (Jassby and Platt 1976) without any photoinhibition term, for simplicity:

$$P = P_{\max} \tanh(\alpha E / P_{\max}) \tag{2}$$

where P = the photosynthetic rate (g C $cell^{-1}$ $time^{-1}$), P_{max} = the maximum photosynthetic rate when photosynthesis is light saturated, α is the initial slope of the *P vs E* curve (g C (µmol photons m^{-2} $s^{-1})^{-1}$), and E is the irradiance (µmol photons m^{-2} s^{-1}).

The productivity of the culture (g C $time^{-1}$ $volume^{-1}$) is a function of the cell density (cells $volume^{-1}$) times the rate of new biomass production (B_{new};). Here I assume that $B_{new} = P$:

$$Productivity = Cell\ Density \times B_{new} \tag{3}$$

Figure 2 shows the results of this model for a 20 cm deep pond using three different surface irradiances (E_0) with the higher irradiances being 2× and 3× the lowest irradiance. In all cases the P_{max} and α (Eq. 2) were constant and the P_{max} was reached at an irradiance less than the surface irradiance. The shape and relationship of the productivity curves show a maximum at a particular cell density (i.e. the optimum cell density, OCD) and this optimum cell density increases with increasing irradiance, similar to what has been observed in several studies (Myers and Graham 1958; Hu et al. 1998). The inclusion of a photoinhibition term (potentially) would reduce further the productivities at the lowest cell densities at high irradiances when photoinhibition would be expected to be greatest.

The above model assumes efficient uniform mixing with the algae being rapidly circulated between the dark and the high light zones of the pond/reactor. The effect of mixing on the areal productivity in raceway ponds has been well

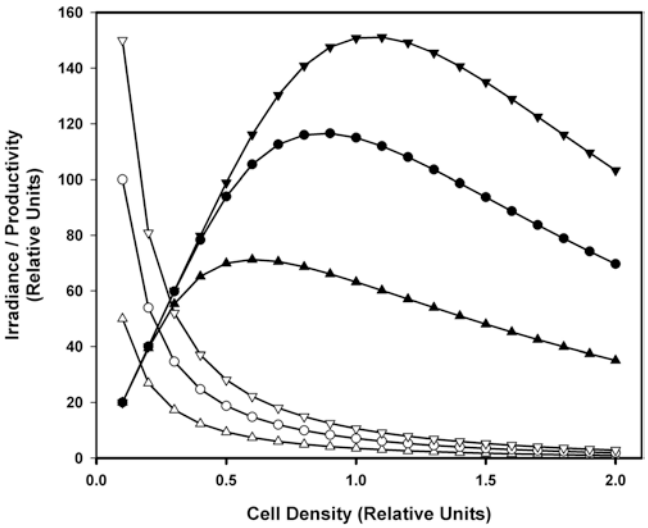

Fig. 2 Illustration of the relationship between cell density, the average irradiance, and the biomass productivity in a 20 cm deep pond. Average irradiance in the pond (*open symbols*) was calculated using Eq. 1 where the *triangle* is the lowest surface irradiance, the *circle* is 2× the lowest surface irradiance, and the *inverted triangle* is 3× the lowest surface irradiance. Biomass productivity (*closed symbols*) was calculated using Eqs. 2 and 3 (*closed symbols*) at the three different irradiances (the three different symbols represent the three different surface irradiances used to calculate the average irradiance in the pond). For the purposes of this model relative units have been used

demonstrated by Richmond and Vonshak (1978) and Richmond and Grobbelaar (1986) for open pond cultures of *Arthrospira*. They found that increasing the flow rate (mixing) increased both the productivity and the OCD. This is particularly evident in large ponds, but also occurs in flat plate reactors at high cell densities (Hu et al. 1996a, b). The magnitude of mixing that can be applied however is potentially limited by two factors: (1) the shear sensitivity of the algae species and (2) the cost of the energy required to provide the mixing. Many species of algae are shear sensitive (Hondzo and Lyn 1999; Leupold et al. 2013), especially species with a thin cell wall or no cell wall (e.g. *Dunaliella*) and species with flagella (Silva et al. 1987; Thomas and Gibson 1990; Gudin and Chaumont 1991; Moheimani et al. 2011). For example, Cózar and Echevarría (2005) found that in high turbulence microcosms the growth of flagellates was markedly reduced compared to diatoms, whereas at low turbulence the flagellates grew well. Diatoms are generally tolerant of high turbulence, with the exception of very large species such as *Ditylum brightwellii* and many chain-forming species (personal observations). However, the effects of turbulence and shear on most algae other than dinoflagellates are still little understood (Berdalet and Estrada 2006). In closed photobioreactors using airlifts, physical interaction between air bubbles and the cells as well as micro eddies can also cause cell damage (Contreras et al. 1998; Garcıa Camacho et al. 2000; Sánchez Mirón et al. 2003), thus reducing productivity. Flagellae are easily sheared-off and the cells then must expend significant energy to resynthesize new flagellae (Reize and Melkonian 1987; Johnson and Rosenbaum 1993), leaving less energy available for growth. In dinoflagellates exposure to damaging levels of shear rate has also been found lead to the production of reactive oxygen species and elevated levels of lipoperoxides (Rodríguez et al. 2009).

The importance of the OCD in maximizing productivity has also been demonstrated in closed photobioreactors (Chen et al. 2011; Michels et al. 2014).

An exception to the above cell density-productivity relationship is algae grown in the sloping thin-layer systems developed by Setlík and coworkers (1970). The current version of these reactors has a culture depth of 6–8 mm with the culture area inclined at 1.6–1.7 % (Doucha and Lívanský 1999, 2006). Doucha and Lívanský (2014) report that, while the optimum areal density for maximal productivity in raceway ponds for *Chlorella* cultures was 38–41 g dry wt m^{-2} (Grobbelaar et al. 1990) and 40–45 g dry wt m^{-2} for *Scenedesmus* (Hartig et al. 1988), they did not observe any decrease in productivity in thin-layer cultures of *Chlorella* up to a cell concentration of 240–300 g dry wt m^{-2}. This is most likely because of the thin culture layer and the high frequency (2.2–2.6 Hz) of the light/dark periods the algae cells experience (Doucha and Lívanský 2014).

The increased productivity of cells exposed to intermittent light/dark cycles observed in several studies often has been attributed to the 'flashing light' effect observed by Kok (1953). This effect has been observed under very short light/dark cycles from less than 40 μs to 1 s (Kok 1953; Phillips and Myers 1954; Terry 1986; Matthijs et al. 1996; Nedbal et al. 1996), and is more pronounced at the shorter cycles in this range. However, for this effect to be observed, the dark period should be three to ten times longer than the light period. At solar irradiation intensities the optimum dark period is about 50 ms (Iluz et al. 2012; Sforza et al. 2012). Thus, if the high light flash duration is very short (<1 ms), it can excite all the 'open' (reduced donor side and oxidised acceptor side) reaction centres. The subsequent dark period then allows the non-photochemical reactions to proceed and, if the duration is long enough, allows for the reaction centres to return to the 'open' state. If the flash duration is long (>1 ms) the electron transfer capacity of the plastoquinone (PQ) pool is exceeded, and then a part of the excitation energy is dissipated as fluorescence or heat. In practical terms, however, the very short flash durations required to achieve this 'flashing light' effect cannot be created in large commercial-scale culture systems.

In outdoor cultures the pattern of intermittent light experienced by the cells depends on the turbulence of the flowing pond culture or in the photobioreactor. Vertical mixing in large raceway ponds is relatively poor (Mendoza et al. 2013; Hreiz et al. 2014; Prussi et al. 2014). Laws et al. (1983)

demonstrated the value of turbulence in enhancing vertical mixing and photosynthetic conversion efficiency by placing aerofoil-like devices in flowing cultures in a flume-type culture system. These foils created vortices of 0.5–1 Hz at a flow rate of 30 cm s^{-1} resulting in an increase in the photosynthetic conversion efficiency from an average of 3.7 % to up to 10 %. However, Grobbelaar et al (1996) showed only minimal enhancement at light/dark cycles of ≤1 Hz. Similarly, several studies in both natural environments and in mass cultures of algae observed no enhancement due to intermittent light/dark cycles (Jewson and Wood 1975; Grobbelaar 1989; Janssen et al. 1999). Laws and Berning (1991) later provided an alternative explanation for the increased productivity observed by them in flumes with foils, suggesting that it was due to re-suspension of settled cells and the breaking down of nutrient gradients rather than the 'flashing-light' effect (see also Grobbelaar 1989).

In large commercial-scale raceway ponds, which usually have a culture depth of 20–30 cm, the flow generally approaches laminar flow in large parts of the channel and the duration of the light/dark cycles due to vertical mixing is much too long for the flashing light effect to take place. On the other hand, in plate-type and tubular photobioreactors the light path generally is too short for all the light to be absorbed by the culture at the normal cell densities used and, therefore, the cells do not experience significant light/dark fluctuations caused by turbulence. However, if the circulation system of the photobioreactor has a 'dark' component such as an airlift and/or degasser, the cells will be exposed to a light/dark light regime with a cycle time of the order of minutes. The longer the culture spends in the 'dark' compartment of the photobioreactor the more productivity is reduced.

Too much light, however, can lead to photoinhibition and photodamage, thus resulting in reduced productivity. *Arthrospira*(*Spirulina*) species are particularly sensitive to high irradiances and Vonshak and Guy (1992) found that the productivity of dense *Arthrospira platensis* cultures grown in outdoor raceways could be enhanced by shading the ponds. The shading of the cultures prevented their exposure to full solar radiation and thus prevented photoinhibition. Using chlorophyll *a* variable fluorescence it was found that F_v/F_m, the maximum PSII photochemical efficiency (quantum yield) of open photosystem II reaction centers, showed a marked decline during the morning reaching its minimum value at noon, after which it partially recovered during the afternoon (Vonshak 1997). When the pond was shaded so as to reduce the irradiance by 25 %, the degree of decline in the F_v/F_m was significantly reduced. Comparison of diurnal changes in F_v/F_m in cultures of different cell densities also showed that dilution of the culture resulted in a greater morning decline in F_v/F_m. As discussed above, better mixing improves the light regime in a pond by reducing laminar flow and enhancing the vertical mixing of the culture and Vonshak (1997) also demonstrated that greater mixing reduced the diurnal decline in F_v/F_m. This phenomenon of the decline in F_v/F_m has been interpreted as photoinhibition due to the high irradiance, and has now been demonstrated as a general phenomenon in dense outdoor algae cultures, even when grown at or near the OCD (Hu et al. 1996b; Vonshak et al. 2001; Masojídek et al. 2003, 2011; Kromkamp et al. 2009). It has also been observed in synchronised cultures of the cyanobacterium, *Prochlorococcus*, under a sinusoidal light field (Bruyant et al. 2005). This photoinhibition, especially around noon when the irradiance is highest, reduces the photosynthetic rate (Rebolloso Fuentes et al. 1999) and thus the daily productivity of the culture.

Grobbelaar (2007) studied the effects of high diurnal irradiance in more detail in raceway ponds of *A. platensis*. He observed a distinct noon depression in both F_v/F_m and the maximum PSII photochemical efficiency in actinic light,[2] F_v'/F_m' or $\Delta F/F_m'$, as had Vonshak and Guy (1992), Vonshak and Torzillo (2004) and Torzillo et al. (2012). Non-photochemical quenching (qN) showed a slight midday increase, which was more evident in a less dense culture, and photochemical quenching (qP) showed no specific trend over the day. The difference between F_v/F_m and Fv'/F_m' was small in the less dense culture, however in the morning the F_v'/F_m' was significantly less than the F_v/F_m with little difference in the afternoon.

The effects of the diurnal changes in irradiance on algal photosynthesis are influenced predominantly by the:

- Cell density and culture depth (optical cross section), which affect the average irradiance the algal cells are exposed to.
- Turbulence, which affects the rate at which individual cells are alternatively exposed to high and low light
- Nutrient supply including supply of CO_2 and pH changes, which are mainly due to photosynthetic CO_2 uptake (note that the supply of CO_2 can be used to regulate the pH)
- Temperature, which will vary over the day in response to air temperature, direct heating due to sunlight, and cooling by evaporation (in open ponds) as a function of the thermal mass (volume) of the pond or reactor.
- Oxygen concentration, which is a function of O_2 production by photosynthesis, O_2 loss to the atmosphere and O_2 uptake by respiration.

Over a 24 h period these factors change dynamically making it difficult to isolate the impact of any single factor clearly. Figure 3 shows the diurnal variation in irradiance, temperature and O_2 concentration in shallow raceway ponds growing the coccolithophorid haptophyte alga, *Chrysotila*

[2]For definition of fluorescence parameters see Cosgrove and Borowitzka (2011).

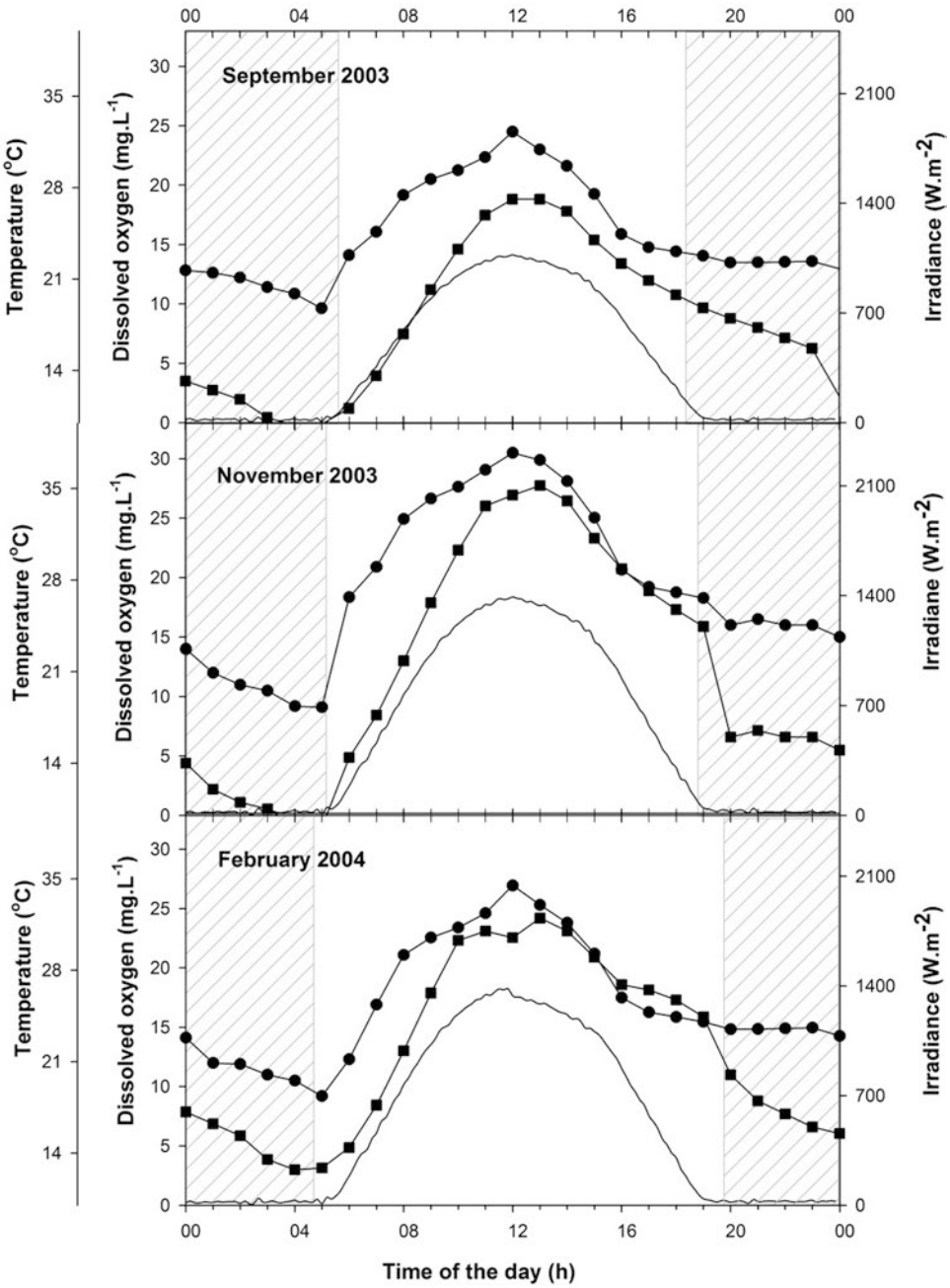

Fig. 3 Diurnal pattern in dissolved oxygen concentration (*circles*), irradiance (*line*), and culture temperature (*squares*) in an outdoor raceway pond culture of the coccolithophorid alga, *Chrysotila* (*Pleurochrysis*) *carterae*, in Perth, Western Australia in winter (September), spring (November) and summer (February). The *shaded areas* show night time (Adapted from Moheimani and Borowitzka (2007))

(*Pleurochrysis*) *carterae*, in Perth, Western Australia, at different times of the year.

The photoinhibition of algal cultures induced by high irradiances is exacerbated by other factors such as sub- and supra-optimal temperatures and high oxygen concentrations (Belay and Fogg 1978); i.e. non-optimal growth conditions. For example, algae cultures generally cool down at night and in the morning the pond temperature is below the optimum temperature for the culture. After sun rise the irradiance increases rapidly, but the increase in the culture temperature lags behind this, especially in open ponds. This means that, in the morning, even relatively low irradiances can result in photoinhibition (Vonshak et al. 2001). When *Arthrospira* was grown in tubular photobioreactors at 25 °C (i.e. about 10 °C below the optimum for the strain used) the F_v/F_m was reduced by about 30 % and F_v'/F_m' decreased by up to 52 % at the same time (Torzillo et al. 1996). By the evening, neither the F_v/F_v nor the F_v'/F_m' had fully recovered to their morning values. This photoinhibition reduced the daily productivity of the culture by 33 % compared to another culture grown at 35 °C. The effect of a low (sub-optimal) morning temperature was studied in another experiment with the

eustigmatophyte, *Monodopsis subterranea* (as *Monodus subterraneus*), by Vonshak et al. (2001). Here they ran two identical raceway cultures where one of the cultures was artificially heated early in the morning and the other was not. Although the two cultures were maintained for most of the day at the same temperature and irradiance, the unheated culture, which was exposed to suboptimal morning temperatures for only a short time, showed a larger decrease in almost all of the photosynthetic parameters. Comparison of the diel changes in maximal photochemical efficiency of PSII, the maximum relative electron transport rate (rETR), and photochemical and non-photochemical chlorophyll fluorescence quenching, led Vonshak et al (2001) to conclude that even a relatively short exposure to suboptimal morning temperatures induced photoinhibitory damage which could not be repaired fully during the day. The effect of this damage also was reflected in the 60 % increase in productivity in the heated culture compared to the non-heated one. A similar improvement on daily productivity as a result of heating the pond early in the morning was also observed by Moheimani and Borowitzka (2007) with the haptophyte, *Chrysotila* (*Pleurochrysis*) *carterae*. Increasing the pond temperature by only 3–5 °C in the morning in winter increased the biomass productivity by 11–21 % and the lipid productivity by 14–25 %. Similarly, heating for 1 h in the morning also markedly increased the productivity of a culture of mixed species of microalgae in hanging bag reactors, whereas heating for the whole day had no additional benefit (Esterhuizen-Londt and Zeelie 2013).

The increased susceptibility to photoinhibition at suboptimal temperatures can be caused by several mechanisms (Torzillo and Vonshak 2013): (i) low temperature slowing the rate of CO_2 fixation (Badger and Collatz 1977), thus causing the over-reduction of electron transport compounds at a given irradiance; (ii) low temperature inhibiting the scavenging of reactive oxygen species (ROS) which normally protects PSII against photoinhibition; (iii) low temperatures inhibiting the repair of the D1 protein which was degraded during photoinhibition (Murata et al. 2007).

Some algae have been shown to acclimate to low temperatures. Vonshak and Novoplansky (2008) studied two strains of *Arthrospira platensis* (strains M2 and Kenya) which had similar growth rates at 30 °C, the temperature considered optimal for this species. The Kenya strain was better able to acclimate to low temperature (15 °C) by down-regulating its photosynthetic activity by (i) decreasing antenna size; (ii) decreasing the reaction center density, and (iii) increasing energy dissipation. There were also differences in the antioxidant enzyme activities between these two strains (Chien and Vonshak 2011). The Kenya strain had a higher superoxide dismutase activity than the M2 strain, and this activity increased with acclimation time. Ascorbate-dependent peroxidase activity of the Kenya strain declined when transferred to the lower temperature, while the peroxidase activity of the M2 strain initially decreased but then increased with time. Similar observations have been made by Gacheva et al. (2013) with the cyanobacterium, *Gloeocapsa* sp. These findings suggest that strains with the ability to increase antioxidant enzyme activities in response to low temperature stress may be better able to respond to low-temperature induced photoinhibition. Such strains might be better suited to culture over the whole year.

Reactive oxygen species (ROS) are inevitable by-products of photosynthesis. The superoxide anion radical ($\bullet O_2^-$), H_2O_2 and hydroxy radical (•OH) are produced as a result of photosynthetic transport of electrons, while $^1O_2$ is produced as the result of the transfer of excitation energy (Asada 1999). Furthermore, the generation of ROS is enhanced when the photosynthetic apparatus absorbs excess light in conjunction with other stressors such as nutrient limitation (Asada 1994). The enhanced photoinhibition caused by ROS is not to be due to photodamage to PSII, but rather due to a reduction in the rate of repair of photodamaged PSII (Murata et al. 2007, 2012; Nishiyama et al. 2011). ROS apparently primarily affect the de novo synthesis of the D1 protein at the translation elongation step, thus inhibiting the repair of PSII (Nishiyama et al. 2004). Similarly, Inue et al. (2011) used a mutant of *Synechococcus* which was unable to synthesise α-tocopherol, a particularly efficient scavenger of $^1O_2$ found both in the chloroplast envelope and the thylakoids, to study the role of α-tocopherol. They found that the activity of PSII in mutant cells was more sensitive to inactivation by strong light compared to wild-type cells. The rate of photodamage to PSII did not differ between the two strains, but in the mutant cells the de novo synthesis of various proteins, including the D1 protein, was inhibited thus suppressing the repair of PSII.

2.1 Changing Antenna Size

One potential way to improve the productivity of algal cultures is to reduce the size of the light-harvesting apparatus and so mitigate the photosynthetic inefficiency associated with the excess absorption of sunlight at the surface of the cultures (Polle et al. 2002; Raven and Ralph 2015). Algae with reduced antenna size absorb less light per cell so that they should be able to tolerate higher irradiances and, furthermore, more light can be transmitted to cells deeper in the culture. This concept was suggested by Benemann (1989) and it was Nakajima and Ueda (1997) who, using phycocyanin-deficient mutants of *Synechocystis* and *Chlorella pyrenoidosa* with reduced chlorophyll content, first demonstrated that reducing the pigment content resulted in greater photosynthesis in dense laboratory cultures. In later studies they further confirmed these findings (Nakajima

and Ueda 1999, 2000). Several studies using either cells with reduced pigment content (Melis et al. 1999), mutants with reduced antenna size (Mussgnug et al. 2007; Bonente et al. 2011; Kwon et al. 2013; Cazzaniga et al. 2014) or transgenic algae with reduced antenna size (Polle et al. 2003; Mitra et al. 2012; Perrine et al. 2012) have further confirmed that this concept works at the laboratory scale.

However, results under outdoor conditions have been mixed. Cazzaniga et al. (2014) produced a truncated antenna mutant (TAM2) of *Chlorella sorokiniana* by UV mutagenesis. The truncated antenna mutant showed a 30 % higher biomass productivity than the wild-type in 7 L outdoor cylindrical hanging bag reactors. On the other hand, de Mooij et al. (2014) compared the performance of four antenna mutants of *Chlamydomonas reinhardtii* which had been created by either UV mutagenesis (strain BF4), RNA interference (strain Stm3LR3, a wall-less strain), or by insertional mutagenesis (strains TLA2 and AS2.2), with a wild-type strain in a small 0.4 L flat-panel airlift photobioreactor illuminated by warm-white LEDs at an incident irradiance of 1500 µmol photons m^{-2} s^{-1} in continuous culture. They found that the highest productivities of the mutant strains were approximately the same as that of the wild-type (1.9 g m^{-2} h^{-1}). Furthermore, the mutant strains exhibited a high sensitivity to abrupt shift in irradiance, indicating a reduced fitness and higher susceptibility to photodamage. The wall-less mutant, Stm3LR3 (Mussgnug et al. 2007), was also more susceptible to shear damage in the photobioreactor. A recent study comparing mutants of *C. reinhardtii* with truncated light harvesting antennae with dark-green mutants with larger antennae size found that the dark green mutants with larger light antennae size were able to grow faster under conditions of increased cell concentration or culture depth, where the average light intensity was lower (Chow and Thung 2015).

Clearly, reduced antenna size alone is not sufficient to produce algae strains with higher productivities in dense outdoor cultures. The production of the reduced antenna size mutants can also result in impaired photoprotection mechanisms induced by antenna complex alterations and/or other unintended side effects of the genetic modifications such as increased respiration (Cazzaniga et al. 2014). These factors can reduce productivity. Melis (2009) and Ort et al. (2011) have proposed the following important criteria that must be met to ensure that truncated antenna mutants are not photosynthetically handicapped in ways other than truncated antenna size: (1) the functional PSII and PSI Chl antenna size should be determined to assess the extent of reduction in antenna size, (2) the number of functional PSII and PSI reaction centres per cell should be determined to ensure that the density of the photosynthetic electron-transport chains remains unaffected, (3) the quantum yield of photosynthesis must remain high to ensure that the efficiency of photosynthesis under limiting light remains unchanged, and (4) the light-saturated rate of photosynthesis should be inversely proportional to the measured Chl antenna size. To this list should be added a high P_{max}/respiration ratio as proposed by Cazzaniga et al. (2014) who found that the productivity of one of their mutant strains of *Chlorella sorokiniana* apparently also had additional mutations which increased its respiration rate thus reducing the productivity of this strain.

2.2 Changing the Light Spectrum

In recent years there have been several suggested ways to improve the economics of algae culture by reducing the energy requirements of the culture system. In open ponds the major energy requirements are for the circulation system (usually paddlewheels) and for pumping the water. For closed photobioreactors the circulation system also is the main consumer of energy, together with the high energy needed for cooling the reactor. However, algae use only part of the photosynthetically active spectrum (PAR; 400–700 nm) for photosynthesis, with red (~660 nm) and blue (~420–470 nm) light being the most effective. The exact spectral requirements of algae depend on the pigment composition of their light-harvesting complexes in their thylakoids (see chapter "Photosynthesis and Light Harvesting in Algae" of this book, (Larkum 2016)). It has, therefore, been suggested that it may be possible to use only part of the available daylight solar spectrum for algal growth and to use the rest of the available spectrum to generate electricity using solar panels placed above the culture and which selectively utilize only part of the spectrum allowing the rest of the spectrum to pass through the panels to the algal culture below (Moheimani and Parlevliet 2013). Alternatively, if artificial lighting is to be used then the light source becomes a major energy consumer. For this scenario it has been proposed to use light emitting diodes (LEDs) as the artificial light source for indoor photobioreactors rather than fluorescent lamps, thus reducing the power requirements for the artificial light source (Schulze et al. 2014). For both of these approaches the spectral requirements for the algae, and not just the total light energy available, are of importance.

Different algal phyla have different pigment systems and therefore also have different light absorption spectra (Millie et al. 2002). The absorption spectra alone are insufficient to predict actual photosynthesis as not all of the photons absorbed are used in photosynthesis ultimately to fix CO_2 and produce organic carbon compounds leading to new biomass productivity (Suggett et al. 2011). For example, the excitation energy of some of the photons absorbed by PSI may be dissipated as heat or re-emitted as fluorescence (Baker 2008). Similarly, some of the absorbed energy may lead to oxygen evolution that is internally recycled via a number of alternative photochemical reactions such as photorespiration or the Mehler reaction (Suggett et al. 2011).

Other electron sinks within the photosynthetic electron chain may also divert energy from O_2 evolution and carbon fixation to functions such as nitrogen assimilation (Holmes et al. 1989). Thus, it is useful to determine the photosynthetic action spectra of the algae when screening algae for the effects of specific wavelengths to identify the wavelengths which are used most effectively in photosynthesis (e.g. Tamburic et al. 2014). Simmer et al. (1994) provide a practical method for evaluating different light sources for their suitability for algae culture.

Specific wavelengths of light affect not only photosynthesis, but also other aspects of algal metabolism and behavior. Algae, including the microalgae, have been shown to contain a diverse range of photoreceptors and light-modulated signaling pathways such as the blue light receptor phototrophin which appears to have a role in gametogenesis and zygote germination in *Chlamydomonas* (Huang and Beck 2003). For example, Abiusi et al. (2014) observed that, in cultures of *Tetraselmis gracilis* grown under red light, almost all of the cells were motile swarmers, whereas under white, green and blue light encysted cells predominated. Similarly, the CrCRYp cryptochrome in *Chlamydomonas* appears to be involved in light-dependent gene expression, while the PtCPF1 cryptochrome in *Phaeodactylum* is involved both in the repair of UV damage and photoprotection by affecting DNA repair and transcription regulation (Coesel et al. 2009; De Riso et al. 2009). A recent comprehensive review of algal photoreceptors and their function provides a detailed summary of our current state of knowledge (Kianianmomeni and Hallmann 2014). The function of these photoreceptors can be very complex and can be cell type specific. For example, the green alga *Volvox carteri* has no less than 13 photoreceptors which are mostly expressed in a cell-type specific manner. Kianianmomeni (2014) found that in response to different light qualities, there were distinct changes in transcript accumulation of genes encoding proteins involved in chlorophyll and carotenoid biosynthesis, light-harvesting complexes, circadian clock and cell cycle control. Blue light tended to be effective in the accumulation of transcripts in the somatic cells; whereas red light led to the accumulation of transcripts predominantly in the reproductive cells. Blue light also induced marked accumulation of two components of circadian rhythms only in the somatic cells, indicating that these clock-relevant components are affected by blue light in a cell-type specific manner. Photosynthesis associated genes were also distinctly regulated among cell types by different light qualities.

There have been a number of studies of the effects of different colour light sources on the growth and photosynthesis of microalgae. Although generalisations can be made, comparison of the results of these studies is complicated by the different methodologies used and the different band-widths and emission peaks of the light sources used in the studies (Schulze et al. 2014). For example, the blue-green InGaN semiconductor used in LEDs emits in the range of 365–550 nm, whereas the orange-red emitting AlGaInP diode emits in the range of 560–650 nm, and the red-infrared GaAlAs chip emits in the 630–940 nm range. White LEDs are obtained by combining different LED chips in colour-mixed LEDs (cm-LEDs) or by coating single blue chips with a photon-converting layer in phosphor-converted LEDs (Pc-LEDs). Most high power LED chips are built up in heterostructures comprising more than one semiconductor material, and the coatings which modify the emission spectrum also vary. Thus it is possible to have red LEDs with different maximum emission wavelengths (e.g. AlGaInP with $\lambda_{max} = 625$ nm, or GaAlAs with $\lambda_{max} = 660$ nm). Unfortunately, several studies only consider the λ_{max} and not the band width of the light sources thus limiting the value of their conclusions (e.g., Lababpour et al. 2005; Das et al. 2011; Kim et al. 2014).

Cyanobacteria (*Arthrospira*, *Synechococcus*, *Synechocystis*) grow better under red light compared to blue or green light (Takano et al. 1995; Wang et al. 2007). On the other hand, the eukaryotic microalgae generally show faster growth under blue light than either red or white light at the same quantum fluxes; e.g. the chlorophytes *Dunaliella tertiolecta* (Wallen and Geen 1971), *Dunaliella salina* (Gorai et al. 2014), *Botryococcus braunii* (Baba et al. 2012), the haptophyte *Isochrysis galbana* (Gorai et al. 2014), the eustigmatophyte *Nannochloropsis* sp. (Das et al. 2011), the diatoms *Thalassiosira pseudonana* (as *Cyclotella nana*) (Wallen and Geen 1971), *Chaetoceros gracilis* (Gorai et al. 2014), *Haslea ostrearia* (Mouget et al. 2004), and the dinoflagellate *Heterocapsa circularisquama* (Gorai et al. 2014). It should be noted however, that this enhancement under blue light is often only observed at low, non-saturating, irradiances (Mouget et al. 2005; Gorai et al. 2014) and this is probably due to blue light (~414 nm) being absorbed more efficiently and directed to PSII more effectively than red light (~679 nm) at light intensities below the photosaturation limit (Tamburic et al. 2014). On the other hand, the rhodophycean unicell *Porphyridium cruentum* grew equally well under red or blue light (You and Barnett 2004). Interestingly, Abiusi et al. (2014) recently also found that *Tetraselmis suecica* grew equally well in either white or red light supplied by LEDs, but that growth was inhibited by blue and green light. It is unknown whether there are any long-term effects of growth under monochromatic light, as almost all studies have been short term. However, in the open ocean phytoplankton in deeper waters grow in a mainly blue light environment (Jeffrey 1984), and one long term (5 months) culture study of *Arthrospira* grown under red light showed no long-term effects (Farges et al. 2009).

The quality of the available light also can affect the composition of the cell, although it is generally unclear whether this is due to changes in growth rates induced by the different light sources or due to other light-mediated metabolic

processes. For example, blue light reduced the growth of *Haematococcus pluvialis*, but increased astaxanthin formation (Katsuda et al. 2004). Lababpour et al. (2005) have used this property of blue light to develop a two-phase batch culture process wherein the biomass is first generated under white light and then astaxanthin synthesis is stimulated under blue light. In the diatom, *Haslea ostrearia*, production of the non-photosynthetic blue pigment, mareninne, is also stimulated by blue light (Mouget et al. 2005). Blue light has been shown to be essential for high light photoacclimation and photoprotection in the diatom *Phaeodactylum tricornutum* (Schellenberger Costa et al. 2013).

The information available to date clearly shows that it is possible to culture microalgae under largely monochromatic light with high biomass productivities, thus reducing the energy costs when using an artificial light source. Alternatively, the culture could be covered or surrounded by solar panels which transmit only those wavelengths of the incoming light to the algal culture which are required for algae growth, while converting the rest of the spectrum to electricity (Moheimani and Parlevliet 2013). Another option is to use photobioreactors where the wall material is covered with a spectrum-shifting layer which converts part of the non-usable solar spectrum to photosynthetically active wavelengths, thus increasing the irradiance available to the algal culture (Delavari Amrei et al. 2014).

An alternate possibility for using more of the available solar irradiation is to extend the wavelengths used by the algae to capture light for photosynthesis (Larkum et al. 2012). For example, Blankenship et al. (2011) have suggested that wavelengths longer than 700 nm could be used by genetically engineering a bacteriochlorophyll *b*-based system into an oxygenic photosynthetic organism, replacing PSI with what they call RC1. Although this is an interesting proposal, Raven and Ralph (2015) have pointed out some significant issues with such a proposal. However, there are some species of algae which absorb in the far-red (i.e. >700 nm). These include the green alga *Ostreobium* (Halldal 1968; Wilhelm and Jakob 2006) and the chlorophyll *d*-containing cyanobacterium *Acaryochloris marina* (Chen et al. 2002; Duxbury et al. 2009). Attempts have been made to clone the gene for chlorophyll *d*-binding light harvesting protein from *A. marina* into the cyanobacterium *Synechococcus* sp. PCC6803 (Yang et al. 2010) and such an approach may eventually lead to other algae with an extended photosynthetic spectrum.

2.3 Cell Size, Photosynthesis and Growth

An interesting finding of studies of phytoplankton is the relationship between cell size and the photosynthetic rate. These findings potentially have implications for the selection of highly productive species for outdoor culture. Many studies have found a general allometric pattern with an inverse relationship between cell size and growth as well as biomass-specific metabolic rates (see reviews by: Geider et al. 1986; Beardall et al. 2009; Finkel et al. 2010). Recently, López-Sandoval et al. (2014) have found that rather than a linear relationship between cell size (volume) and photosynthetic rate in log-log plots, the data were better represented by a quadratic model. The unimodal nature of the relationship between cell size and photosynthetic rate is even more evident if the photosynthetic carbon fixation data are divided by the cell biomass (i.e. the biomass-specific photosynthetic rate). Furthermore, and probably not surprisingly, the specific growth rate also shows the same unimodal pattern. Thus, the highest biomass-specific photosynthetic rate and growth rates are achieved by microalgae of intermediate cell size with cell volumes of the order of about 100 μm^3. Unimodal patterns in growth rate with respect to cell size have also been observed in natural populations of phytoplankton (Bec et al. 2008; Chen and Liu 2010).

López-Sandoval et al. (2014) have suggested that these findings are the results of different constraints on cell metabolism operating at the opposing ends of the cell size range studied (i.e. ~10^{-1}–10^{6} μm^3). The small species had lower carbon to nitrogen ratios so that they were comparatively more nitrogen-rich, and therefore had higher nitrogen requirements (Marañón et al. 2013). Furthermore, as pointed out by Raven (1998), the cell volume occupied by non-scalable components (e.g., membranes and nucleic acids) increases with decreasing cell size, which means that picophytoplankton cells have a more limited amount of space to accommodate the catalysts required for the synthesis of new biomass. In the small to medium size range, biomass-specific photosynthesis would increase with cell size because nitrogen requirements decrease and also because more space becomes available for the enzymes involved in biomass synthesis. In the medium to large size range, metabolism would slow down, in spite of the large nutrient uptake ability of the cells, as a result of the increasingly larger intracellular distances for the transport of resources from the cell membrane (Banavar et al. 2002).

3 Temperature

Temperature influences all metabolic processes and the temperature optimum of a particular algae strain will have a pronounced effect on the achievable productivity of a culture. One important characteristic of the temperature tolerance of microalgae is that the lethal temperature is usually only slightly higher than the optimum temperature (Butterwick et al. 2005; Bernard and Rémond 2012). Figure 4 shows the effect of temperature on the growth of several species of

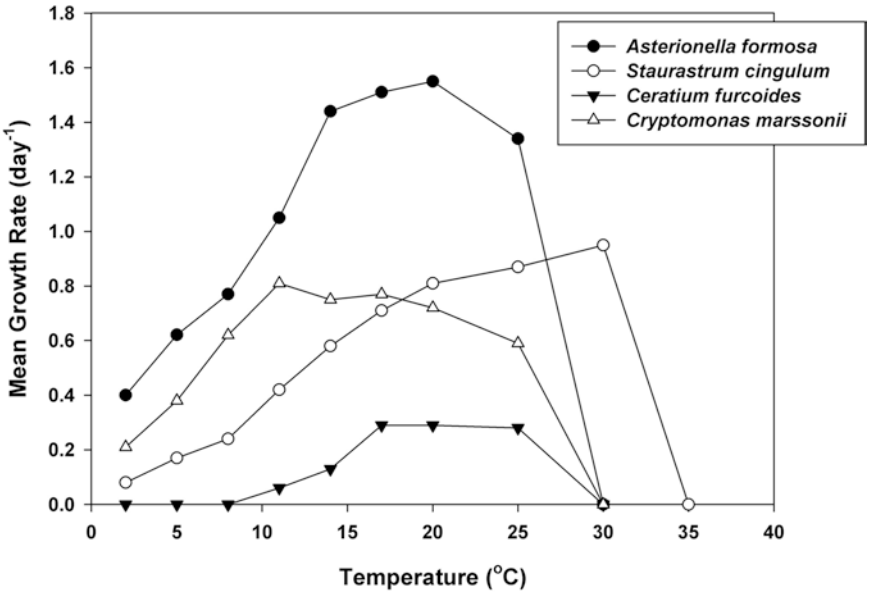

Fig. 4 Growth vs temperature curves for several species of freshwater algae (Data from Butterwick et al. (2005))

freshwater algae. When at their optimum temperature, algae are better able to utilise the available light and are less likely to be significantly photoinhibited (Torzillo and Vonshak 1994; Borowitzka 1998). Figure 5 shows the interaction between the effects of temperature and irradiance on growth rate for three species of algae. *Planktothrix agardhii* (as *Oscillatoria agardhii*) is a planktonic filamentous cyanobacterium typical of cold temperate waters, *Ankistrodesmus falcatus* is a unicellular chlorophyte found in temperate waters, and *Phormidium bohneri* is a benthic filamentous cyanobacterium found in warm to tropical climates. The temperature responses of these algae generally reflect the temperature conditions found in their natural environments. *P. agardhii* and *A. falcatus* have a wide temperature optimum for growth, whereas *P. bohneri* is clearly adapted to high temperatures and at low temperatures (less than ~15 °C) can only grow in low light. Temperature-specific adaptation to the conditions of the environment from which the algae were isolated is a common phenomenon (Oh-hama and Miyachi 1988; Degerlund et al. 2012; Stamenković and Hanelt 2013; Teoh et al. 2013) and it is important to select strains with temperature optima in the temperature range that the large-scale outdoor cultures will be experiencing (Payer et al. 1980). The benefits of using strains adapted to the prevailing temperatures and, potentially, culturing different strains at different times of the year has been well demonstrated for the culture of *Arthrospira* at Earthrise Farms in California, USA (Belay 1997). By using a strain better adapted to lower temperatures the growing season at this production plant could be extended for several months. Similarly, the very broad temperature tolerance of *Dunaliella salina* (Borowitzka and Borowitzka 1988a) means that this species can be grown for the whole year at the production plants in Australia (Borowitzka 2013a).

The detrimental effect of sub-optimal or supra-optimal temperatures on photosynthesis has been discussed earlier. The night-time temperature is also important as it affects the respiration rate of the algae at night (see section "Respiration").

4 Acclimation and Regulation

Acclimation, as defined by Raven and Geider (2003), is the change of the macromolecular composition of an organism that occurs in response to variation of environmental conditions. Algae acclimate to changed irradiances and to temperature, and this has implications both for the maximum potential productivities that can be achieved in outdoor cultures and for culture management strategies.

Photoacclimation (acclimation to different irradiances) has been demonstrated in many studies of natural phytoplankton (Robinson et al. 1997; Stambler 2006), laboratory cultures (Fisher et al. 1996b; Mouget et al. 1999; Müller and Schagerl 2004; Bonente et al. 2012) and in outdoor algae cultures (Moheimani and Borowitzka 2007; Torzillo et al. 2012). Photoacclimation by phytoplankton has been reviewed by MacIntyre et al. (2002), Raven and Geider (2003), and Dubinsky and Stambler (2009). The immediately visible change occasioned by photoacclimation is an increase in the pigment content of cells grown under dim light (Cunningham et al. 1989; Herzig and Dubinsky 1992), a feature already observed by Myers in 1946 in cultures of *Chlorella* (Myers 1946). The change in pigment content can be quite large. For example, the chlorophyll content per cell in *D. salina* increased sixfold from 0.03 pmol cell^{-1} at 1700 µmol photons m^{-2} s^{-1} to 0.18 pmol cell^{-1} at 500 µmol photons m^{-2} s^{-1} (Smith et al. 1990). With decreasing irradiance both

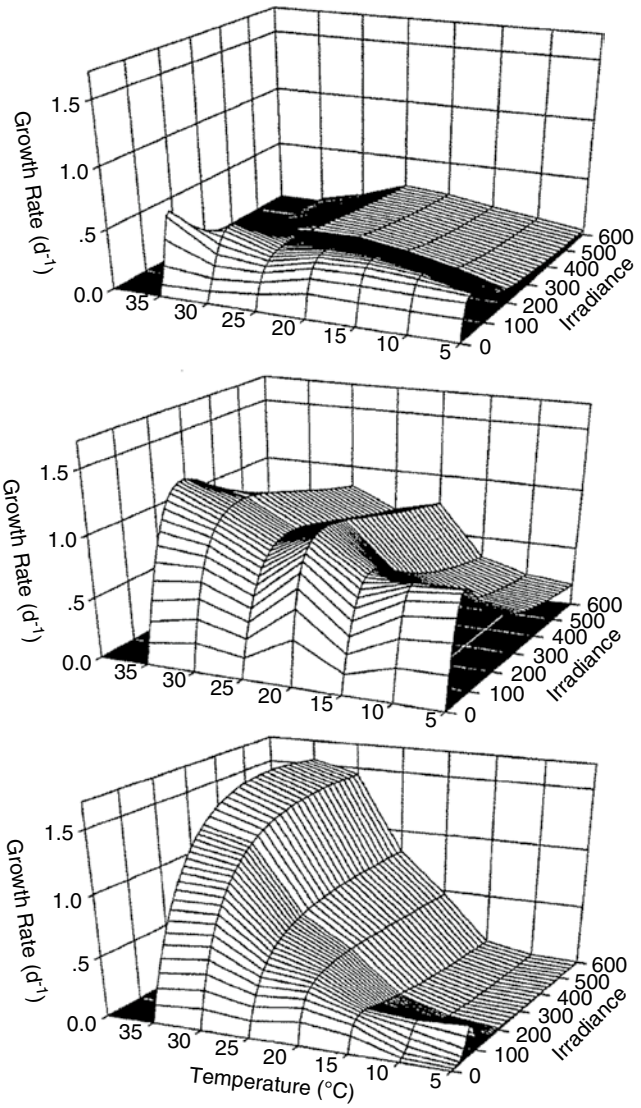

Fig. 5 Effects of temperature and irradiance on the growth rates of three species of microalgae, *Top* = *Planktothrix agardhii*, *Middle* = *Ankistrodesmus falcatus*, *Bottom* = *Phormidium bohneri*. Irradiance units are µmol photons m^{-2} s^{-1} (Figure from Borowitzka (1998), based on data from Talbot et al. (1991))

chlorophyll *a* and phycoerythrin contents per cell increased in the red alga, *Porphyridium cruentum* (Jahn et al. 1984; Cunningham et al. 1989) and, in the cyanobacterium *Arthrospira*, chlorophyll *a*, phycocyanin and allophycocyanin contents increased markedly (Tomaselli et al. 1997; Nomsawai et al. 1999). Not only does the pigment content change, but photoacclimation also results in other coordinated changes in the composition and functioning of the photosynthetic apparatus. These changes include changes in the composition and cellular abundance of light-harvesting pigment-protein complexes, the abundance and ratios of PSI:PSII reaction centres and other catalysts within the electron transport chain, and Calvin-Benson cycle activities, especially of Rubisco, per cell (Richardson et al. 1983; Dubinsky and Stambler 2009). Decreasing irradiance also causes structural changes in the chloroplast such as increases in the number of thylakoids (Cunningham et al. 1989; Janssen et al. 2001).

Of particular interest for outdoor cultures of microalgae is the acclimation to fluctuating irradiances and, ultimately, the effect of this on productivity. Although algae respond to rapid variations in the light environment via several regulatory responses (van de Poll et al. 2010; Giovagnetti et al. 2014), over the longer term microalgae acclimate to an irradiance which is near the average irradiance over the diurnal period (Kromkamp and Limbeek 1993; Flameling and Kromkamp 1997; Havelková-Doušová et al. 2004). In fact, dense outdoor cultures of microalgae in raceway ponds growing near or above their OCD are generally shade adapted (Kromkamp et al. 2009) which is not surprising, because most of the cells spend a significant amount of the time lower in the water column where irradiance is reduced. Raven and Geider (2003) propose that the algae appear to be acclimated to variations in the irradiance in a manner which protects against photoinhibition at high irradiances rather than maximising performance at the low irradiance extreme of the variation. Figure 6 illustrates the first order time constants for the main regulatory and acclimation processes in response to changes in irradiance. Some of these occur very rapidly, with time constants of seconds, effectively allowing photosynthesis to track the naturally occurring changes in irradiance almost instantaneously. For example, the light-dark activation/deactivation of fructose 1,6-bisphosphate-1-phosphatase occurs over seconds. Other regulatory mechanisms, such as Rubisco activation/deactivation and state transitions, occur with time constants of minutes and therefore may constrain rates of photosynthesis and photoprotection in rapidly changing light environments such as can be found in outdoor algae cultures (MacIntyre et al. 2000).

The acclimation of algae cultures to outdoor conditions is well illustrated by the study of Moheimani and Borowitzka (2007) with the coccolithophorid alga, *Chrysotila carterae*. They compared an indoor grown culture (25 °C, 300 µmol photons m^{-2} s^{-1}, 12 h light:12 h dark) with a freshly inoculated culture in an outdoor raceway pond over a period of 6 weeks. The photosynthetic rate was measured weekly in an oxygen electrode under constant conditions (28 °C, O_2 concentration 7.5 mg O_2 L^{-1}, cell concentration 1×10^5 cells mL^{-1}) at two high irradiances in the range observed outdoors (1800 and 2300 µmol photons m^{-2} s^{-1}). The samples were taken at 10:00 a.m. while the cells outdoors were still not photoinhibited. The photosynthetic rates of the indoor culture did not change over the 6 week period. However, in the outdoor culture the algae had fully acclimated to the high-light outdoor conditions after 4 weeks, as indicated by the higher mean photosynthetic rate (Fig. 7). Furthermore, the outdoor cultured cells had

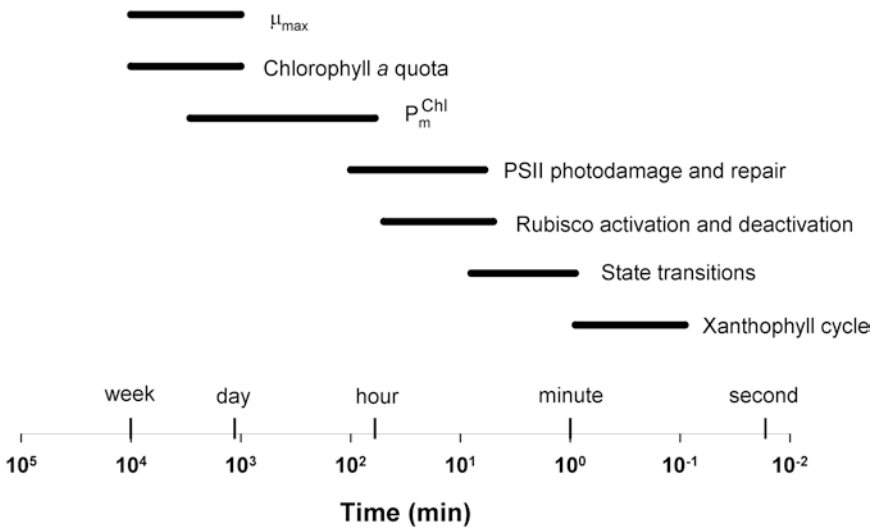

Fig. 6 First order time constants for acclimation of algal photosynthesis to step changes in irradiance (Redrawn from Raven and Geider (2003) as modified from MacIntyre et al. (2000))

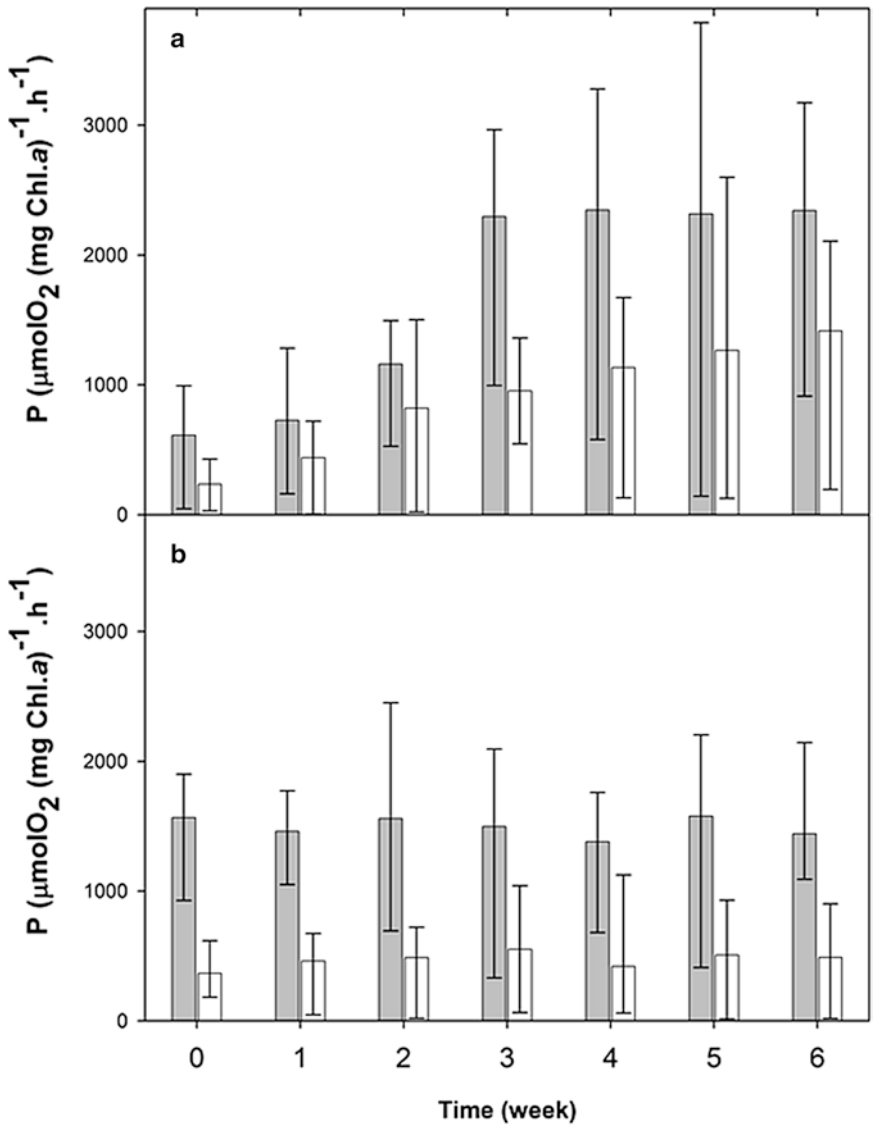

Fig. 7 Changes with time following inoculation in the photosynthetic rate of *P. carterae* at irradiances of 1800 µmol photons.$m^{-2}.s^{-1}$ (*grey bars*) and 2300 µmol photons.$m^{-2}.s^{-1}$ (*white bars*). The algae were (**a**) grown in an outdoor raceway pond and (**b**) cultured indoors under a 12:12 *light/dark* cycle at an irradiance of 300 µmol photons.$m^{-2}.s^{-1}$ and at 25 °C. All measurements were taken under constant conditions (see text) (Data are mean and range, n = 3 (From Moheimani and Borowitzka 2007))

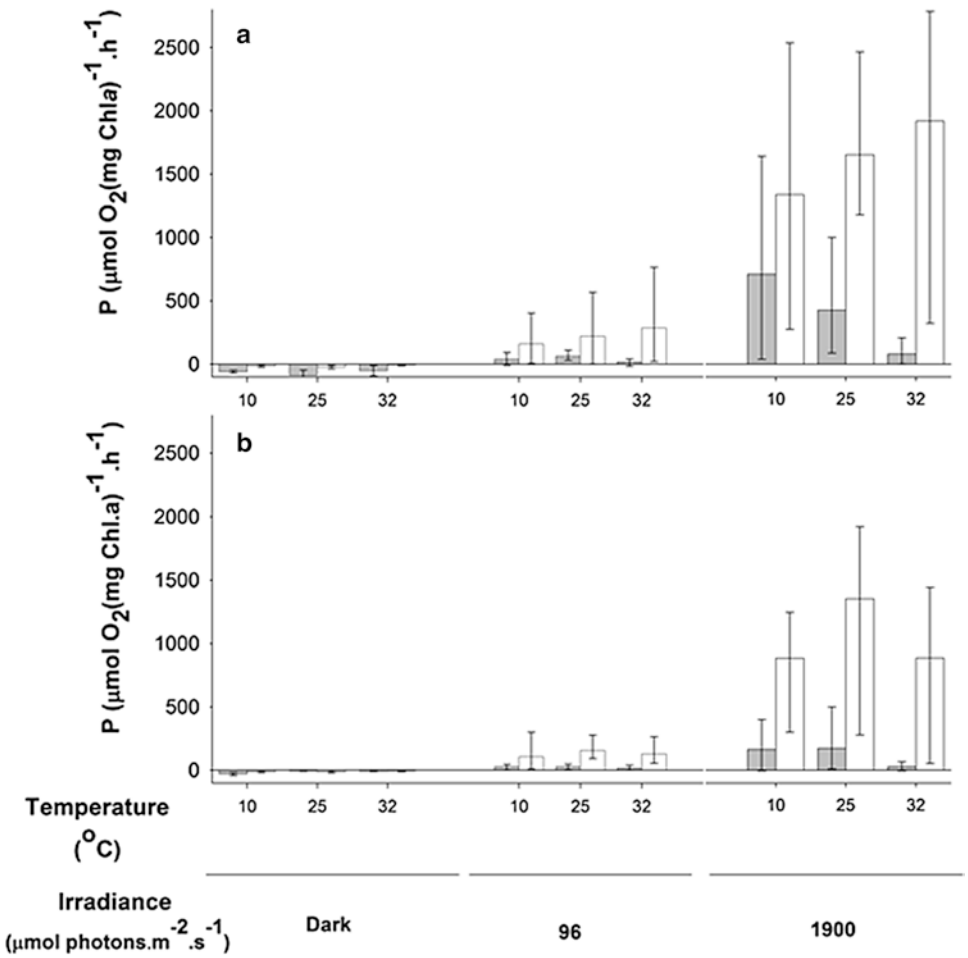

Fig. 8 Effect of irradiance and oxygen concentration on photosynthetic rates of *Chrysotila* (*Pleurochrysis*) *carterae* grown in (**a**) an outdoor raceway pond and (**b**) indoors under a 12:12 *light/dark* cycle at 300 µmol photons. $m^{-2}.s^{-1}$ and at 25 °C. *White bars* = 6–10 mg $O_2.L^{-1}$ and *Grey bars* = 26–32 mg $O_2.L^{-1}$ (Data are mean ± range, n = 3 (From Moheimani and Borowitzka 2007))

apparently also acclimated to higher O_2 concentrations and to the higher temperatures outdoors (Fig. 8) with the highest photosynthetic rates observed at 25 °C in the indoor grown culture, and at 32 °C for the outdoor culture. The cells in the outdoor culture also had a higher chlorophyll content. These results clearly illustrate the importance of evaluating the performance of algae under the conditions they are actually exposed to in outdoor cultures and after they have fully acclimated to these conditions. Results based on cultures acclimated to laboratory conditions are likely to be misleading.

5 Oxygen and Inorganic Carbon

Algal cultures, especially dense cultures, are exposed to very high concentrations of O_2, produced as a result of photosynthesis. This increase in O_2 concentration is generally accompanied by a decline in the CO_2 concentration, as a result of both CO_2 uptake for photosynthesis and of the increase in pH (due to CO_2 uptake). These two changes alter the $[O_2]$:$[CO_2]$ ratio. An increase in O_2 concentration results in inhibition of photosynthesis, mainly due to photorespiration, and is a major factor in reducing productivity (Ogawa et al. 1980; Livansky 1995; Kliphuis et al. 2011; Sousa et al. 2013). Photorespiration is the light stimulated oxidation of the products of photosynthesis to CO_2 and is due to the oxygenase activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39) (Beardall et al. 2003). At high O_2 concentrations and low CO_2 Rubisco catalyses the conversion of ribulose-1,5-bisphosphate to 3-phosphoglyceric acid and 2-phosphoglycolate (Hough and Wetzel 1978; Beardall et al. 2003). At high $[O_2]$:$[CO_2]$ ratios the Mehler reaction can also occur, especially at high irradiances and low temperatures (Badger et al. 2000). The Mehler reaction occurs when the NADP/NADPH pool, as well as other components of the photosynthetic apparatus, is reduced. At low irradiances it has clearly been shown that the O_2 inhibition effect is purely due to the O_2 effect on Rubisco (Raso et al. 2012). Temperature and irradiance further modulate the effect of high O_2 concentration and the effect is species specific and related to the optimum temperature range of a particular species/strain. For example, Fig. 9 shows how temperature at different irradiances interacts with increasing O_2 concentration impacts on the photosynthetic rate of

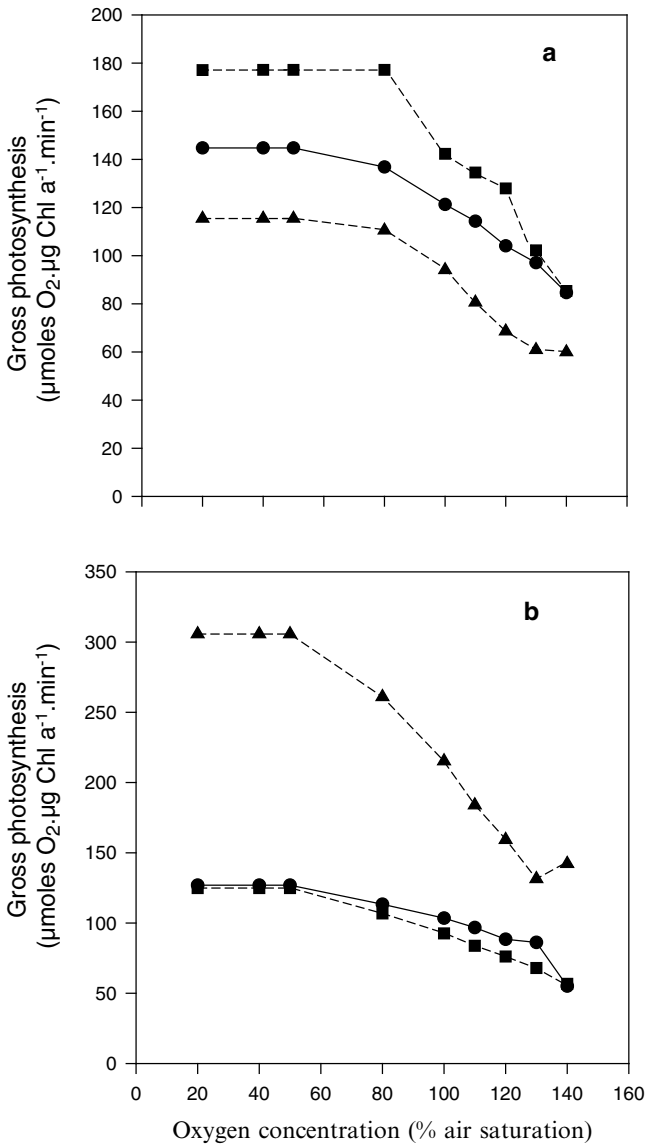

Fig. 9 The effect of oxygen concentration on gross photosynthesis in *Isochrysis galbana* at different irradiances and temperatures. (**a**) 1200 µmol photons m^{-2} s^{-1} (**b**) 2500 µmol photons m^{-2} s^{-1}. *Circles* = 20 °C, *squares* = 23 °C, *triangles* = 26 °C

Isochrysis galbana. The gross photosynthetic rate decreased with increasing O_2 concentration above 50 % air saturated water (sat_{air}) for all conditions except for the 23 °C treatment at 1200 µmol photons.m^{-2}.s^{-1} which was inhibited at concentrations above 80 % sat_{air}. The rate of decrease in gross photosynthetic rate (O_2 inhibition rate) was affected by both irradiance and temperature. The gross photosynthetic rate at 1200 µmol photons.m^{-2}.s^{-1} was highest at 23 °C, whereas at 2500 µmol photons.m^{-2}.s^{-1} the highest temperature-specific maximum gross photosynthetic rate was observed at 26 °C. In *Ettlia* (*Neochloris*) *oleoabundans* grown at near saturating irradiances in a photobioreactor the specific growth rate decreased with increasing O_2 concentrations, and increasing the partial pressure of CO_2 could not reduce this inhibition (Sousa et al. 2013), unlike what is observed at sub-saturating irradiances (Raso et al. 2012; Sousa et al. 2012). The chlorophyll content of *E. oleoabundans* grown at 200 µmol photons m^{-2} s^{-1} was about 1.9 times higher than when grown at 500 µmol photons m^{-2} s^{-1}, whereas the carotenoid content was about 1.5 times lower showing that the algae had photoacclimated. Elevated O_2 concentrations do not affect the pigment content at either of these irradiances indicating that the increased O_2 does not contribute to photooxidative damage at the light conditions that are generally experienced by algae in closed photobioreactors and that the growth inhibition observed at high O_2 is mainly due to the increased irradiance (Sousa et al. 2013). On the basis of these observations Sousa et al. (2013) propose that, for large-scale outdoor cultivation of microalgae, reactor configurations that allow spatial dilution of light should be used, in combination with addition of CO_2. In these types of photobioreactors the algae grow at sub-saturating light conditions and, with the addition of CO_2, the photorespiration effects will be minimized.

The relative rates of the carboxylase and oxygenase functions of Rubisco can be defined by the selectivity factor S_{rel} (Beardall and Raven 2013):

$$S_{rel} = \frac{K_{\frac{1}{2}}(O_2) \times k_{cat}(CO_2)}{K_{\frac{1}{2}}(O_2) \times k_{cat}(CO_2)} \quad (4)$$

Where $k_{cat}(CO_2)$ = the CO_2-saturated specific rate of carboxylase activity of Rubisco (mol CO_2 mol^{-1} active site s^{-1}); $K_{1/2}(CO_2)$ = the concentration of CO_2 at which the CO_2 fixation rate is half of $k_{cat}(CO_2)$; $k_{cat}(O_2)$ = the O_2-saturated specific rate of oxygenase activity of Rubisco (mol O_2 mol^{-1} active site s^{-1}); and $K_{1/2}(O_2)$ = the concentration of O_2 at which the O_2 fixation rate is half of $k_{cat}(O_2)$ (Beardall and Raven 2013).

There is significant variation in the S_{rel} of different algae (see Table 3 in Badger et al. 1998). The lowest S_{rel} values are found in cyanobacteria and in dinoflagellates, such as *Amphidinium* sp., and the highest values are found in red algae, such as *Porphyridium* and *Cyanidium* (Uemura et al. 1997; Badger et al. 1998).

At the normal levels of CO_2 and O_2 the competition between CO_2 and O_2 at the active site of Rubisco often limits carbon fixation. For example, for green algae the half saturation of photosynthesis occurs at ~30 µM CO_2, whereas the concentration of CO_2 in water in equilibrium with air is ~12 µM. Thus, if algae rely only on diffusive CO_2 entry to the cell, Rubisco fixation of CO_2 would be C-limited. However, many algae have evolved an active transport system for dissolved inorganic carbon (DIC) based on active transport of HCO_3^- and/or CO_2. This active transport, called the carbon concentrating mechanism (CCM), increases the

CO_2 concentration - and thus also the CO_2/O_2 ratio – at the active site of Rubisco, and significantly enhances the carboxylation activity of Rubisco. CCMs in algae and their function are discussed in detail in chapter "Carbon Acquisition by Microalgae" of this book (Beardall and Raven 2016) and also by Giordano et al. (2005). The activity of CCMs is light dependent, with cells grown at lower light having a lower affinity for CO_2 in photosynthesis (Shiraiwa and Miyachi 1983; Beardall 1991; Young and Beardall 2005). However, there have been no studies on CCM activity under fluctuating light, as occurs in dense cultures. Similarly, the addition of CO_2 to avoid DIC limitation in cultures can lead to potential downregulation of the CCMs (Beardall and Raven 2013). The implications of this for algal productivity, especially at low irradiances, are not well understood.

In open ponds little can be done to reduce the build-up of O_2 during the day and O_2 concentrations of over 300–400 % of air saturation are commonly observed (Weissmann et al. 1988; Jiménez et al. 2003). In tubular photobioreactors the O_2 concentration increases while the CO_2 concentration decreases as the culture flows through the tubular photostage of the reactor, until some of the O_2 is stripped from the medium in the manifold to which the tube is connected (Camacho Rubio et al. 1999). Thus, in order to achieve optimum biomass productivities, there is a flow rate dependent practical limit to the length of the tube between the manifolds, so as to avoid excessive O_2 concentrations and CO_2 limitation. Molina Grima et al. (1999) have recommended that tube lengths not exceed 80 m when the flow rate is between 0.3 and 0.5 m s^{-1}. Recently Muller-Feuga et al. (2012) have described a new type of horizontal photobioreactor in which the medium and a gas phase above the medium co-circulate. This provides improved gas exchange and reduces the build-up of oxygen in the medium, although there are still limitations to the scale-up of this system (Valiorgue et al. 2014).

In almost all dense algal cultures addition of an inorganic C-source, either as CO_2 or HCO_3^-, will enhance productivity (Yang and Gao 2003; Moheimani 2013; Sutherland et al. 2015). Addition of CO_2 can also be used to maintain the optimum pH of the culture, thus further optimizing productivity. The inorganic carbon system is the main buffering system in water and can be described as follows:

$$CO_{2(gas)} + H_2O \leftrightarrow CO_{2(aq)} / H_2CO_3 \overset{pK1}{\leftrightarrow} HCO_3^- + H^+ \overset{pK2}{\leftrightarrow} CO_3^{2-} + 2H^+ \quad (5)$$

The apparent dissociation constants of the inorganic carbon system for freshwater at 25 °C are: pK1 = 6.35 and pK2 = 10.33 (Harned and Scholes 1941; Harned and Davis 1943), and for seawater of 35‰ salinity at 25 °C they are: pK1 = 6.00 and pK2 = 9.12 (Mehrbach et al. 1973).

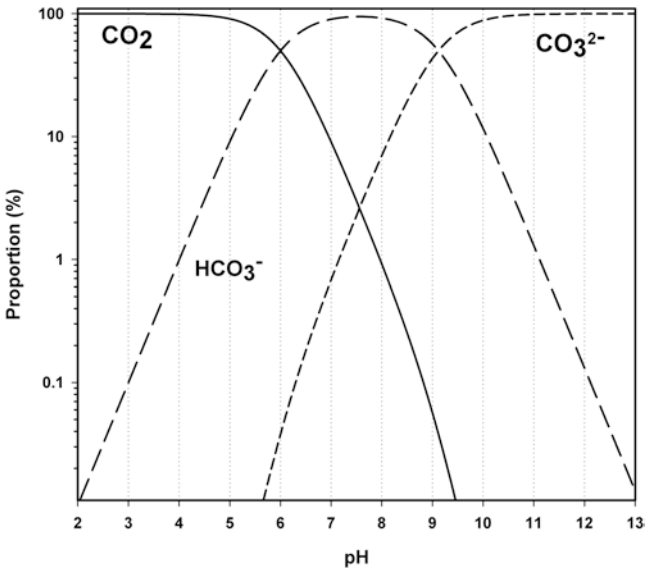

Fig. 10 Semi-log plot showing the relative proportions of the inorganic carbon species (CO_2, HCO_3^-, CO_3^{2-}) at different pH in seawater with salinity 35‰, at 25 °C

Figure 10 shows the effect of pH on the relative proportions of the three inorganic carbon species in seawater. As can be seen from Eq. 2, CO_2 uptake during photosynthesis will shift the equilibrium to the right resulting in an increase in pH (Shiraiwa et al. 1993). This pH shift due to CO_2 uptake is commonly observed in algal cultures during the day (Moheimani 2013). The uptake of CO_2 will not only reduce the total inorganic carbon (TIC) in the medium, but because of the pH rise of the medium, will also reduce the proportion of the TIC available as CO_2. Due to the photosynthetic activity, even in relatively dilute and well aerated cultures, the level of DIC is rarely as high as might be expected from the air-equilibrium. For example, Johnston and Raven (1992) observed a tenfold drop in TIC over a 6.5 day growth period for cultures of *Phaeodactylum tricornutum*. Similarly Young and Beardall (2005) found that semi-continuous cultures of *Dunaliella tertiolecta* growing in buffered medium aerated at a rate of one culture volume per minute, still exhibited a 50 % decrease in TIC and a 0.4 unit rise in pH. Williams and Coleman (1996) also showed that in *Chloroidium saccharophilum* (as *Chlorella saccharophila*) cultures with a low aeration rate the TIC concentration dropped from 450 µM (air equilibrium) to below 30 µM over 3 days. Thus microalgae in cultures are very likely to become C-limited over time. The CO_2 compensation point is often determined in the laboratory by using pH-drift experiments (i.e. when algal C-uptake ceases at a certain pH where there is insufficient available CO_2 and HCO_3^- for uptake to occur) (Bañares-España et al. 2006; Maberly et al. 2009). In dense outdoor cultures the pH often reaches the value equivalent to the pH at which CO_2 compensation point is reached in pH-drift

experiments, indicating that algal photosynthesis in these cultures is now effectively inhibited by the lack of a supply of inorganic carbon. The addition of CO_2 to a culture therefore not only serves to increase the TIC but, as it also reduces the pH, it ensures that more of the TIC is available to the algal cells in the form of CO_2 and HCO_3^-. This is even more important for the culture of algae, such as *Dunaliella salina*, growing in hypersaline media (several times the salinity of seawater) and at a high temperatures, because the solubility of CO_2 is reduced both by increasing salinity and by increasing temperature (Duan et al. 2006).

CO_2 addition, however, is not necessarily beneficial to the growth of all algae (Moheimani and Borowitzka 2011). In the coccolithophorid alga, *Emilianea huxleyi*, addition of CO_2 lowered both biomass and coccolith production.

The importance of mixing to maximize inorganic C uptake also should not be underestimated. Diffusion of CO_2 in water is about 10,000 times slower than in air (see Table 20.1 in Larkum et al. 1989; Zeebe and Wolf-Gladrow 2001). Diffusional transport of inorganic C species to the phytoplankton cell surface is dominated by molecular diffusion, because turbulent shear is insignificant at phytoplankton cell sizes that are small compared with the Kolmogorov length scale (Lazier and Mann 1989). The external diffusion boundary layer thickness is predicted to be equal to the radius of the cell for a spherical cell, although the boundary layer thickness is smaller than this prediction for cells with radii >50 μm (Raven 1998). This relationship is modified for motile cells, and non-spherical and chain-forming algae (Karp-Boss et al. 1996; Wolf-Gladrow and Riebesell 1997; Popp et al. 1998; Guasto et al. 2012). Thus the rate of supply of CO_2 and/or HCO_3^- to the cell surface can become limiting in dense cultures, especially if no additional CO_2 is supplied (Riebesell et al. 1993). Mixing (turbulence) may also reduce the thickness of the boundary layer (Lazier and Mann 1989; Karp-Boss et al. 1996; Warnaars and Hondzo 2006) and thus reduce the diffusion limitation of inorganic carbon supply.

Most microalgae can take up both CO_2 and HCO_3^-, but there are a few species which can take up only CO_2 or only HCO_3^- (Colman et al. 2002). Interestingly, the small number of known species with a single inorganic carbon uptake system includes several which are of particular interest with respect to large scale culture for potential commercial applications. These are the chlorophytes *Picochlorum* (*Nannochloris*) *atomus* and *Picochlorum* (*Nannochloris*) *maculata* and the eustigmatophyte *Monodopsis subterrarea* (as *Monodus subterraneus*) which can take up only CO_2 and not HCO_3^-(Huertas and Lubián 1998; Huertas et al. 2000a, 2002), and the eustigmatophytes *Nannochloropsis gaditiana* and *Nannochloropsis oculata*, which can only take up HCO_3^- (Huertas and Lubián 1998; Huertas et al. 2000b). This clearly has implications as to the pH at which the culture should be maintained in order to achieve optimum productivity. For most species which can take up both CO_2 and HCO_3^- a pH of between 7 and 8 is optimal, but for a few species, such as the coccolithophorid alga, *Chrysotila carterae*, the pH must be higher than 8.5 (Moheimani and Borowitzka 2011). Maintaining the pond pH at 8.5 or lower by CO_2 addition leads to culture collapse for this species. It is not known whether this collapse is due to peculiarities of the carbon uptake system or whether it is a direct effect of pH.

Addition of CO_2 changes the pH but not the alkalinity of the medium. An alternative method of increasing the DIC content without having a marked effect on the pH is the addition of bicarbonate (Pimolrat et al. 2010; Gardner et al. 2012; White et al. 2013). However, bicarbonate addition changes the alkalinity and is more likely to lead to the precipitation of hydroxides such as brucite ($Mg(OH)_2$), phosphates, and carbonates in the presence of algae (Hartley et al. 1997; Olsen et al. 2006; Tesson et al. 2008). Another potential issue for large scale cultures is that bicarbonate addition will also increase the concentration of other ions such as Na^{2+} (from sodium bicarbonate), which ultimately may affect the cultures negatively. Bicarbonate addition is generally only used for the culture of *Arthrospira* which grows best at high alkalinity and high pH (Richmond 1988) and can tolerate elevated levels of Na^+ (Iltis 1968). Bicarbonate addition has also been shown to enhance the lipid content of species such as *Scenedesmus* sp., *Chlamydomonas reinhardtii*, *Tetraselmis suecica*, *Nannochloropsis salina* and *Phaeodactylum tricornutum* (Gardner et al. 2012, 2013; White et al. 2013), although the reason for this is not well understood.

Most studies on the effect of CO_2 on microalgae have focused on relatively low CO_2 concentrations of <5 % CO_2, as would be expected in the current environment (i.e. present-day atmospheric CO_2 is about 0.04 % v/v) or in future environments with increased atmospheric CO_2 concentrations (Raven et al. 2011, 2012). However, if the algae are to be cultured for CO_2 bioremediation the ability to grow using gas streams with high CO_2 content (>5 % CO_2) is desirable. However, not all algae are equally tolerant of high CO_2 concentrations (Table 2). Algae can be classified into CO_2-sensitive (intolerant) species which are inhibited by <2–5 % CO_2, CO_2-tolerant species which can cope with up to 20 % CO_2, and extremely CO_2-tolerant species which can withstand even higher CO_2 concentrations (Miyachi et al. 2003; Solovchenko and Khozin-Goldberg 2013). For example, *D. salina* is a CO_2-sensitive species and growth is inhibited at 10 % CO_2 and completely blocked at 25 % CO_2 (Sergeenko et al. 2000). On the other hand, the extremely CO_2-tolerant chlorophyte *Chlorococcum littorale* continues to grow rapidly at CO_2 concentrations up to 60 % (Kodama et al. 1993). It is also important to remember that bubbling cultures with high-CO_2 containing air not only increases the CO_2 concentration, but also acidifies the medium.

Table 2 Reported CO_2 tolerance of microalgae species (Ono and Cuello 2003)

Species	Known maximum CO_2 concentration tolerated (% v/v)	Optimum CO_2 concentration for growth (% v/v)	References
Cyanidium caldarium	100		Seckbach et al. (1970)
Scenedesmus sp. K34	80	20	Hanagata et al. (1992)
Chlorococcum littorale	60		Kodama et al. (1993) and Satoh et al. (2001)
Synechococcus elongatus	60		Miyairi (1995)
Euglena gracilis	45	5–40	Nakano et al. (1996)
Nostoc flagelliforme	40[a]	20	Lv et al. (2014)
Chlorella sp. K35	60	10	Hanagata et al. (1992)
Chlorella sp. UK001	40		Satoh et al. (2001)
Chlorella minutissima	>40	25–30	Papazi et al. (2008)
Parachlorella (*Chlorella*) *kessleri*	<18	12	de Morais and Costa (2007)
Chaetoceros muelleri	30[a]	10	Wang et al. (2014)
Botryococcus braunii 765[b]	20[a]		Ge et al. (2011)
Dunaliella tertiolecta	15[a]		Nagase et al. (1997)
Nannochloris sp. NANNO2	15[a]		Negoro et al. (1991)
Nannochloropsis sp.	15[a]		Negoro et al. (1991)
Phaeodactylum tricornutum	15[a]		Negoro et al. (1991)
Dunaliella salina	<25	<10	Sergeenko et al. (2000)
Dunaliella salina	20	5	Ying et al. (2014)

[a]Highest concentration tested

[b]Growth measured as increase in dry weight

The transfer of extremely CO_2-tolerant microalgae such as *Chlorococcum littorale*, from low CO_2 conditions to high-CO_2 conditions often leads to a temporary decline in growth, CO_2 fixation, O_2 evolution, and quantum yields of PS II, before the cells recover. At the same time PSI activity (mainly cyclic electron transport around PS I) is greatly enhanced (Pesheva et al. 1994; Iwasaki et al. 1996, 1998). The shift to high-CO_2 results in a downregulation of linear electron transport between the two photosystems; i.e. a state-II transition (Demidov et al. 2000; Sergeenko et al. 2000). The increase in PSI cyclic electron transport due to this state transition has been suggested to generate additional ATP to support pH homeostasis in the cell (Miyachi et al. 2003). In contrast, CO_2-intolerant species such as *Dunaliella salina* lack the state transition response and show signs of PSI damage under high CO_2 conditions (Muradyan et al. 2004). The growth inhibition caused by extremely high CO_2 concentration appears to be associated with cytoplasmic acidification and possibly also with acidification of the chloroplast stroma. Satoh et al. (2001) found that intracellular acidification occurred upon exposing low-CO_2 acclimated cells of *C. littorale* to 40 % CO_2 for 1 h. This intracellular acidification did not occur when the cells were exposed to ethoxyzolamide, a membrane-permeable inhibitor of carbonic anhydrase, indicating that this intracellular acidification might be due to the function of carbonic anhydrase. High-CO_2 grown cells of *C. littorale* have neither extracellular nor intracellular carbonic anhydrase activity and, when these were transferred to 40 % CO_2 conditions they grew without any lag period. Additionally, there is also an increase in the number and size of vacuoles in *C. littorale* cells when exposed to extremely high CO_2 conditions (Kurano et al. 1998), and the development of these vacuoles and the increased activity and induction of vacuolar H^+-ATPase (V-ATPase) is well correlated with the recovery of photosynthetic activity during the acclimation of the cells to the extremely high CO_2 conditions (Sasaki et al. 1999). Vacuolar proton pumps such as V-ATPase maintain cytoplasmic pH homeostasis by generating an electrochemical potential difference across the vacuolar membrane (Maeshima 2001).

The above observations indicate that high CO_2 tolerance requires the ability to maintain intracellular pH. Pronina et al. (1993) showed that the intracellular pH in *Chlorococcum littorale* is maintained at about pH 7 in both high- and low-CO_2 grown cells. This requires the ability to shut down the CCM mechanism. Even in *Chlamydomonas reinhardtii*, a species not tolerant of extremely high CO_2 conditions, the cultivation of low-CO_2 adapted cells at elevated CO_2 levels results in a decline in carbonic anhydrase (Baba and Shiraiwa 2012) and an inhibition of active transport of bicarbonate (Bhatti and Colman 2008; Raven 2010). Similarly, in *Isochrysis galbana* grown at high (3 %) CO_2, external carbonic anhydrase activity is completely repressed (Bhatti et al. 2002). Interestingly, acidophilic microalgae such as *Synura petersenii*, *Tessellaria volvocina* and *Palmellopsis* sp. lack a CCM (Bhatti and Colman 2008; Diaz and Maberly

2009). They also lack a plasmalemma-bound carbonic anhydrase and are unable to take up bicarbonate. Furthermore, their Rubisco is characterized by a high affinity for CO_2 (Bhatti and Colman 2008).

6 Respiration

The respiration rate is important as respiration effectively reduces the net production of biomass. A general feature of microalgae is that the respiration to photosynthesis ratio is reasonably constant between taxa and over a wide range of cell sizes. Under constant laboratory conditions, López-Sandoval et al. (2014) found that dark respiration was 9 ± 7 % of the photosynthetic rate in the exponential growth phase, and 22 ± 23 % in the stationary growth phase, in batch cultures of 22 marine phytoplankton species from 5 Phyla, covering a range of 7 orders of magnitude in cell size,. However, environmental conditions can have a marked effect on the dark respiratory rate (see chapter "Dark Respiration and Organic Carbon Loss" in this book – Raven and Beardall 2016). Higher temperatures at night usually mean higher respiratory rates and a greater loss of biomass at night (Grobbelaar and Soeder 1985). The rate of respiration at night is also influenced by the levels of irradiance experienced by the algae during the day, with high daytime irradiances increasing respiration at night (Banse 1976; Raven 1981; Beardall et al. 1994). This is presumed to be due to the need for extra energy at night to repair any photodamage resulting from the high irradiances. Torzillio et al. (1991a, b), studying *Arthrospira platensis* grown in a tubular photobioreactor, observed higher night-time respiration at 25 °C (leading to a loss of about 7.6 % of the total dry weight) than at 35 °C (with a loss of 5 % of total dry weight). The optimum temperature for this strain is 35 °C and more carbohydrate was synthesised when the culture was grown at high light or at the suboptimal temperature of 25 °C. The higher respiration rate as night was attributed to the suboptimal temperature and the high light causing photodamage during the day and its repair at night, with the carbohydrates serving as the substrates for respiration. If the cell density is maintained at near optimal, the night biomass loss was minimal (Hu et al. 1996b). Ogbonna and Tanaka (1996) observed similar results with *Chlorella pyrenoidosa*. They also found that the biomass loss at night was predominantly due to the decline in carbohydrate content. High irradiances can also increase dark respiration during the day (Falkowski et al. 1985).

The respiration rate is influenced by the growth rate of the algae; i.e. faster growing algae respire more (Laws and Caperon 1976; Geider and Osborne 1989; Gilstad et al. 1993; Michels et al. 2014). This is because faster growing algae have a higher maintenance requirement.

In algae which can use organic substrates heterotrophically at night provision of these substrates (e.g. glucose, ethanol or acetate) reduces the loss of biomass at night as they provide additional substrates for respiration (Esterhuizen-Londt and Zeelie 2013).

7 Mixotrophy

Many microalgae can take up and utilise organic compounds such as acetate or glucose for growth in the light (Ogawa and Aiba 1981; Chen and Zhang 1997; Ip et al. 2004; Cheirsilp and Torpee 2012). This is known as mixotrophy. Although most current commercial-scale outdoor algal cultures are grown photoautotrophically to avoid problems with excessive bacterial load, mixotrophy and heterotrophy are inevitable for algae grown on wastewaters for the treatment of these. The consortia of microalgae in oxidation ponds and high-rate algal wastewater treatment ponds include many species which can utilise available organic compounds either through mixotrophy and/or heterotrophy (Abeliovich and Weisman 1978; Fruend et al. 1993). Several studies however have shown that these ponds are still C-limited and that provision of additional CO_2 improves algal growth (Park and Craggs 2011; Craggs et al. 2012; Prathima Devi and Venkata Mohan 2012).

8 Circadian Rhythms and Diel Variations

An understudied, and generally underappreciated, aspect of algal physiology in outdoor cultures is the occurrence and role of circadian rhythms. Algal cultures growing under a light-dark cycle usually exhibit distinct circadian rhythms in photosynthesis and other physiological, biochemical and behavioral processes (Hastings et al. 1961; Palmer et al. 1964; Owens et al. 1980; Østgaard and Jensen 1982; Ramalho et al. 1995; Mittag 2001; Corellou et al. 2009); however, the importance of these metabolic rhythms in outdoor cultures has been little investigated, although they have been widely studied in natural phytoplankton populations (Harding et al. 1981; Brand 1982; John et al. 2012). Circadian rhythms are controlled by 'clocks', which are autonomous internal timekeeping mechanisms that allow organisms to adapt to external daily rhythms of light, temperature, and other environmental factors (Wijnen and Young 2006). Circadian clocks oscillate with periods close to 24 h and continue to run in the absence of environmental cues (i.e., they continue to operate in constant continuous light) and they significantly affect other biological functions. Physiological processes of microalgae shown to be under the influence of a circadian biological 'clock' include photosynthesis (Hastings

et al. 1961; Prézelin et al. 1977; Samuelsson et al. 1983; Sayed and El-Shahed 2000; Mackenzie and Morse 2011; Sorek et al. 2013), chloroplast ultrastructure (Herman and Sweeney 1975), phototaxis (Mergenhagen 1984), pigment synthesis (Ragni and d'Alcalà 2007; Braun et al. 2014), TCA cycle enzymes (Akimoto et al. 2005), nitrogen assimilation and nitrate reductase activity (Ramalho et al. 1995; Vergara et al. 1998; Brown et al. 2009; Mackenzie and Morse 2011; Wang et al. 2015), and nitrogen fixation (Grobbelaar and Huang 1992; Chen et al. 1993) (see Table 3). In *Chlamydomonas reinhardtii* and *Tetraselmis subcordiformis*, stickiness to glass (and presumably to other surfaces) also has been demonstrated to be under control of a circadian rhythm (Grant and Vadas 1976; Straley and Bruce 1979). This has implications for the management of large-scale cultures.

The molecular basis of the circadian clock has been extensively studied in *Chlamydomonas*, *Ostreococcus* and *Synechococcus*. Circadian control of transcriptional rate has been found for a number of genes, resulting in rhythms of RNA abundance over the circadian cycle (Mittag 2001; Werner 2002; Mittag and Wagner 2003; Bouget et al. 2014). However, there is also evidence for non-transcriptional clocks (O'Neill et al. 2011; Bouget et al. 2014), highlighting the complexity of the control of circadian rhythms.

A very important observation is that the cell cycle of most microalgae is also under the control of the light/dark cycle, with cell division occurring at night in most species (Sweeney and Hastings 1962; Bruce 1970; Senger 1970; Williamson 1980; Chisholm and Brand 1981; Jacquet et al. 2001; Ragni and d'Alcalà 2007). The cell cycle can continue for some time in continuous light, with cell division still occurring in the putative 'night' (de Winter et al. 2013); however, there is evidence that, in at least some algae such as *Chlamydomonas reinhardtii*, the cell cycle is not under the control of an endogenous 'clock' as such, but rather is strongly dependent on the cell's rate of growth (Vítová et al. 2011). This phased cell division has implications for the possible optimisation of the harvesting time to maximise productivity and yield in semi-continuous cultures.

Cultures grown outdoors show a synchronized cell cycle similar to those found in laboratory cultures synchronized using a light/dark regime. For example, Fig. 11 illustrates phased cell division occurring in an outdoor raceway pond culture of *Chrysotila carterae*. Early studies showed that the cell cycle in light/dark synchronized cultures strongly modulate the photosynthetic activity of the algae. For example, in *Chlorella pyrenoidosa* (= *Chlorella vulgaris*) the photosynthetic activity reaches a maximum during the early phase of the light period, remains high for a few hours, and then steadily declines until the end of the light period where the minimum coincides with the onset of cell division (Sorokin 1957). Respiration also shows a similar periodic modulation (Sorokin and Myers 1957). A similar modulation of photosynthetic activity has also been found in synchronized cultures of the diatom, *Cylindrotheca fusiformis* (Claquin et al. 2004). Using *Scenedesmus communis* (*as S. quadricauda*) Kaftan et al. (1999) found that the rate of O_2 evolution increased two to threefold during the first 3–4 h of the light period, remained high for the next 3–4 h, and then declined during the last half of the light period. This study showed that these changes in O_2 evolution were due to PSII heterogeneity. There are two populations of PSII, active centres that contribute to carbon reduction and inactive centers that do not. Measurements of PSII antenna sizes also revealed two populations, $PSII_\alpha$ and $PSII_\beta$, which differ from one another by their antenna size. During the early light period the photosynthetic capacity of the cells doubled, the O_2-evolving capacity of PSII was nearly constant, the proportion of $PSII_\beta$ centres decreased to nearly zero, and the proportion of inactive PSII centers remained constant. During the period of minimum photosynthetic activity 30 % of the PSII centres were insensitive to the inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), and this insensitivity may be related to reorganization of the thylakoid membrane. They concluded that the decline in photosynthetic activity observed during the last half of the light period could be accounted for by limited PSII activity. Electron micrographs showed significant changes in the organization of the thylakoid membranes during cell division (see also Matusiak-Mikulin et al. 2006), including an increase in membrane stacking, and Kaftan et al. (1999) propose that this reflects dramatic changes in the thylakoid membrane resulting in an impaired PSII repair cycle and leading to an increase in inactive PSII centres. Cell-cycle dependent changes in antenna size also have been observed in *Euglena gracilis* (Winter and Brandt 1986).

9 Salinity, Osmoregulation, and Compatible Solutes

In nature, microalgae grow in waters of varying salinity, ranging from freshwater to hypersaline lakes. The salinity range tolerated by an individual species is a function of its osmoregulatory system and is dependent on the type of osmoregulatory solute(s) the alga utilises (Borowitzka 1981b; MacKay et al. 1984; Oren 2007). Both organic and inorganic solutes play a role in osmoregulation and often algae use a combination of solutes in osmoregulation. The osmoregulatory solutes serve to maintain water availability for metabolic processes at a steady state where there is no net water flux to or from the cell despite the water permeability of the cell membrane. These solutes have particular properties (Borowitzka 1981b):

- They must be highly soluble as high concentrations are often required in osmoregulation. For example K^+ and

Table 3 Circadian rhythms in microalgae

	Alga	Details	References
Photosynthesis			
Photosynthetic capacity (P_{max})	*Lingulodinium polyedra*	Peaks about 8 h after the start of the light period (12:12 h L:D cycle; 140 µmol photons m^{-2} s^{-1})[a]	Hastings et al. (1961)
	Lingulodinium polyedra	Peaks about 6 h after start of the light period (12:12 h L:D cycle; 1000 µW cm^{-2})	Prézelin and Sweeney (1977)
	Phaeodactylum tricornutum	Peaks during day (12:12 h L:D cycle; 88 µmol photons m^{-2} s^{-1})[a]	Palmer et al. (1964)
	Chlorella vulgaris	Peaks about 6 h after start of light period (12:12 h L:D cycle; 200 µmol photons m^{-2} s^{-1})	Sayed and El-Shahed (2000)
Initial slope of P/E curve (α)	*Lingulodinium polyedra*	Peaks about 6 h after start of the light period (12:12 h L:D cycle; 1000 µW cm^{-2})	Prézelin and Sweeney (1977)
	Nannochloris sp.	Peaks about 8 h after start of the light period (16:8 h L:D cycle; 314 µmol photons m^{-2} s^{-1})	Raateoja and Seppälä (2001)
O_2 evolution	*Lingulodinium polyedra*	Peaks about 6 h after start of the light period (12:12 h L:D cycle, 60 µmol photons m^{-2} s^{-1}). Associated with changes in Rubisco distribution inside the chloroplast	Nassoury et al. (2001)
PSII activity	*Lingulodinium polyedra*	Peaks early in the day (12:12 h L:D cycle; 900 µW cm^{-2})	Samuelsson et al. (1983)
F_v/F_m	*Symbiodinium* sp. (free-living)	Lowest value ~8 h after start of light, highest value just before start of light (12:12 h L:D cycle; 100 µmol photons m^{-2} s^{-1})	Sorek et al. (2013)
Chlorophyll content	*Nannochloropsis gaditana*	Increases during the day (12:12 h L:D cycle; 100 µmol photons m^{-2} s^{-1})	Braun et al. (2014)
	Skeletonema costatum	Increases during the day (14:10 h L:D cycle; 70 µmol photons m^{-2} s^{-1})	Østgaard and Jensen (1982)
In-vivo chlorophyll fluorescence and DCMU enhanced chlorophyll fluorescence	Range of diatoms, dinoflagellates, haptophytes and chlorophytes	Persistent rhythm in fluorescence in continuous low light. Fluorescence peaks during day (14:10 h L:D cycle; 70 µmol photons m^{-2} s^{-1})	Brand (1982)
Carbon metabolism			
Activity of glyceraldehyde-3-phosphate dehydrogenase	*Lingulodinium polyedra*	Peaks late night, early morning (12:12 h L:D cycle; 150 µmol photons m^{-2} s^{-1})	Fagan et al. (1999)
Activity of NADP-dependent isocitrate dehydrogenase	*Lingulodinium polyedra*	Peaks in dark phase (12:12 h L:D cycle; 50 µmol photons m^{-2} s^{-1})	Akimoto et al. (2005)
Periplasmic carbonic anhydrase gene *Cah1* mRNA	*Chlamydomonas reinhardtii*	Shows strong diurnal rhythm under a 12:12 h light cycle. *Cah2* mRNA does not	Fujiwara et al. (1996)
Nitrogen metabolism			
Methylammonium uptake	*Chlamydomonas reinhardtii*	Peaks in day phase (12:12 h L:D cycle; 25 µmol photons m^{-2} s^{-1})	Byrne et al. (1992)
Nitrate reductase activity	*Lingulodinium polyedra*	Peaks about 6 h after start of light period (12:12 h L:D cycle; 150 µmol photons m^{-2} s^{-1})	Ramalho et al. (1995)
Ferredoxin-nitrite reductase activity	*Chlamydomonas reinhardtii*	Peaks about 6 h after start of light period (12:12 h L:D cycle; 50 W m^{-2})	Pajuelo et al. (1995)
Cell behavior			
Stickiness to glass	*Chlamydomonas reinhardtii*	Peaks in early night phase (12:12 h L:D cycle; 43 µmol photons m^{-2} s^{-1})[b]	Straley and Bruce (1979)
	Tetraselmis subcordiformis	Peaks in early night phase (12:12 h L:D cycle; 104–134 µmol photons m^{-2} s^{-1})[b]	Grant and Vadas (1976)

[a]Irradiance converted from foot-candles to µmol photons m^{-2} s^{-1}
[b]Irradiance converted from lux to µmol photons m^{-2} s^{-1} (Thimijan and Heins 1983)

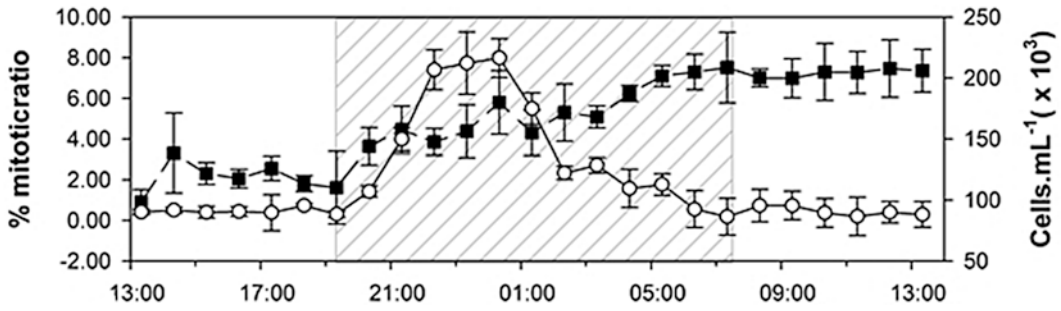

Fig. 11 Mitotic ratio (*open circles*) and cell concentration (*filled squares*) in over 24 h for *Chrysotila* (*Pleurochrysis*) *carterae* grown in a raceway pond (mean ± SE, n = 5) showing the diurnal pattern in cell division where cell division occurs during the night (*shaded portion*) (From Moheimani (2005))

Na^+ are relatively soluble. Among the organic osmoregulatory solutes glycerol is 'infinitely' soluble (i.e. glycerol never crystallises), and glycinebetaine is more soluble than sucrose and trehalose.

- The solutes should be uncharged at physiological pH, as high concentrations of charged solute would present a charge balance problem to the cell. Accumulated inorganic ions must be appropriately balanced by charges on macromolecules and by counterions. Glycinebetaine is a zwitterion, carrying no net charge at neutral pH, whereas glucosylglycerol, sucrose and trehalose are uncharged.
- The solutes must be retained against large concentration gradients across the plasma membrane and this retention can be the result of passive and/or active processes.
- The solutes must be 'compatible' (Brown and Simpson 1972), meaning that at high concentrations they should cause minimal inhibition of enzyme and membrane dependent processes. Some compatible solutes actually have a stabilising influence on enzyme activity (Incharoensakdi et al. 1986; Gabbay-Azaria et al. 1988; Warr et al. 1988) and on the photosystem II complex (Papageorgiou and Murata 1995).

Some compatible solutes protect the cells not only from osmotic stress, but also from other stresses such as temperature extremes, presumably due to their enzyme stabilising capacity (Paleg et al. 1981; Welsh 2000), although this aspect has been very little studied in algae.

In large-scale cultures, especially open air cultures, the salinity of the medium will vary due to evaporation, rainfall and/or the accumulation of salts when the medium is recycled. Thus a degree of tolerance to changing salinity conditions is highly desirable to maintain high productivities in these cultures. Borowitzka and Moheimani (2013b) have also argued that, in order to use saline water in the extremely large scale culture of microalgae for biofuels, it is essential to use algae such as the strains of *Tetraselmis* isolated by Fon Sing and Borowitzka (2015), which have a very wide salinity tolerance as evaporation leads to an increase in salinity in the ponds. Identification of the compatible solute(s) used by a particular alga can be a useful tool in preliminary screening of new strains, as the type of compatible solute is an excellent indicator of the salinity range over which the alga should be able to grow (Reed et al. 1986).

Several species of microalgae of particular interest for large-scale cultures are euryhaline (i.e. they have a wide salinity tolerance) and their growth rates are little affected by major changes in salinity. These include *Dunaliella salina* (salinity range ~40–350‰; Borowitzka and Borowitzka 1988a), *Nannochloris* sp. (salinity range ~5–30‰; Witt et al. 1981) and some *Tetraselmis* spp. (salinity range ~50–120‰; Strizh et al. 2004; Fon-Sing and Borowitzka 2016). Salinity changes in the ponds are usually gradual allowing osmotic adjustment by the algal cells. However, rapid changes in salinity as would occur when ponds are topped up with fresh water should be avoided, as they will result in a temporary cessation of growth while the algal cells acclimate to the new salinity (Borowitzka et al. 1990), thus reducing the overall productivity.

10 Nutrients

Other than CO_2 algae also require an adequate supply of nutrients, especially N and P, to achieve high growth rates. The proximate composition of algal cells can also be manipulated by nutrient concentrations and the forms of the nutrients supplied. The average elemental composition, normalised on C, of freshwater microalgae is $C_{1.0}N_{0.15}P_{0.0094}$ (Oswald 1988b) and for marine phytoplankton it is $C_{1.0}N_{0.129}P_{0.008}$ (Ho et al. 2005), although the N content in particular will vary somewhat with the environmental conditions and nutrient status of the algae (e.g., Beardall et al. 2001; Berges et al. 2002; Geider and Roche 2002). The stoichiometry of other elements is more variable and depends on the taxonomy, cell size and irradiance (Ho et al. 2005; Finkel

et al. 2006; 2010). Since nutrients make up a significant part of the cost of the production of microalgae (Stephens et al. 2010; Acién et al. 2012), a good understanding of the nutrient requirements of each algal species being produced in large-scale cultures is important if nutrient costs are to be reduced. However, in most cases to date, nutrients are supplied on the basis of the same media formulae as used for laboratory cultures, meaning that nutrients are not optimized and are generally in excess. For long-term, semi-continuous cultures the nutrient supply needs to be regularly 'topped up' to sustain ongoing high productivities. Again, this 'top up' of nutrients is not optimized in most operations (Hadj-Romdhane et al. 2012). One other important point is that the nutrient requirements of microalgae grown outdoors in dense cultures under varying environmental conditions may not be the same as those of laboratory cultures grown at low irradiances in a constant environment, and they may vary with season (see for example: Olofsson et al. 2014). Most attempts at medium optimization so far have been based only on laboratory studies and therefore may not be of particular relevance to large-scale outdoor cultures (e.g., Gong and Chen 1997; Mandalam and Palsson 1998; Fabregas et al. 2000; Cogne et al. 2003; Song et al. 2012; Welter et al. 2013).

As several of the products from microalgae of commercial interest are secondary metabolites (e.g. carotenoids) or storage compounds (e.g. triacylglycerols) which are predominantly accumulated by the cells under nutrient-limiting conditions, a two-stage cultivation mode has been proposed for their production (Ben-Amotz 1995; Aflalo et al. 2007; Su et al. 2011). Here, in the first stage, the algae are grown in nutrient-rich medium in order to generate the biomass, and then the algae are transferred to a nutrient-limiting (often N-limiting) medium to stimulate accumulation of the desired secondary metabolite by the cells while growth is greatly reduced or totally inhibited. Since the 2-stage process involves more handling of the cultures, and usually results in lower productivity of the desired product despite the higher per-cell content, it is generally uneconomical.

The various nutrients are treated in detail in other chapters of this volume, and here I will consider briefly only aspects most pertinent to large-scale cultures.

10.1 Nitrogen

Nitrogen is the main nutrient required by algal cultures. Nitrogen is usually supplied as nitrate, ammonium or urea, although nitric acid is also been used in some media. Algae can also utilise nitrite as an N-source (Grant 1967; Luque et al. 1994). When provided with a combination of these nitrogen sources algae generally utilise them in the order: ammonium > nitrate > urea. However, both nitrate and ammonia appear to support similar growth rates when supplied as the sole N source under optimal laboratory culture conditions and only a few studies have shown enhanced growth rates of cells growing on NH_4^+ versus NO_3^- under saturating growth irradiances (e.g., Thompson et al. 1989 and references therein; Herndon and Cochlan 2007).

Light stimulates nitrate and nitrite uptake by microalgae (Grant 1967; Grant and Turner 1969) and there is a general requirement for CO_2 for effective nitrate uptake (Grant 1968; Thacker and Syrett 1972). In freshwater algae in particular, nitrate uptake leads to an alkalinisation of the medium (Eisele and Ullrich 1975), whereas NH_4^+ uptake leads to acidification (Fuggi et al. 1981; Goldman et al. 1982). Uptake of urea has little effect on the pH of the medium. In *Chlamydomonas reinhardtii* several different plasmalemma nitrate transporter genes have been identified, including one bispecific nitrate/bicarbonate transporter (Fernandez and Galvan 2008). Other algae, such as the diatom *Thalassiosira pseudonana*, also have multiple plasmalemma nitrate uptake systems (Bender et al. 2012). The presence of NH_4^+ or nitrite inhibits the assimilation of nitrate (Thacker and Syrett 1972; L'Helguen et al. 2008), however (at least in some algae) the inhibiting effect of NH_4^+ is influenced by temperature, nitrate concentration and other factors, probably because of the relative action of the biphasic high-affinity and low-affinity nitrate transport systems (Dortch 1990; Flynn 1999; Lomas and Glibert 1999).

More details on the physiology of N in microalgae can be found in chapter "Combined Nitrogen" of this book (Raven and Giodano 2016).

As pointed out earlier, caution must be exercised in translating the results of laboratory experiments to outdoor experiments. For example, several laboratory studies have found that ammonium is a suitable N source for the growth of *Dunaliella salina* (Kosmakova and Prozumenshchikova 1983; Dhanam and Dhandayuthapani 2013). However, Borowitzka and Borowitzka (1988b) found that, under laboratory conditions equivalent to those prevailing in outdoor cultures (i.e., very high irradiance, high temperatures, high cell density), the use of NH_4Cl or NH_4NO_3 rather than $NaNO_3$ led to a very rapid acidification of the medium to ~pH 4 and to cell death. The optimum conditions for photosynthesis, as well as the high cell density, greatly amplified the acidification of the medium as compared to what is observed under normal laboratory culture conditions. Furthermore, ammonium also inhibited the production of β-carotene, the desired cell product. Similarly, in *Haematococcus pluvialis* grown on NH_4Cl, the formation of the astaxanthin-containing aplanospores was inhibited compared to cultures grown on KNO_3, although there was little difference in overall growth on the two different N-sources (Borowitzka et al. 1991).

The changes in pH of the medium, as well as associated changes in alkalinity (Brewer and Goldman 1976) due to uptake of NO_3^- and/or NH_4^+, will have effects on the avail-

ability of inorganic C and potentially other nutrient ions, and thus also on the potential productivity of the culture. Supply of nitrogen as NO_3^- and NH_4^+ to enhance productivity is possible through the use of fed batch addition of stiochiometrically-balanced media. Scherholz and Curtis (2013) used a strategy where they supplied small amounts of NH_4^+ periodically in the presence of NO_3^- to maintain the pH in a culture of *Chlorella vulgaris*. This strategy not only maintained pH but also resulted in higher biomass yield.

10.1.1 Nitrogen Metabolism and Greenhouse Gas Emissions

One often overlooked aspect of culturing algae on nitrate or nitrite is the possibility of the production and emission of nitrous oxide (N_2O), a potent greenhouse gas. This has important implications for the use of large-scale microalgal cultures for CO_2 bioremediation and the reduction of greenhouse gases (Flores-Leiva et al. 2010; Ferrón et al. 2012) and the use of algae for wastewater treatment (Mezzari et al. 2013). N_2O production in the presence of nitrite was first demonstrated for microalgal and cyanobacterial cultures by Weathers (Weathers 1984; Weathers and Niedzielski 1986). The mechanism is not well understood and many authors have suggested that these N_2O emissions are due to associated bacteria and not the algae (e.g., Ferrón et al. 2012; Harter et al. 2013). However, recently Guieysse et al. (2013) have demonstrated that axenic cultures of *Chlorella vulgaris* emit N_2O, especially in the dark in the absence of photosynthesis. Mezzari et al. (2013) have also provided evidence that N_2O emissions from lab-scale photobioreactors using *C. vulgaris* to treat ammonia-rich swine wastewater digestate was linked to *Chlorella* metabolism and not to bacterial activity. These findings are in contrast to that of Fagerstone et al. (2011) who found that N_2O production stopped in cultures of *Nannochloropsis salina* when antibiotics were added to the culture to inhibit denitrifying bacteria. These contrasting observations suggest that there may be differences in N_2O forming activity between taxa.

Guieysse et al. (2013) have proposed two possible mechanisms for N_2O formation by *Chlorella vulgaris*. N_2O production is greatly enhanced when nitrite is the sole N source in the dark or in the presence of DCMU (Weathers 1984; Weathers and Niedzielski 1986; Guieysse et al. 2013; Alcántara et al. 2015). Pre-incubating *Chlorella vulgaris* with the nitrate reductase inhibitor tungstate repressed N_2O production, suggesting a key role for nitrate reductase in N_2O production. The enzyme, nitrate reductase (NR), which catalyses the reduction of nitrate to nitrite using NAD(P)H as electron donor, is located in the cytoplasm, whereas nitrite reductase (NiR), which catalyses the reduction of nitrite to ammonium using reduced ferredoxin as electron donor, is localized in the chloroplast (Fischer and Klein 1988). NR can reduce nitrate to nitrite in both the light and the dark, but NiR cannot carry out nitrite reduction if photosynthesis does not regenerate the reduced ferredoxin. Thus NiR repression results in nitrite accumulation in the cytoplasm and its reduction to nitric oxide (NO) mediated by the NR (Sakihama et al. 2002). The NO may then act as a precursor for N_2O formation by a pathway similar to that found in bacterial denitrifiers (Averill 1996). Alternatively, the NR may reduce nitrite to nytroxyl (HNO) which may then dimerise to N_2O in hydrophobic cell areas (Miranda et al. 2003).

10.2 Phosphorus

In large-scale cultures phosphorus is usually supplied in the form of phosphate salts, although in cultures using wastewaters P may also be available in the form of polyphosphates and organic P-containing compounds (Cembella et al. 1982). Phosphate uptake is by a number of different $Na^+/H_2PO_4^-$ symporters (Ullrich and Glaser 1982; Valiente and Avendano 1993; Weiss et al. 2001) and/or H^+/P_i symporters (Gauthier and Turpin 1994). It is therefore not surprising that pH affects P-uptake (Ullrich-Eberius 1973; Ullrich-Eberius and Yingchol 1974). See Dyhrman (2016) for a detailed review of P uptake and metabolism in microalgae.

As inorganic phosphate (P_i) has an important role in cellular energy transduction, limitation of growth by phosphate availability can affect various aspects of metabolism. Large-scale microalgal cultures are usually supplied with sufficient P so that P-limiting conditions should not occur. However, an often overlooked factor is that, as a result of the high pH reached by algal ponds during the day, precipitation of phosphate salts can occur, especially in seawater-based media (Murphy et al. 1983; Sylvestre et al. 1996; Olsen et al. 2006; Larsdotter et al. 2007) and this potentially can lead to P-limitation in the culture. One of the main phosphate precipitates is struvite ($MgNH_4PO_4.6H_2O$) which redissolves slowly, even when the pH of the culture becomes less alkaline during the night (Doyle and Parsons 2002).

Of course, many algae are known to store excess P within the cells as polyphosphate (Miyachi et al. 1964; Cembella et al. 1982; Powell et al. 2009) and this stored 'luxury P' may ameliorate any temporary shortage of P in the medium due to phosphate precipitation, although mobilisation of the intracellular polyphosphate can be slow (Bolier et al. 1992).

10.3 Silicon

Silicon addition to the medium is generally only required for the culture of diatoms which require silicon for the formation of their frustules (chapter "Micronutrients" in this book,

(Finkel 2016)). The silicon requirement of diatoms varies greatly between species (Brzezinski et al. 1990) as does the response of the algae to silicon limitation. Thus, for example, growth of *Phaeodactylum tricornutum* is not affected by silicon limitation, whereas in species such as *Conticribra* (*Thalassiosira*) *weissflogii*, *Cylindrotheca fusiformis* and *Chaetoceros muelleri* growth ceases, and in species such as *Chaetoceros simplex* and *Skeletonema costatum* silicon limitation leads to cell death (D'Elia et al. 1979; Brzezinski et al. 1990).

Unlike nitrogen and phosphorus uptake, which are closely linked to photosynthetic metabolism, the energy for silicic acid uptake in diatoms comes from aerobic respiration (Martin-Jézéquel et al. 2000). Silicic acid transport and cell wall silicification are also closely coupled to the cell cycle (Sullivan and Volcani 1981). Diatom growth is also more rapidly inhibited under Si starvation than under nitrogen or phosphorus formation (Parslow et al. 1984; Harrison et al. 1990).

10.4 Iron

Iron limitation of phytoplankton has long been recognised as a major factor in the oceans (Behrenfeld et al. 2009) and iron is an important and essential component of all algae media (McLachlan 1973; Nichols 1973). In photosynthetic organisms iron is essential to maintain the Fe-rich electron transport chains (Raven et al. 1999; Marchetti and Maldonado 2016). Thus, it is important to supply Fe to large-scale cultures. In most laboratory media Fe is supplied in a chelated form such as ferric EDTA, ferric citrate or ferrous ascorbate (Andersen et al. 2005; Botebol et al. 2014). However, these chelated Fe sources are expensive and experience with large-scale cultures has shown that ferric chloride can be used to supply Fe for these cultures (personal observations). Recently iron nanoparticles have been shown to be an excellent source of Fe (Kadar et al. 2012), but whether their use is possible in large scale cultures remains to be determined. The uptake of Fe by microalgae is an active process, and a wide variety of uptake mechanisms has been described for different microalgae taxa (see chapter "Iron" in this book, (Marchetti and Maldonado 2016)). The Fe requirements of microalgae are also species-specific (Ryther and Kramer 1961).

Although iron is essential for algae, iron is also highly reactive and potentially toxic because of the Fenton reaction which produces hydroxyl radicals (Halliwell and Gutteridge 1992). Irrespective of the Fe-source, too high concentrations of Fe can, however, result in reduced growth and potentially in cell death (Belyanin et al. 1979; Wang et al. 2013b; Huang et al. 2014). Therefore the addition of Fe to algal media, especially if the medium is recycled, must be carefully managed.

10.5 Trace Elements

Microalgae also require a range of trace elements such as Cu, Se, Ni, Bo, Br, Zn, Mo etc. (see chapter "Micronutrients" this book, (Quigg 2016)). However, the exact requirements for most species are not well known, if at all. There have been only a few studies on the micronutrient requirements of microalgae (Song et al. 2012).

The need to supply these trace elements is dependent, in part, on the composition of the water source used in the cultures. For example, if seawater is used there is generally no need to supply additional trace elements to large-scale cultures. However, depending on the freshwater source used, freshwater algae cultures may need to be supplemented with specific trace elements. However, if the medium is recycled (or in very high density cultures) some trace elements may become a limiting factor to growth. For example, when urea is used as an N source, Ni is required for the functioning of the urease enzyme (Price and Morel 1991; Dupont et al. 2008). A Ni requirement has also been shown for *Graesiella* (*Chlorella*) *emersonii*, although this species lacks a urease (Soeder and Engelmann 1984). Similarly, Zn is a co-factor for carbonic anhydrase (Moroney et al. 2001), although in some cases the Zn can be replaced by Mo (Yee and Morel 1996; Lane and Morel 2000).

10.6 Vitamins

More than 50 % of cultured microalgae species require an exogenous supply of vitamin B_{12} (cobalamin), over 20 % require vitamin B_1 (thiamine) and only about 5 % require vitamin B_7 (biotin) (Croft et al. 2006). They key requirement for cobalamin seems to be as a cofactor for methionine synthase (METH) which catalyses the C1 transfer from methyltetrahydrofolate to homocysteine to make methionine. An alternative, B_{12}-independent, form of methionine synthase (METE) is also found (Helliwell et al. 2011). The presence of these enzymes varies between algal species so that, for example, the green alga *Chlamydomonas reinhardtii* and the diatom *Phaeodactylum tricornutum* have both METE and METH and are B_{12} independent, whereas another diatom, *Thalassiosira pseudonana*, which contains METH only, is B_{12} dependent. On the other hand, the B_{12}-independent red alga *Cyanidioschyzon merolae* has METE only (Helliwell et al. 2011). Interestingly, or fortuitously, most of the species currently cultured on a large-scale, such as *Dunaliella salina*, *Haematococcus pluvialis* and *Chlorella* spp., do not have a vitamin requirement (see supplementary table in Croft et al. 2006); however, as the species to be cultured expand, the provision of supplemental vitamins may have to be reconsidered although this requirement potentially may be met by associated heterotrophic bacteria which could supply the vitamin requirements in non-axenic cultures

of algae which are auxotrophic with respect to certain vitamins (Croft et al. 2005; Droop 2007; Kazamia et al. 2012). However it is still possible that vitamins may become limiting in very high density cultures.

10.7 Recycling of the Culture Medium

For cost-effective production of microalgae, recycling of the growth medium after harvesting the biomass is desirable. This can present potential problems due to the build-up of metabolites excreted by the cells (Zlotnik and Dubinsky 1989; Burkiewicz and Synak 1996; Kirpenko et al. 2012), organic matter resulting from cell death and/or build-up of other chemicals which may be used in the harvesting process, such as flocculant chemicals. There may also be an accumulation of certain ions such as the counter ions of NH_4^+, CO_3^{2-}, PO_4^{3-} in salts such as NH_4Cl, $NaHCO_3$ and KH_2PO_4 which are not, or are only poorly, assimilated by the algae (Hadj-Romdhane et al. 2012). This build-up may impact negatively on the growth of the algae (Khatri et al. 2014). Several studies have found that some algae produce compounds, generally termed autoinhibitors, which inhibit their own growth (Imada et al. 1992; Lívanský et al. 1996; Richmond et al. 2003), or cell wall remnants which remain after harvesting and which apparently can also inhibit growth (Rodolfi et al. 2003). However, almost all short-term laboratory studies on recycling of media have not shown any effects on growth as long as the nutrients consumed (including trace elements) during the previous growth cycle are replaced (Kim et al. 2011; Rwehumbiza et al. 2012; Wu et al. 2012; Hadj-Romdhane et al. 2013). The one exception is the study by Issarapayup et al. (2011) which found that recycling the growth medium of *Haematococcus pluvialis* resulted in reduced growth, despite careful replacement of all nutrients.

Long-term, repeated recycling may, however, give different results compared to short term recycling. The few long term studies have shown either some growth inhibition when the medium was recycled after harvesting (Lívanský et al. 1996; culture of *Scenedesmus* or *Chlorella*) or no detrimental effects of medium recycling (Samson and Leduy 1985 – culture of *Arthrospira*; but see Belay et al. 1994). In at least one case, the recycled medium gave better growth than fresh medium (Fon Sing et al. 2014; culture of a halophilic *Tetraselmis* sp.), possibly because dissolved organic matter released during harvesting by electroflocculation provided an additional carbon source allowing mixotrophic growth. The effects of the recycled medium on algal growth are also affected by the method of harvesting. For example, Kim et al. (2011) harvested *Scenedesmus* by flocculation and observed no inhibitory effect of the recycled medium, whereas Lívanský et al. (1996) harvested the algae by centrifugation, which is more likely to cause some cell breakage, and did observe growth inhibition by the recycled medium. In the current commercial culture of *Arthrospira* spp. and *Dunaliella salina* long-term recycling of the medium is commonly practiced without any detrimental effects (Borowitzka and Borowitzka 1990; Belay 2008). Whether the differences observed are mainly species-specific or are related to the method of harvesting is still unknown.

At least one species, *Desmodesmus* (*Scenedesmus*) *subspicatus*, releases compound(s) which affect the cell cycle and enhance its growth and photosynthesis (Grabski and Tukaj 2008; Grabski et al. 2010). One would therefore expect stimulation of growth if recycled medium is used, although this has not been demonstrated as yet. Pheromones which induce and control the sexual cycles of species of green algae and diatoms have also been identified (Frenkel et al. 2014). The potential action of such compounds when using recycled medium however will be greatly dependent on how labile these compounds are.

11 In-Culture Evolution

It is often implicitly assumed that algal cultures originating from a single cell (clonal cultures) remain genetically uniform over time. However, as well illustrated in the review by Lakeman et al. (2009), the occurrence of genetic changes and associated changes in phenotype are common in cultures of microalgae. Spontaneous mutation is an inevitable consequence of DNA replication and thus is unavoidable in any growing culture. Many studies have shown that spontaneous mutants arise at non-trivial rates in microalgae cultures (Reboud and Bell 1997; Costas et al. 1998; Kassen and Bell 1998; Goho and Bell 2000; Colegrave et al. 2002; Collins and Bell 2004; Lakeman and Cattolico 2007; Perrineau et al. 2014a). Because of the rapid growth rates and high cell densities in laboratory and outdoor cultures, rare mutations that affect the phenotype are likely to arise (e.g., Lakeman and Cattolico 2007). Not only can point mutations and small-scale insertions/deletions produce mutations as a result of DNA replication errors, but large genome modifications can be made by the movement of mobile genetic elements (transposons). Various classes of transposons have been found in most species of microalgae that have been sequenced, such as the diatoms *Thalassiosira pseudonana* (Armbrust et al. 2004) and *Phaeodactylum tricornutum* (Bowler et al. 2008), and the green algae *Dunaliella viridis* (Sun et al. 2006), *Chlamydomonas reinhardtii* (Merchant et al. 2007) and *Ostreococcus tauri* (Derelle et al. 2006). Even larger genomic rearrangements have been found in cultures of *Conticribra weissflogii*, where whole and partial genome duplication (polyploidy and aneuploidy) events were inferred from differences in the cellular DNA content and the ratio of gene copy number

to genomic DNA quantity among an assortment of strains and sub-clones (von Dassow et al. 2008). Importantly, the copy number of three separate genes varied inconsistently among sub-clones of a single strain. These sub-clones had been cultured less than 3 years since being established from a single progenitor clone. Another factor which can introduce genetic variation to cultures is recombination during sexual reproduction. Sexuality has been described for many, but not all, species in culture (Blackburn and Parker 2005; Coleman and Pröschold 2005; von Dassow and Montresor 2011) and cryptic sexuality may also be present (Grimsley et al. 2010) leading to further genetic diversity.

In culture algae undergo several changes due to the changed environmental conditions in the culture environment as compared to the original habitat. The first and immediate change is physiological; i.e. acclimation to the new conditions of irradiance, temperature and medium composition. Over longer time frames epigenetic adaptation may take place, allowing heritable changes in gene regulation or expression to occur. This, though heritable, happens without any change in the DNA itself. Finally, genetic adaptation arises through the differential inheritance of genetic variation arising from the processes described in the preceding paragraph. This may lead to evolutionary change in the culture due to selection driving the phenotype of the culture to a new optimum. The phenotypes that determine optimum temperature, irradiance, nutrient level or salinity needed for growth are 'quantitative' traits (Mackay 2001), meaning that variation in the traits occurs across a continuous spectrum and has a polygenetic basis. Table 4 lists some of the changes that have been observed in long-term cultures of microalgae. Ultimately the laboratory population may show quite different properties from the originally isolated alga.

As in laboratory cultures, the long-term culture of algae outdoors will lead to the development of a cell population consisting of a variety of genotypes which have arisen by in-culture evolution. However, to date there has been only one study attempting to compare the evolutionary changes in outdoor cultures with those in indoor cultures (Pais 2011). This study compared physiological changes in cultures of a halophilic *Tetraselmis* sp. grown indoors and also outdoors in raceway ponds for 14 months in semi-continuous culture. It was found that the outdoor culture had apparently lost the ability to acclimate to low irradiances.

The observations that algae undergo evolutionary changes in culture have implications for the management of industrial, large-scale, production of microalgae. First, if it is desired to ensure the fidelity of a particular algae strain with highly desirable characteristics it will be necessary to periodically restart the cultivation, using an inoculum from a stock culture. To avoid the possibility of genetic changes in the stock culture this must be cryopreserved rather than being maintained in liquid cultures (Day and Brand 2005; Hédoin et al. 2006). Alternatively, one can use a strategy of using specific culture conditions to apply selective pressure to the culture in the ponds or photobioreactors to select for and maintain the desired phenotype (Borowitzka 2013d). Several recent studies have shown that natural populations have a wide genetic and phenotypic diversity (Liang et al. 2005; Iglesias-Rodríguez et al. 2006; Kashtan et al. 2014), and through the use of selective environments such as can be created in large-scale culture systems, selection of a strain or population with the desirable characteristics is potentially possible. This strategy has been used successfully for the production of *Dunaliella salina* in the extremely large open ponds at Hutt Lagoon, Western Australia. The large pond size and long residence times preclude the introduction of specific strains of *D. salina*. Therefore the strategy has been to use selective culture conditions which favour the growth of highly a carotenogenic strains of *D. salina* from the autochthonous population over less productive strains as well as over the other non-carotenogenic *Dunaliella* species such as *D. viridis* and *D. bioculata* which co-occur in these ponds (Moulton et al. 1987).

Table 4 Changes in long-term cultures of algae attributed to genetic or epigenetic evolution

Change	Species	Reference
Photoautotrophy	*Poterioochromonas malhamensis* CCAP933/1c	Jones et al. (1996)
Temperature range tolerated	*Chloromonas augustae* (as *Chlamydomonas augustae*) UTEX LB1969	Hoham et al. (2002)
	Chloromonas rosae UTEX1337	
Shear tolerance	*Lingulodinium polyedrum*	Von Dassow et al. (2005)
Increased salt tolerance	*Chlamydomonas reinhardtii*	Perrineau et al. (2014a)
Improved growth rate on acetate	*Chlamydomonas reinhardtii*	Perrineau et al. (2014b)
Adaptation to liquid/solid medium	*Chlamydomonas reinhardtii*	Goho and Bell (2000)
Loss of nitrate reductase	*Chlamydomonas reinhardtii*	Harris (2009)
Loss of toxicity	*Alexamdrium minutum* (as *A. lusitanicum*)	Martins et al. (2004)
	Alexandrium tamarense	Cho et al. (2008)
Loss of circadian rhythm	*Lingulodinium polyedrum*	Sweeney (1986)
Loss of sexuality	*Pandorina*	Coleman (1975)

12 Culture Monitoring and Management

A key requirement of commercial large-scale cultures is the reliability of the culture. This is achieved, in the first instance, by the selection of the algal strain and a well-designed and operated culture system. However, regular monitoring of the culture is also important to detect early signs of possible problems, so that remedial action can be taken. Relatively simple monitoring methods, based on the physiological behavior of the algae, are commonly used. These include monitoring the diurnal changes in photosynthesis by measuring changes in pond O_2 concentrations or pH, or by monitoring the CO_2 consumption, if the culture is being operated at constant pH regulated by the addition of CO_2. Any deviation from the normal pattern will indicate changes in the culture. Furthermore, changes in chlorophyll concentration per cell can indicate changes in the physiology and 'health' of the cultures. Of course, microscopic observation of the algae culture is very important and can be very informative. Newer methods, such as on-line measurement of fluorescence using pulse amplitude modulated (PAM) fluorometry (Masojidek et al. 2011) and a range of multispectral methods, are currently being developed (Reichardt et al. 2012; Murphy et al. 2014; Reichardt et al. 2014; Sarrafzadeh et al. 2015). These methods can provide data, not only on biomass and pigment composition, but also on aspects of the physiological state of the algae (Ralph et al. 2011; Shelly et al. 2011). PAM fluorometry measurements (i.e. F_v/F_m, rETR, quenching analysis) can be very important and informative, however they do require a good understanding of algal physiology and how this affects the fluorescence signal to be best able to interpret the information gathered (Masojidek et al. 2011).

The options for management of large-scale cultures are relatively limited due to the large volumes of culture involved. Open pond cultures are also more limited in the factors which can be controlled compared to closed photobioreactors. However, closed photobioreactors are more expensive to construct and operate and have reduced scalability (Lehr and Posten 2009). Both open systems and closed photobioreactors can also experience fungal infections, especially from parasitic chytrid fungi (Abeliovich and Dickbuck 1977; Gutman et al. 2009) and the management of these infections is, as yet, little understood and studied (Carney and Lane 2014). The infectivity of the parasites, at least in some cases, appears to be influenced by factors such as irradiance, temperature, the nutrient status of the algal culture, and the population density of the algal culture (Bruning 1991a, b, c, d; Kühn and Hofmann 1999; Gsell et al. 2013). Different strains of the same species may also be differentially susceptible to infection (De Bruin et al. 2004). Whether any of these factors affecting chytrid infections can be used in culture management of such infections to reduce them remains to be seen.

As light is the most important factor limiting productivity, the available irradiance must be used efficiently. In order to achieve the most effective use of the available irradiance one can adjust the cell density and/or the pond depth or reactor thickness (i.e. the length of the light path), in order to achieve the OCD (see section "Light"). This method was first proposed by Azov et al. (1980) to optimize high-rate sewage oxidation ponds, but is equally applicable to 'clean' algae cultures. Clearly the OCD will change throughout the year with the change in irradiance, and therefore the cell density and/or pond depth will need to be adjusted.

In open ponds it is practically and economically impossible to regulate the temperature – thus the only practical and the easiest option available is the selection of strains with suitable temperature optima (Borowitzka 2013d). In closed photobioreactors temperature regulation is possible, but it does come at a cost and may not be economically feasible in all cases. However, cooling of the photobioreactors during the day may be essential. Similarly, although increased mixing stimulates growth, there are economic constraints on the amount of mixing that can be applied due to energy costs. Similarly, the salinity of the culture can be controlled, but this can require very large amounts of fresh water (Borowitzka and Moheimani 2013b).

Aside from maintaining the culture as close as possible to the OCD, the main factor that can be used to optimize productivity is the amount and nature of the nutrients supplied. Thus, the determination of the nutrient requirements and their optimization is an important task for efficient large-scale culture. However, this is not easy to do as the nutrient requirements of large-scale outdoor cultures generally are not the same as those determined using small-scale laboratory cultures due to the different environmental conditions. The provision of an adequate supply of inorganic carbon for photoautotrophic culture is also extremely important as almost all large-scale algal cultures are CO_2-limited. Unless a 'free' supply of waste CO_2 from a power station or cement plant or similar source is available nearby, something which is rarely the case (Darzins et al. 2010), the design of the CO_2 supply system becomes very important in order to maximize the absorption of the CO_2 in the culture medium and to minimize losses to the atmosphere.

13 Using Culture Conditions to Manipulate Cell Composition

Large-scale microalgal cultures are grown to produce algal biomass as a source of specific compounds, or with a particular biochemical composition for specific applications. Algae species and strains are selected not only for their ability to be cultured on a large scale, but also for their composition, and the composition can be manipulated to some degree by cul-

ture conditions. However, it must be recognised that only a limited number of culture factors can be manipulated, practically and cost effectively, in large-scale cultures.

The organic components of the cell can be classified broadly as either primary or secondary metabolites. A primary metabolite is one that is directly involved in normal growth, development, and reproduction, generally performing an intrinsic physiological function. A secondary metabolite is one that is not essential for the normal functioning of the cell, but one which may have a supporting role in cell survival under certain conditions. Secondary metabolites are usually derived from primary metabolites or, as in the case of some of the carotenoids, may be the same as primary metabolites but have a different physiological function.

Most of the high-value products of microalgae, such as the carotenoids β-carotene and astaxanthin, are secondary metabolites. Similarly, the triacylglycerols produced by microalgae under nutrient-limiting conditions are also secondary metabolites. A common feature of secondary metabolites is that they are accumulated by the cells under conditions where growth is inhibited (often called 'stress' conditions). A number of different culture strategies are being used, or have been proposed, to optimize the productivity of these secondary metabolites in cultures. These strategies are often species-specific.

Here I describe two examples of these strategies for selected high value secondary metabolites: β-carotene production by *Dunaliella salina*, astaxanthin production by *Haematococcus pluvialis*, as well as a brief consideration of the production of lipids and fatty acids.

13.1 *Dunaliella Salina* and β-Carotene

Dunaliella salina, the best source of the carotenoid β-carotene, with the cell content reaching up to 14 % of dry weight, is cultured in Australia in very large shallow unmixed ponds of several hundreds of hectares in area and, in Israel, China and India, in paddle wheel driven raceway ponds of up to 3000 m^2 in area (Borowitzka 2013a). *D. salina* is the most salt tolerant eukaryotic organism known, growing over a salinity range of about 10–35 % (w/v) NaCl (Borowitzka and Borowitzka 1988a). *D. salina* also has a very wide temperature tolerance, ranging from less than 0 °C to over 40 °C (Post 1977; Wegmann et al. 1980), and the tolerance of high temperature is increased when the algae are grown at higher salinities (Henley et al. 2002). The optimum growth temperature for *D. salina* is between 20 and 40 °C and is somewhat dependent on the strain and the growth irradiance (Gibor 1956; Borowitzka 1981a). The main reason for this extreme environmental tolerance is that this alga uses glycerol as the compatible solute (Brown and Borowitzka 1979; Ben-Amotz 1980) coupled with very efficient Na^+ transport systems, which enable the maintenance of a fairly constant and low cytoplasmic Na^+ concentration over a wide external salinity range (Pick et al. 1986; Katz and Pick 2001).

The accumulation of β-carotene occurs in lipid droplets in the chloroplast and is a function of the salinity and the total irradiance received by the cells (Loeblich 1982; Ben-Amotz and Avron 1983). With increasing irradiance the rate of carotenoid accumulation is increased and the maximum level of carotenoid that can be accumulated is greatly influenced by the salinity, with higher levels achieved at higher salinities (Borowitzka and Borowitzka 1990). Interestingly, if the PAR irradiance (400–700 nm) supplied by cool-white fluorescent lights is supplemented with a small amount of UV-A (320–400 nm) light, growth and carotenogenesis are both slightly stimulated (Salguero et al. 2005). The redox state of the plastoquinone pool in the photosynthetic electron transport chain is thought to be the sensor for the induction of carotenogenesis (Pfannschmidt 2003), similar to the green alga *Haematococcus pluvialis* where inhibition of electron flow from photosystem II to plastoquinone prevents carotenogenesis by the downregulated expression of carotenoid biosynthesis genes (Steinbrenner and Linden 2003). In *D. salina*, UV-A exposure also stimulates increased β-carotene accumulation (Jahnke 1999; Salguero et al. 2005), possibly as a response to the formation of photosynthetically produced oxygen radicals (Shaish et al. 1993), but the exact mechanism of this UV-A effect is still unclear (Jahnke et al. 2009). Nutrient limitation – especially nitrogen, phosphorus, and/or sulphate limitation – also stimulates carotenogenesis (Lerche 1937; Ben-Amotz and Avron 1983), presumably via an effect on lipid synthesis, as the accumulation of β-carotene in the chloroplast requires triacylglycerol synthesis for the formation of the chloroplast lipid droplets in which the β-carotene is localised (Rabbani et al. 1998; Mendoza et al. 1999; Lamers et al. 2010). Interestingly, the ratios of the major ions in brines, i.e. Ca^{2+}:Mg^{2+} and Cl^-:SO_4^{2-} ratios, also affect growth and carotenoid formation (Mil'ko 1962; Ben-Amotz and Avron 1983).

The high levels of carotenoid have a photoprotective effect. Comparison of the photosynthetic rate of a low-carotenoid (green) culture with a high-carotenoid (red) culture of the same *D. salina* strain showed that both cultures had the same maximum photosynthetic rate (P_{max}) of ~600 μmol O_2 mg^{-1} chl *a* h^{-1}; however photosynthesis saturated at about 1300 μmol photons m^{-2} s^{-1} in the 'green' culture and higher irradiances caused marked photoinhibition, whereas in the 'red' culture photosynthesis saturated at 2300 μmol photons m^{-2} s^{-1} (Borowitzka et al. 1984). In later study, Gômez Pinchetti et al. (1992) obtained similar results.

The combination of a high content and productivity of a valuable metabolite, β-carotene, as well as the ability to grow under extreme environmental conditions made *D. salina* an ideal candidate for commercial-scale production and the first

pilot-scale trials were started in the USSR in 1966 (Massyuk 1966; Massyuk and Abdula 1969), with the first commercial plants starting operations in the early 1980s in Israel, Australia, and the USA (Ben-Amotz and Avron 1990; Borowitzka and Borowitzka 1990; Schlipalius 1991).

The best environmental conditions to achieve high β-carotene productivities are high irradiance and high temperatures. Laboratory studies showed that the highest biomass productivities were obtained at salinities less than 24 % NaCl, but the highest cell β-carotene content was obtained at the highest salinities (i.e. greater than 24 % NaCl) when the growth rate is reduced. Thus β-carotene is a typical secondary metabolite. On the basis of these laboratory studies the optimum salinity for maximum carotenoid productivity was determined to be about 25 % NaCl (Borowitzka et al. 1984) (Fig. 12). In practice, however, in the Hutt Lagoon production plant in Australia the salinity in the algal ponds ranges from about 25 % NaCl to NaCl saturation (~35 % NaCl). These higher, suboptimal, salinities are a compromise as the very large size of the ponds and the high evaporation rate in summer means that the ponds cannot be maintained at the lower salinity in practice since seawater is used as the water source used to replace evaporative losses. The higher salinity has the advantage, however, of minimising problems with predators such as the protozoans *Fabrea salina* and *Heteroamoeba* sp. (Post et al. 1983) as well as contamination by other algae species which can occur at salinities below 20 % (Moulton et al. 1987). This is particularly important in winter when rain can reduce the salinity in the ponds; the higher salinity means that rain does not reduce the salinity below the critical value allowing significant protozoan growth.

An important aspect of the relationship between salinity and carotenoid content is that although an increase in salinity leads to a rapid rise in cell carotenoid content, the decline in carotenoid content following a similar scale decrease in salinity is very slow, and is mainly due to partitioning of the carotenoid between the cells as they divide, rather than carotenoid breakdown (Borowitzka et al. 1984, 1990).

The high salinity of the brines, high temperatures (often >35 °C), and the high pH of the ponds greatly reduce the solubility of CO_2 in the ponds; the solubility of CO_2 at 15 °C in 2 M NaCl is 1.2 g L^{-1}, while at 40 °C it is only 0.53 g L^{-1} (Lazar et al. 1983). The algae are therefore very C-limited and the addition of CO_2 (impractical at this scale) or HCO_3^- markedly stimulates growth. *Dunaliella salina* has an unusual salt-resistant external α-type carbonic anhydrase (Premkumar et al. 2003) and increased carbonic anhydrase activity at high salinities (Booth and Beardall 1991; Fisher et al. 1996a) allowing efficient use of HCO_3^- as a carbon source for photosynthesis. The solubility of iron is also limited at high salinities and *D. salina* has an unusual mechanism for Fe uptake, mediated by a transferrin-like protein (Katz et al. 2009).

The large ponds used in Australia are unmixed and therefore the biomass productivity is low, although the *Dunaliella* cells have a high β-carotene content because of the high irradiance. Biomass productivity is significantly higher in the well mixed raceway ponds used in Israel (Ben-Amotz 1999). Decreasing pond depth from the 20–30 cm depths commonly used and thus increasing the irradiance received by the algal cells would also increase the carotenoid productivity. The higher irradiances would not be a problem for the carotenoid-containing 'red' cells as they are not photoinhibited even at very high irradiances; however, in large raceway ponds and in the very large ponds in Australia very low pond depths are not achievable due to hydraulic limitations of these systems.

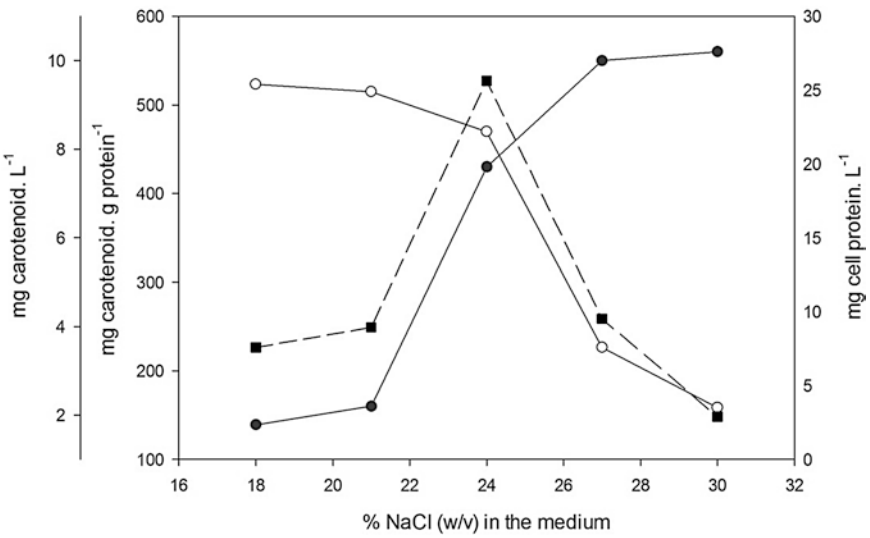

Fig. 12 Biomass production (*open circle*) and carotenoid content in cells (*filled circle*) in *Dunaliella salina* W5 at different salinities. The calculated carotenoid production per liter of culture is also shown (*filled square*). Cultures were grown in modified Johnson's medium (Borowitzka 1988a) at an irradiance of 400 µmol photons m^{-2} s^{-1} with a 12:12 h light:dark cycle at 33 °C during the day and 28 °C at night. Early stationary phase cultures were sampled (Redrawn from Borowitzka et al. (1984))

13.2 *Haematococcus pluvialis* and Astaxanthin

The study of the physiology and biochemistry of the freshwater green alga *Haematococcus pluvialis* presents some challenges unique amongst the microalgae. First, there is great variation in the physiological responses to various environmental factors and in the nutrient requirements (Stross 1963; Borowitzka 1992; Howieson 2001) and productivity (Zhang et al. 2009) between different strains of *H. pluvialis*. This diversity is also reflected in molecular phylogenetic studies (Mostafa et al. 2011; Buchheim et al. 2013). Second, as the alga undergoes major morphological changes during growth and carotenogenesis (see below for details), the appropriate design of experiments and the interpretation of the results are complicated. Unfortunately, not all researchers carefully monitor the specific cell types present in their cultures during the experiments focusing on only one or a few parameters such as the astaxanthin content, sometimes making the comparison of data from the literature extremely difficult.

Haematococcus pluvialis is grown in large-scale cultures as a commercial source of the oxygenated carotenoid, astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione). Unlike *Dunaliella salina*, and all other microalgae grown at a large scale, *H. pluvialis* needs to pass through several stages of its life cycle during the culture process (Elliott 1931; Boussiba 2000). The green flagellated motile zoospores are the fast-growing cells which produce the required biomass in culture. Once the medium becomes nutrient limited or due to other environmental stresses such as high temperature, high light or increased salinity cell division slows and the green flagellated cells start to accumulate small, but visible, quantities of carotenoids in the cytoplasm near the nucleus. The cells then lose their flagella, round up and begin to develop a thick cell wall, eventually forming the thick walled, non-motile aplanospores (haematocysts). Concomitant with the formation of aplanospores accumulation of astaxanthin continues until the astaxanthin-containing droplets completely fill the cytoplasm. Astaxanthin in *Haematococcus* is mainly in the form of monoesters of astaxanthin linked to 16:0, 18:1 and 18:2 fatty acids. All of the free astaxanthin and its monoesters and diesters have an optically pure 3*S*,3′*S* chirality (Renstrøm et al. 1981; Grung et al. 1992). The aplanospores can accumulate up to about 6 % of dry weight as astaxanthin, although in commercial production the content is usually about 2–4 %.

The optimum temperature for most *H. pluvialis* strains is between 25 and 28 °C and only slightly higher temperatures (i.e. 30 °C) can lead to cessation of cell division and the onset of aplanospore formation (Fan et al. 1994; Tjahjono et al. 1994). Strains with significantly lower temperature optima are also known (Borowitzka et al. 1991; Klotchkova et al. 2013). As pointed out above, nutrient limitation retards growth and stimulates aplanospore formation and astaxanthin synthesis. Both nitrogen limitation and phosphorus limitation are effective (Borowitzka et al. 1991; Boussiba and Vonshak 1991; Boussiba et al. 1999), although nitrogen limitation has a greater effect (Harker et al. 1996b). Fe^{2+}, Mn^{2+} and Cd^{2+} also increase the cellular carotenoid content but, with the exception of Fe^{2+}, these ions also inhibit growth (Harker et al. 1996b) possibly via the generation of reactive oxygen species, especially hydroxyl radicals (Kobayashi et al. 1993). Salinity stress (0.1–0.5 % w/w NaCl) also induces astaxanthin accumulation (Harker et al. 1996a; Kobayashi et al. 1997b).

Haematococcus pluvialis can grow photoautotrophically, mixotrophically and heterotrophically (Droop 1961; Kobayashi et al. 1992b; Olaizola 2000). Currently the major commercial producers culture the algae photoautotrophically. The algae are grown in a 2-stage process: First the algal biomass in the form of the flagellate cells is produced in closed photobioreactors such as bubble columns, plate reactors or tubular photobioreactors at the optimal temperature and in a nutrient replete medium, usually with CO_2 addition using a pH-stat system. This is then followed by a carotenoid accumulation step in medium with reduced N and P in high light (usually daylight), either in raceway ponds (e.g. by Cyanotech, Hawaii) or tubular photobioreactors (Algae Technologies, Israel), or in internally lit, closed photobioreactors (e.g. by AstaReal, Sweden and USA). This whole growth process takes several weeks (Boussiba et al. 1997; Lorenz and Cysewski 2000). A single-stage method for producing the astaxanthin-containing, non-motile, palmelloid cells has also been developed (Del Rio et al. 2005; García-Malea et al. 2009); however, the astaxanthin productivity of this process is lower than in the 2-step process (Aflalo et al. 2007).

As the cells change from the motile flagellate stage to the aplanospore stage they undergo changes in morphology, metabolism and composition as outlined below.

The Green Flagellates These pear-shaped cells are biflagellate, actively motile with the cell covered by an extracellular matrix of variable thickness with an external trilamellar crystalline layer (Hagen et al. 2002). The extracellular cell matrix (ECM) is traversed by cytoplasmic strands. As these cells are very shear sensitive (Vega-Estrada et al. 2005; Powtongsook et al. 2006) the method used to circulate (mix) the culture is very important to achieve high growth rates. Cell division is highest in these cells and *Haematococcus* can be maintained in this cell stage, apparently indefinitely, when grown heterotrophically in the dark (Kobayashi et al. 1997a). The duration of the flagellate stage can also be extended in media with a low N/P ratio (Tocquin et al. 2012). According to Lee and Ding (1994) the flagellated cells divide only a maximum of

about five times before they turn into the non-motile palmelloid cells, however this observation may be specific to the culture conditions used.

The Green/Reddish Palmelloid Cells At the onset of nutrient limitation or under other 'stress' conditions the flagellate cells lose their flagella and the cells become spherical and increase in size. The first indication of carotenoid accumulation can also be seen, usually near the nucleus at the center of the cell. As with the flagellate cells, irradiance is an important factor affecting cell growth and survival, and the rate of astaxanthin accumulation (Lee and Soh 1991). However, high irradiances can lead to severe photoinhibition of photosynthesis or photooxidative damage to the cells, especially the 'green' flagellate cells and the palmelloid cells (Hu et al. 2008; Li et al. 2010). This detrimental effect of high irradiances can be reduced by adjusting the cell density in the photobioreactor so the cells receive an optimal irradiance (Wang et al. 2013a).

The astaxanthin-containing flagellate and palmella cells have a higher photosynthetic rate on a chlorophyll basis (Hagen et al. 2000), a higher P_{max} (Wang et al. 2003) and a higher F_v/F_m (Hagen et al. 1994; Wang et al. 2011) than the green flagellate cells.

The Red Aplanospores With ongoing nutrient limitation or other stresses cell division is reduced markedly and the palmelloid cells begin to form a new cell wall within the extracellular matrix and develop into the thick walled aplanospores. The cell wall of the aplanospores consists of 70 % carbohydrates, (66 % hexoses), 3 % cellulose and 6 % proteins, and contained 3 % acetolysis-resistant material (algeenan). Analysis of the monosaccharides shows mannose as the main part of the carbohydrate dry mass (89 ± 4 %), with only small amounts of other sugars (glucose, 6 ± 4 %; arabinose, 1 ± 6 %; xylose, 1 ± 3 %) (Hagen et al. 2000). Secondary carotenoid formation (mainly astaxanthin) continues rapidly in the developing aplanospores with the astaxanthin-containing oil droplets eventually occupying much of the cytoplasmic volume and over 50 % of the total cell volume (Collins et al. 2011; Wayama et al. 2013).

The red aplanospores remain photosynthetically active, although they have a lower photosynthetic rate (Tan et al. 1995; Fan et al. 1998; Qiu and Li 2006), a lower P_{max} (Zlotnik et al. 1993; Tan et al. 1995), and a slightly lower F_v/F_m (Fan et al. 1998; Chen et al. 2012) and an associated reduction in cytochrome *f* (Tan et al. 1995), than the palmelloid cells. Furthermore, the aplanospores also have an extremely high temperature tolerance in excess of 50 °C (unpublished results).

There are also other physiological changes associated with the development of the different cell types. For example, the activity of Rubisco changes with cell type, being highest in the astaxanthin containing palmelloid cells (Chen et al. 2012), and lowest in the aplanospores (Tan et al. 1995). The chlorophyll *a*/*b* ratio increases as the cells start to accumulate carotenoids in the cytoplasm and the total chlorophyll content of the red aplanospores is markedly reduced compared to the green flagellate cells (Solovchenko et al. 2011).

In *Haematococcus*, N-starvation initially leads to accumulation of carbohydrates (possibly starch), before the onset of increased lipid synthesis and astaxanthin accumulation (Recht et al. 2012). In electron micrographs of astaxanthin-accumulating early stage aplanospores (Fig. 4a) a large number of starch granules can be seen in the chloroplast and a starch sheath also surrounds the pyrenoid (see Fig. 4a in Wayama et al. 2013). It has been suggested that at least part of the accumulated carbohydrate is used for astaxanthin synthesis (Recht et al. 2012). As in other algae, N-starvation results in a reduced protein content and there is no increase in biomass (Dong et al. 2007). However, the protein content per g dry weight also declines in N-replete cultures under high irradiance but not under low irradiance, although the biomass increases in both conditions. Based on the correlation between protein decline and astaxanthin increase, Dong et al. (2007) proposed that protein catabolism provides carbon to carotenoid synthesis, as the increase in astaxanthin in the high light cultures is accompanied by an increase in NH_4^+ in the medium, although it is also possible that the proteins have a role in the cell wall formation which accompanies astaxanthin formation.

Light is very important for astaxanthin formation, and the rate of formation is light dependent (Goodwin and Jamikorn 1954; Yong and Lee 1991; Kobayashi et al. 1992a, 1997a; Dong et al. 2007), but there can be astaxanthin synthesis at a reduced rate in the dark if an organic carbon source is available (Droop 1955; Kobayashi et al. 1992b). When cells are exposed to increased irradiance and low nitrogen concentration the concentrations of isopentyl pyrophosphate isomerase (Sun et al. 1998) and phytoene desaturase (Grünewald et al. 2000) increase. The enzyme, phytoene synthase (PSY) is the first committed step in carotenoid synthesis and is under photosynthetic control (Steinbrenner and Linden 2001). In green flagellate cells transcript levels of lycopene synthase, phytoene synthase, phytoene desaturase and carotenoid hydroxylase increased in response to increased light. Using inhibitors, Steinbrenner and Linden (2003) showed that the photosynthetic plastoquinone pool functioned as a redox sensor for the up-regulation of these carotenoid biosynthesis genes.

The synthesis of astaxanthin requires not only the activation of the genes and the corresponding enzymes of the carotenoid synthesis pathway, but also the availability of carbon precursors, both for pigment synthesis and for the synthesis of the fatty acids of the oil droplets in which the carotenoids are located (Schoefs et al. 2001; Zhekisheva et al. 2002). Chaumont and Thepenier (1995) monitored

growth and astaxanthin formation in flagellate cells grown in a tubular photobioreactor outdoors, operated as a chemostat, over a 24 h period. They found that astaxanthin synthesis was initiated at daybreak and there was a correlation between irradiance and astaxanthin synthesis while cell division proceeded. Later in the day, the period of the most heightened synthesis of astaxanthin was correlated with the maximum irradiance. At this time dry weight formation slowed down in viable cells and total dry weight actually decreased due to some cell lysis. It is likely that available C was directed away from growth and towards the synthesis of the photoprotectant astaxanthin. When the carotenoid level was high enough to provide adequate photoprotection (estimated at 1 % w/w), dry weight again increased. Astaxanthin concentration later decreased with decreasing irradiance in the afternoon. The rapidity of the response to changes in irradiance suggests a dynamic reallocation of available carbon between biomass generation and carotenogenesis. The importance of sufficient carbon for astaxanthin biosynthesis is also shown by the study of Kang et al. (2005) who showed that provision of an additional carbon source, either as CO_2, bicarbonate, or acetate, enhanced astaxanthin synthesis, with CO_2 being the best carbon source. However, to achieve cells with a high astaxanthin content and productivity it is important that the cultures are not light-limited (Kang et al. 2007).

If the aplanospores are returned to favorable conditions of nutrients, temperature and irradiance mitosis occurs and biflagellate zoospores are formed and released. Although the zoospores may contain a portion of the astaxanthin of the parent aplanospore, this astaxanthin is rapidly metabolised and decreases markedly within hours, leading to the formation of the 'green' flagellate cell stage (Elliott 1931; Lee and Ding 1994; Borowitzka personal observations; Fábregas et al. 2003). Only when the zoospores remain exposed to high irradiance is the decline in astaxanthin content reduced (Wang et al. 2003). In this aspect *H. pluvialis* differs markedly from *D. salina* where, when the cells are transferred to non-carotenogenic optimal growth conditions, the total β-carotene content in the culture declines only very slowly at a time-scale of weeks and the per cell β-carotene content declines mainly due to cell division and the partitioning of the β-carotene between the daughter cells (Borowitzka et al. 1984). Unfortunately the biochemical and physiological changes occurring during aplanospore germination and the excystment of the zoospores in *H. pluvialis* are almost totally unstudied. However, a possible reason for the differences in the rate of carotenoid breakdown between *H. pluvialis* and *D. salina* may lie in the location of the carotenoid-containing lipid bodies; i.e. in the cytoplasm in *H. pluvialis* and within the chloroplast stroma in *D. salina*. The catabolism of the lipids and associated carotenoids in the carotenoid-containing lipid bodies requires the action of lipases. Clearly the lipase activity in *H. pluvialis* must be greater than that in *D. salina*, and high lipase activity in the chloroplast could have undesired effects on the structure and photosynthetic function of the thylakoids. However, as the natural habitats of *H. pluvialis* are mainly shallow temporary pools in which the alga survives in the form of the aplanospores when they dry out, rapid mobilisation of the significant carbon reserves which the astaxanthin-containing lipid droplets represent, can be critical in allowing a rapid population increase before growth conditions again become unfavorable. The implication for large-scale cultures of *Haematococcus* is that there is potential for rapid loss of carotenoids if culture conditions change.

13.3 Lipids and Fatty Acids

Microalgae have long been of interest as sources of lipids and hydrocarbons for biofuels and in applications such as cosmetics, as well as specific fatty acids such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (AA) as nutritional supplements. There is great variation between taxa in their lipid and fatty acid composition (Borowitzka 1988b; Guschina and Harwood 2006; chapter "Lipid Metabolism in Microalgae" in this book; Khozin-Goldberg 2016), reflecting the diverse evolutionary origins of the various algal phyla. Many microalgae can have very high lipid contents, with total lipid contents ranging from about 10 % of ash-free dry weight, to over 70 % in cells which have been in stationary phase for a long time (Aach 1952; Guschina and Harwood 2013). In general, the total lipid content of actively growing algae is in the range of 10–20 %, and in most microalgal species this can be increased under conditions where growth is inhibited, especially by N-limitation (Shifrin and Chisholm 1981; Griffiths et al. 2012), or by Si limitation in diatoms (Roessler 1988; Jiang et al. 2015). Selecting the correct species and strain clearly is the first critical step to producing algal biomass with a desired lipid and fatty acid composition and content. Many laboratory studies have shown that culture conditions such as irradiance, temperature, pH, salinity, nutrients etc. have important effects on the lipid content and the lipid composition of the cell and, therefore, also on the fatty acid composition (Guschina and Harwood 2013 and chapter "Lipid Metabolism in Microalgae", this book; Khozin-Goldberg 2016).

In large-scale cultures the fatty acid composition and the productivity of specific fatty acids can be manipulated to some degree. For example, Chrismadha and Borowitzka (1994) showed how the EPA content and productivity of the diatom *Phaeodactylum tricornutum* cultured in a tubular photobioreactor can be optimised by manipulating both cell density and irradiance. The improved EPA productivity was a function of both a higher EPA content in the biomass and a faster growth rate. In laboratory studies of *Nannochloropsis* spp. low temperatures increased the cell content of EPA (Sukenik et al. 1993); however, in outdoor studies the EPA

content appears to be relatively temperature insensitive and the EPA productivity is primarily a function of the biomass productivity (Chini Zitelli et al. 1999; Camacho-Rodríguez et al. 2014). Similar results have been obtained with other species of algae such as *Pavlova lutheri* and *Chaetoceros muelleri* (Jacobsen et al. 2012). These results reflect the fact that the *n*-3 polyunsaturated fatty acids (PUFAs) such as EPA and DHA are predominantly located in the polar lipids (mostly phospholipids and glycolipids) which are common membrane components (Dunstan et al. 1993) that change relatively little with growth conditions.

On the other hand, the *n*-3 PUFA, arachidonic acid (AA) is a significant component of triacylglycerols (TAGs) which are storage products mainly accumulated in stationary phase cells. For example, in the red alga *Porphyridium cruentum* N-starvation enhances the accumulation of TAG from 21 to 61 % of total lipids (Cohen 1990) and the proportion of AA in the TAG increases from 20 to 31 % (Cohen et al. 1988). Similarly, N-starvation in the trebouxiophycean alga *Lobosphaera* (*Parietochloris*) *incisa* leads to TAG production in the form of cytoplasmic oil droplets and an increase in the proportion of AA in the neutral lipids of up to 64 %. The AA in the TAG constituted over 90 % of the cellular AA, mainly in the form of triarachidonylglycerol (Khozin-Goldberg et al. 2002). AA production is also enhanced in high density light-limited cultures (Cohen and Khozin-Goldberg 2005). Interestingly, EPA is also a significant component of TAGs in the xanthophyte *Trachydiscus minutus* and the proportion of EPA can be manipulated by nitrogen and phosphorus starvation (Řezanka et al. 2011).

The differences between the lipids which contain the PUFAs of interest (i.e. membrane lipids for EPA and DHA, and storage lipids for AA) mean that different culture strategies are required to optimize their productivity. For maximum EPA and DHA productivity the prime objective is to increase biomass productivity, whereas for AA, which is effectively a secondary metabolite, the productivity will be highest at some intermediate biomass productivity at a high AA cell content (Sukenik 1991; Solovchenko et al. 2008; Cepák et al. 2014). This has been shown in outdoor cultures in plate photobioreactors with different light path lengths (Cheng-Wu et al. 2002) and in vertical tubular photobioreactors at different cell densities and nutrient contents (Tababa et al. 2012).

Sukenik and Carmeli (1990), using *Nannochloropsis*, showed that storage triacylglycerols are synthesized in the light and rapidly metabolized in the dark (Fig. 13a). Polar lipids such as MGDG are also synthesized in the light, but they turn over slowly in the dark. Thus, in a phased (partially synchronized) culture, as might be expected in an outdoor culture, the cell content of EPA increased during the day and decreased at night, mainly due to cell division during the night. The total mass of EPA per unit culture volume increased during the day but remained fairly stable during the night. Furthermore, the consumption of neutral storage lipids for cell maintenance during the night caused an increase in the relative proportion of EPA at the end of the night period (Fig. 13b). Thus, if the production aim is to produce lipids with a high EPA content, harvesting of the algae at around the end of the night period would be advantageous.

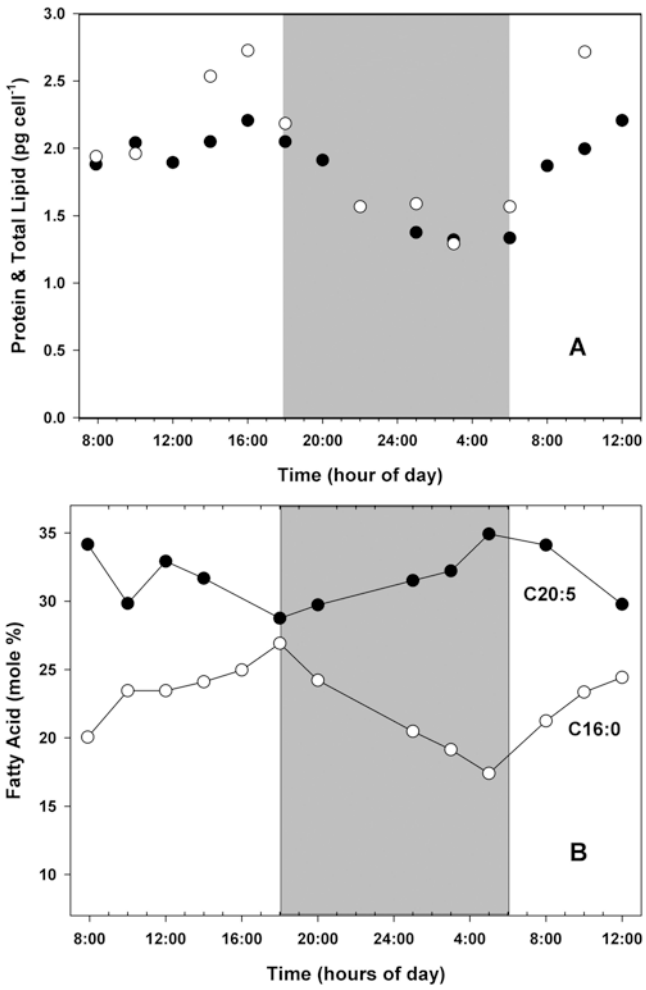

Fig. 13 Diurnal changes in composition in a phased culture of *Nannochloropsis* sp. grown on a light:dark cycle. The shaded area indicates the dark period. (**a**) Changes in cellular content of total lipids (*open circles*) and protein (*closed circles*); (**b**) changes in the proportion of the major fatty acids EPA (*C20:5*) and palmitic acid (*C16:0*) (Data from Sukenik and Carmeli 1990)

14 Conclusions

The large-scale commercial culture of microalgae is now a well-established industry and several new species and products are currently under development. However, unlike land-based intensive agriculture, our understanding of the basic biology, physiology and biochemistry of microalgae in out-

door production systems is still very limited and is based on only a very small number of species. Furthermore the extreme evolutionary diversity of microalgae which include both prokaryotic and eukaryotic organisms from a number of phyla, as well as the diversity of habitats from which the algae are isolated, means that much remains yet to be discovered. Although the principal factors which limit algal productivity are well defined (i.e. light, temperature, nutrients etc.) the evolutionary diversity of the microalgae also means that different taxa have evolved different adaptations to the availability of these factors. The understanding of the interactions of factors, both physical and chemical, also remains a challenge.

This chapter has highlighted aspects of the physiology of microalgae as it relates to the large-scale outdoor culture of these algae. Understanding the physiology and biochemistry of the algae can be used in the process of selecting species and strains potentially suitable for large-scale outdoor culture, to optimise productivity, as a means of monitoring the cultures and to manipulate the biochemical composition of the algal cells. For efficient and effective strain selection it is essential to understand the environmental conditions prevailing in large-scale outdoor cultures and in the culture system to be used. This allows screening of newly isolated strain for those physiological characteristics which make the strain suitable for outdoor culture. As pointed out by Borowitzka (2013d), some part of the screening should be carried out outdoors wherever possible, especially to test the capability of the strain(s) to acclimate to outdoor conditions.

As outdoor conditions are fundamentally different from conditions in the laboratory conclusions based purely on laboratory studies must be considered carefully and, where possible, confirmed by outdoor trials. Similarly, in order to understand the physiological processes underlying the observations on outdoor cultures growing under what are very variable conditions, it is necessary to return to the laboratory where conditions can be controlled. To date, the number of published studies on large-scale outdoor cultures is limited and they are for only a very small number of species. Most studies are either small scale or only indoor laboratory studies. Scale-up, in particular, presents an important problem and the outcomes are still somewhat unpredictable because we do not adequately understand the physiological responses to the changes in the environmental conditions the algae experience as ponds and photobioreactors get larger. However, great progress has been made since the initial isolation of microalgae into pure cultures in the 1890s by Beijerinck (1890) and Miquel (1892) and the beginning of larger-scale outdoor culture in the 1950s (Burlew 1953a; Tamiya 1957). Our understanding of the physiology and ecology of natural algae populations and algae blooms also provides important insights which can aid in optimising and managing the artificial 'blooms' which constitute outdoor algae cultures.

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Part VII

Systematics and Taxonomy

Systematics, Taxonomy and Species Names: Do They Matter?

Michael A. Borowitzka

1 Introduction

In this book considerable effort has gone into ensuring, as far as possible, that the currently accepted names for algae species are used. Why is this important?

It is important to consider first what we mean the name/term **algae**.[1] The term 'algae' (singular 'alga') is not a taxonomic term, but is a common collective name and a term of convenience for all the, apparently primitive, plant-like organisms which contain chlorophyll *a*, have oxygenic photosynthesis (usually) and are not specialised land plants (the embryophytes) like mosses, ferns, coniferous trees and flowering plants. Bold and Wynne (1978) distinguished algae from other chlorophyllous plants where in the algae, (i) when unicellular, every cell may function directly as gametes, (ii) when multicellular, producing either unicellular or multicellular gametangia and, in the latter case, where every gametangial cell is fertile. This distinction separates the algae from other green plants, but only partially defines the group as it excludes many asexual taxa and those taxa which have lost the ability to photosynthesise. Clearly, it is very difficult to provide an accurate and comprehensive definition of 'algae' since the algae are not monophyletic and are currently placed in four kingdoms: Bacteria, Plantae, Chromista, and Protozoa. The simple alternative, but circular, definition of algae suggested by Entwhistle and Huisman (1998) as: '*those organisms studied by phycologists*' [Phycologists being those people that study algae !] may thus be quite practical. Microalgae (the subject of this book) are broadly defined as those algae where the individual organism generally requires a microscope to be recognised. Microalgae can be unicellular, colonial or filamentous.

2 What's in a Name?

Biologists currently use a system of naming based on the Linnaean system and, in order to minimise confusion (and avoid anarchy), the naming of biological organisms is regulated by four widely accepted codes of nomenclature. Formal biological names have a number of important characteristics that are shared only partially, or not at all, by common names. Valdecasas et al. (2014) list these characteristics as: (i) individuation; (ii) hypothesis of relationship; (iii) retrieval of information; (iv) explanatory power, (v) testable predictions, (vi) conceptual power, and (vii) language.

The species[2] name is a shorthand identifier for a taxonomic entity and provides an important information retrieval tool, even more so in this age of electronic on-line searching and large-scale databases. However, the species name also has limitations. Firstly, the species must have been correctly identified and the correct name applied. Correct identification is not a trivial effort and seeking the help of experienced taxonomists (unfortunately these are a rare breed) is encouraged to ensure the most accurate identification possible. It is always also good practice to provide information on how the specimen was identified and to lodge reference specimens (including samples of DNA if available) or culture in a recognised repository such as a herbarium or culture collection and to lodge gene sequences in Genbank or a similar recognised database. This will allow later confirmation (or otherwise) of the identification. Secondly, because of new insights and data on species, there may be a reappraisal of

[1] The word 'alga' comes from the Latin meaning 'seaweed'. The study of algae is called 'phycology' rather than 'algology' as 'algology' can be confused with the Greek word άλγολογία which is the study of pain. Phykos (φῦκος) is the Greek word for seaweed.

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[2] What constitutes a species is actually quite difficult to define and the species concept varies with taxonomic group. A discussion of this can be found in Guiry (2012).

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the species and its relationship to other taxa leading to possible changes in the name. Therefore one needs to be aware of these changes if one wishes to retrieve all the information available on the organism. Although one of the important aims of the codes of nomenclature is to encourage the stability of names, taxonomy, as other areas of science, does not stand still; new knowledge about species and their phylogenetic relationships will result changes in species delineations and species concepts and thus in the need to reclassify species resulting in name changes. In recent years major advances have been made in the study of the systematics of algae, especially the microalgae where taxonomies based on the limited morphological characters available are clearly unsatisfactory. Molecular analyses are providing new characters well beyond the morphology-based systematics of the past, leading to a much better understanding of what may constitute a species, as well as the phylogenetic and evolutionary relationships between species. In the future developments in proteonomics and metabolomics will also impact on our understanding of what constitutes a species and will enhance our understanding. However, thanks to several taxonomic databases, especially AlgaeBase (http://www.algaebase.org/) for the names of algae it is now relatively easy for a researcher to check the current as well as the past names (synonyms) of a particular species.

In publications the species name (binomial) alone is not sufficient, especially for the microalgae. For effective and accurate data retrieval and communication the strain designator (strain number) is also extremely important. Two strains may initially have been identified as the same species, but later studies may show them to belong to different species or, even if they are the same species, to show some genetic and important phenotypic differences in physiology, some of which may have arisen in culture over time. An excellent example of this is the very widely studied green alga *Chlamydomonas reinhardtii*. The genealogy of the standard laboratory strains of *C. reinhardtii* has been reconstructed. These 'standard *C. reinhardtii*' strains are all allegedly derived from the meiotic products of a single zygote isolated by G.M. Smith in 1945 from a site in Massachusetts, USA (Harris 1989). Based on the studies of Harris (1989) and Kubo et al. (2002), Pröschold et al. (2005) used use analyses of the nuclear internal transcribed spacer (ITS) regions and other genetic traits to resolve inconsistencies in the earlier studies and provided a more detailed genealogy of the commonly used *C. reinhardtii* strains with three basic sublines (see Figure 3 in Pröschold et al. 2005). Although these *C. reinhardtii* strains comprise one morphological species and one biological species of high sexual intercompatability, with essentially identical ITS sequences (except the tip of helix I of ITS2), they show some physiological differences. For example, subline II strains can grow on nitrate, whereas subline III strains cannot. This difference appears to be the result of unrecorded sexual crosses, probably of the original Smith isolate with another compatible mating strain.

Important physiological and biochemical variations in isolates and clones of the same species have also been shown in diatoms (Happey-Wood and Hughes 1980; Terry et al. 1983; Liang et al. 2005; Van den Wyngaert et al. 2015), dinoflagellates (Costas et al. 1998; Loret et al. 2002; Cho et al. 2008) haptophytes (Alonso et al. 1992), and cyanobacteria (Vonshak et al. 1996) etc.

The above examples show the importance of being able to clearly track strains as well as species in order to compare results of different researchers. All studies of microalgae cultures therefore should always clearly identify the strain used, so that later studies can account for any inconsistencies in results and to allow reinterpretation of the available information. Unfortunately, many journals do not insist on a clear strain designation in published papers, thus potentially limiting the usefulness of the results of the published work. An example of the importance of strain numbers is the recent determination that the widely studied strain *Chlorella vulgaris* UTEX2714 is actually *Chlorella sorokiniana* (Bashan et al. 2015). Similarly, *Chlorella vulgaris* C-169 which has been used as a model organism in pioneering studies on green algal chromosome architecture (Noutoshi et al. 1998; Yamamoto et al. 2003), was reclassified in a different Class on the basis of sequence data as *Coccomyxa subellipsoidea* (Blanc et al. 2012). Another widely used alga is *Isochrysis* aff. *galbana* T-Iso (strain numbers from different culture collections = CCAP927/14; CCMP1234, CS-177 etc.) often also referred to as 'Tahitian *Isochrysis*' which is extensively used in aquaculture. It has now been found not to be an *Isochrysis* and has been reclassified in a new genus as *Tisochrysis lutea* (Bendif et al. 2013). The fact that the 'Tahitian *Isochrysis*' as well as another tropical isolate (strain C-ISO, now CCMP 463 and also a *T. lutea*), differed from other *Isochrysis* strains with respect to the sterol and long-chain alkenones composition was already noted by Wikfors and Patterson (1994) but the importance of this observation was not recognised. Even though several authors have arbitrarily changed the name from *Isochrysis* aff. *galbana* to *I. galbana* or *Isochrysis* sp. in their publications (e.g., Pernet et al. 2003; Rocarati et al. 2004; Lin et al. 2007; Sayegh and Montagnes 2011), the actual species can be tracked because they also included the strain designation. However, where the strain number has not been provided it is impossible to be certain which species was actually used. Typographical mistakes also occur; for example in their paper Timmermans et al. (2005) refer to *Prasinomonas capsulatus* (CCMP 1617), however the genus *Prasinomonas* does not exist and the correct name of this strain is *Prasinococcus capsulatus*. As the strain number was provided the error in the publication can be corrected.

New taxonomic tools such as molecular studies lead to new insights into the speciation of algae. An excellent example of this is the diatom *Skeletonema costatum*, which has been found to show extensive cryptic speciation. Using light microscopy, transmission electron microscopy, scanning electron microscopy, and by comparing these morphological features with sequence data from small subunit rDNA and partial large subunit rDNA, Sarno et al. (2005) identified eight distinct entities (species) in strains which had previously been identified generally as *S. costatum*. Further analysis of *Skeletonema* species by Kooistra et al. (2008) has led to the updating of the taxonomy of *Skeletonema* strains available in culture collections. These findings are further reflected in important physiological variations between strains (Balzano et al. 2011). *Skeletonema costatum* (sensu lato) probably represents an extreme example of variation within what had been considered a single species; the other 'extreme' is the diatom *Thalassiosira pseudonana*, where a recent examination of publicly available strains showed that they clearly all belonged to the same species (Rad-Menéndez et al. 2015).

Many researchers are now using gene sequences and Blast searches in Genbank to identify new strains of algae isolated. Although Genbank is a very useful source of information, it is not necessarily reliable as there is little quality control applied to the information lodged in this database (Vilgalys 2003; Bidarttondo et al. 2008; Kang et al. 2010). It was never created to be a taxonomic reference source. The species names associated with sequences may not be correct or may have changed over time and therefore, as a minimum, the original references to these sequences should be checked to confirm that the name associated with them is a correct and valid one. The ability to trace the sequence(s) back to a specific strain is critically important.

Good taxonomy and species names are also very important in the mundane world of commerce. In many countries of the world the use of algal products as nutraceuticals, health foods or pharmaceuticals requires approval and registration (Gellenbeck 2012; Borowitzka 2013). This approval is usually for one or more named species of algae. Incorrectly applied names and/or name changes due to taxonomic reassignment potentially can create problems for the commercial use of the algal product (see for example Champenois et al. 2015).

To assist in reconciling species names and to find the taxonomic affiliation of species this chapter includes two tables at the end. Table 1 is a quick reference source and lists the commonly used species names which have changed and their currently accepted names based on records in AlgaeBase (Guiry and Guiry 2015). Table 2 lists all the currently accepted algae names cited in this book according to their taxonomic placement. The higher taxonomic levels are those used in AlgaeBase. Other higher taxonomic classifications which attempt to best reflect phylogeny are under development (e.g., Adl et al. 2005, 2012), and they are largely in agreement with the classification used here.

In the scientific literature there are also names for species which have not been validly described according to the International Code of Nomenclature for algae, fungi, and plants (Melbourne Code) (McNeill et al. 2012) or described at all. For example the *Ostreococcus lucimarinus* for the isolate of *Ostreococcus* CCE9901 (Worden et al. 2004) is first mentioned in the paper by concerning the fully sequenced genome of this alga (Palenik et al. 2007). Similarly, *Dunaliella bardawil* was never validly described and named, however since the original paper first mentioning this name (Ben-Amotz et al. 1982) many papers have been published using this name. The strain is actually an isolate of *Dunaliella salina* (Borowitzka and Siva 2007). This misuse of species names can cause a lot of confusion for researchers new to the field. Another 'orphan' name is *Chloromorum toxicum*. This name is mentioned in Giner et al. (2008), where it is noted that this new genus and species are being described in a 'submitted' paper. However, it appears that this 'submitted' paper has as yet not been published, and all we know that it appears to be a raphidophyte based on pigment analysis and molecular phylogenetic analysis (Thomas et al. 2004; Edvardsen et al. 2007). Another, as yet not described, genus is '*Euhalothece*'. Garcia-Pichel et al. (1998) first used this name for an extremely halophilic cluster of cyanobacteria in a phylogenetic analysis based on 16S rRNA gene sequences. This cluster name seems then to have been adopted as a genus name by Mikhodyuk et al. (2008) and others, however no description of the genus can be found in the literature.

3 Conclusion

Good science relies on effective transmission of information. One aspect of this is good taxonomy and the use of a commonly agreed system of nomenclature. Thus, although taxonomy at times does not seem to be a very exciting field of research, it underpins almost all of biological research. Furthermore, where the algae are in culture, the ability to track a particular strain via the strain number or code through the literature means that, even if the original identification is ultimately shown to be incorrect or the name is changed due to taxonomic revision, the research findings pertaining to this strain can be retrieved and compared, and knowledge is not lost. Ultimately, a research paper is only as good as the identification of the organisms being studied and if the identity of the organism is not verifiable it is hard to impossible to extend or verify the findings of the research.

Table 1 Summary of older microalgae species names and the currently accepted name mentioned in this book

Older name/synonym	Currently accepted name	Reference for synonym
Anabaena variabilis	*Trichromus variabilis*	Komárek and Anagnostidis (1989)
Astasia longa	*Euglena longa*	Marin et al. (2003)
Chlamydomonas mexicana	*Chlamydomonas oblonga*	Ettl and Gärtner (1995)
Chlamydomonas sajao	*Lobochlamys segnis*	Pröschold et al. (2001)
Chlorella emersonii	*Graesiella emersonii*	Nozaki et al. (1995)
Chlorella kessleri	*Parachlorella kessleri*	Krienitz et al. (2004)
Chlorella zofingiensis	*Chromochloris zofingiensis*	Fucíková and Lewis (2012)
Chroomonas salina	*Rhodomonas salina*	Hill and Wetherbee (1989)
Chrysochromulina polylepis	*Prymnesium polylepis*	Edvardsen et al. (2011)
Chrysotila lamellosa	*Ruttnera lamellosa*	Andersen et al. (2014)
Crocosphaera watsonii[a]	*Cyanobium waterburyi*	Komárek et al. (1999)
Cylindrotheca closterium	*Ceratoneis closterium*	Jahn and Kusber (2005)
Cymbella microcephala	*Encyonopsis microcephala*	Krammer (1997)
Dictyosphearium pulchellum	*Mucidosphaerium pulchellum*	Bock et al. (2011)
Dunaliella bardawil	*Dunaliella salina*	Borowitzka and Siva (2007)
Goniotrichum elegans	*Stylonema alsidii*	Yoon et al. (2006)
Karlodinium micrum	*Karlodinium veneficum*	Bergholtz et al. (2006)
Microcoleus chthonoplastes	*Coleofasciculus chthonoplastes*	Siegesmund et al. (2008)
Microcoleus sociatus	*Trichocoleus sociatus*	Anagnostidis (2001)
Monodus subterraneus	*Monodopsis subterranea*	Hibberd (1981)
Neochloris oleoabundans	*Ettlia oleoabundans*	Deason et al. (1991)
Nitzschia closterium	*Ceratoneis closterium*	Jahn and Kusber (2005)
Ochromonas malhamensis	*Poteriochromonas malhamensis*	Peterfi (1969)
Parietochloris incisa	*Lobosphaera incisa*	Karsten et al. (2005)
Pavlova salina	*Rebecca salina*	Green (1976) and Edvarsen et al. (2000)
Pediastrum simplex	*Monactinus simplex*	Corda (1839)
Plectonema boryanum	*Leptolyngbya boryana*	Anagnostidis and Komárek (1988)
Pleurochrysis carterae	*Chrysotila carterae*	Andersen et al. (2014)[b]
Pleurochrysis haptonemofera	*Chrysotila haptonemofera*	Andersen et al. (2014)
Prorocentrum minimum	*Prorocentrum cordatum*	Dodge (1975) and Velikova and Larsen (1999)
Porphyra perforata	*Pyropia perforata*	Sutherland et al. (2011)
Rhodella reticulata	*Dixoniella grisea*	Scott et al. (1992)
Scenedesmus acutus	*Scenedesmus communis*	Hegewald and Hanagata (2000)
Scenedesmus obliquus	*Acutodesmus obliquus*	Hegewald and Hanagata (2000)
Scenedesmus communis	*Desmodesmus communis*	Hegewald (1977, 2000a)
Scenedesmus subspicatus	*Desmodesmus subspicatus*	Hegewald (2000b)
Spirulina maxima	*Arthrospira maxima*	Komárek and Anagnostidis (2005)
Spirulina platensis	*Arthrospira platensis*	Komárek and Anagnostidis (2005)
Synechococcus bacillaris	*Cyanobium bacillare*	Komárek et al. (1999)
Thalassiosira weissflogii	*Conticribra weissflogii*	Stachura-Suphoples and Williams (2009)

Note that not all species names found in this book where the name has changed are listed. Only the more commonly occurring names are listed here

[a]According to M Guiry (2015) this species was never described and no authority for the names is known. However, the name is widely used in the literature

[b]Note that the name in this paper was misspelled as *C. carteri* in the paper due to an editorial error (R.A. Andersen, Personal communication)

Table 2 Listing of all species names in this book organised according to their taxonomic placing. Only the currently accepted names are listed (the information is predominantly based on AlgaeBase (Guiry and Guir 2015)

Empire: Prokaryota
Kingdom: **Eubacteria**
Phylum Cyanobacteria
Class Cyanophyceae
Chroococcales
Aphanothecaceae
Aphanothece halophytica Frémy
Aphanothece nidulans Richter
Chroococcaceae
Asterocapsa nidulans (N.L.Gardner) Komárek & Komárková-Legnerová
Chroococcus minutus (Kützing) Nägeli
Chroococcus submarinus (Hansgirg) Kovácik
Entophysalidaceae
Johannesbaptistia pellucida (Dickie) W.R.Taylor & Drouet
Gomphospheriaceae
Snowella Elenkin
Microcystaceae
Gloeocapsa gelatinosa Kützing
Microcystis aeruginosa (Kützing) Kützing
Microcystis flosaquae (Wittrock) Kirchner
Microcystis ichthyoblabe (G.Kunze) Kützing
Microcystis panniformis Komárek, Komárková-Legnerová, Sant'Anna, M.T.P.Azevedo, & P.A.C.Senna
Microcystis viridis (A.Braun) Lemmermann
Microcystis wesenbergii (Komárek) Komárek ex Komárek
Radiocystis fernandoi Komárek & Komárková-Legnerová
Gloeobacterales
Gloeobacteraceae
Gloeobacter kilaueensis J.H.W. Saw et al.
Gloeobacter violaceus Rippka, Waterbury & Cohen-Bazire
Nostocales
Aphanizomenonaceae
Aphanizomenon ovalisporum Forti
Chlorogloeopsidaceae
Chlorogloeopsis A.K.Mitra & D.C.Pandey
Hapalosiphonaceae
Fischerella maior Gomont
Fischerella muscicola Gomont
Hapalosiphon fontinalis Bornet
Mastigocladus laminosus Cohn ex Kirchner
Microchaetaceae
Hassallia byssoidea Hassall ex Bornet & Flahault
Nostocaceae
Anabaena aequalis Borge
Anabaena augstumalis Schmidle
Anabaena cylindrica Lemmermann
Anabaena flosaquae Brébisson ex Bornet & Flauhault
Anabaena oscillarioides Bory de Saint-Vincent ex Bornet & Flahault
Anabaena sphaerica Bornet & Flahault
Anabaena torulosa Lagerheim ex Bornet & Flahault
Cyanospira capsulata Florenzano, Sili, Pelosi & Vincenzini
Cylindrospermum licheniforme Kützing ex Bornet & Flahault

(continued)

Table 2 (continued)

Cylindrospermum majus Kützing ex Bornet & Flahault
Cylindrospermopsis raciborskii (Woloszynska) Seenayya & Subba Raju
Desmonostoc muscorum (C.Agardh ex Bornet & Flahault) Hrouzek & Ventura
Dolichospermum lemmermannii (*Richter*) *P.Wacklin*, *L.Hoffmann & Komárek*
Dolichospermum sigmoideum (Nygaard) Wacklin, L.Hoffmann & Komárek
Dolichospermum spiroides (Klebhan) Wacklin, L.Hoffmann & Komárek
Nodularia baltica Komárek, M.Hübel, H.Hübel & Smarda
Nodularia harveyana Thuret ex Bornet & Flahault
Nodularia spumigena Mertens ex Bornet & Flahault
Nostoc calcicola Brébisson ex Bornet & Flahault
Nostoc carneum C.Agardh ex Bornet & Flahault
Nostoc commune Vaucher ex Bornet & Flahault
Nostoc flagelliforme Komárek
Nostoc insulare Borzì
Nostoc linckia Bornet ex Bornet & Flahault
Nostoc punctiforme Hariot
Nostoc spumigena (Mertens) Drouet
Trichormus dolium (Bharadwaja) Komárek & Anagnostidis
Trichormus fertilissimus (C.B.Rao) Komárek & Anagnostidis
Trichormus variabilis (Ralfs ex Bornet & Flahault) Komárek & Anagnostidis
Rivulariaceae
Calothrix parietina Thuret ex Bornet & Flahault
Rivularia C.Agardh ex Bornet & Flahault
Scytonemataceae
Diplocolon Nägeli ex Bornet & Flahault
Scytonema hofmannii C.Agardh ex Bornet & Flahault
Scytonema javanicum Bornet ex Bornet & Flahault
Scytonema ocellatum Lyngbye ex Bornet & Flahault
Tolypothrichaceae
Tolypothrix tenuis Kützing ex Bornet & Flahault
Pseudoanabaenales
Schizotrichaceae
Schizothrix Kützing ex Gomont
Trichocoleus sociatus (West & G.S.West) Anagnostidis
Scytonemataceae
Scytonema hofmannii C. Agardh ex Bornet & Flahault
Scytonema javanicum Bornet ex Bornet & Flahault
Oscillatoriales
Coleofasciculaceae
Geitlerinema amphibium (C.Agardh ex Gomont) Anagnostidis
Gloeotrichaceae
Gloeotrichia echinulata P.G.Richter
Microcoleaceae
Microcoleus autumnalis (Gomont) Strunecky, Komárek & J.R.Johansen
Microcoleus lacustris Farlow ex Gomont
Microcoleus paludosus Gomont
Pseudophormidium battersii (Gomont) Anagnostidis
Pseudophormidium golenkinianum (Gomont) Anagnostidis
Oscillatoriaceae
Lyngbya aestuarii Liebman ex Gomont

(continued)

Table 2 (continued)

Lyngbya confervoides C.Agardh ex Gomont
Oscillatoria corallinae Gomont ex Gomont
Phormidiaceae
Arthrospira maxima Setchell & N.L.Gardner
Arthrospira platensis Gomont
Coleofasciculus chthonoplastes (Thuret ex Gomont) M.Siegesmund, J.R.Johansen & T.Friedl
Phormidium bohneri Schmidle
Phormidium minimum O.C.Schmidt
Phormidium pseudopriestleyi Anagnostidis & Komárek
Planktothrix agardhii (Gomont) Anagnostidis & Komárek
Trichodesmium erythraeum Ehrenberg ex Gomont
Trichodesmium thiebautii Gomont ex Gomont
Tychonema bourrellyi (J.W.G.Lund) Anagnostidis & Komárek
Wilmottia murrayi (West & G.S.West) Strunecký, Elster & Komárek
Synechococcales
Acaryochloridaceae
Acaryochloris marina H.Miyashita & M.Chihara
Leptolyngbyaceae
Halomicronema excentricum Abed, Garcia-Pichel & Hernández-Mariné
Halomicronema hongdechloris Chen, Li, Birch & Willows
Leptolyngbya boryana (Gomont) Anagnostidis & Komárek
Leptolyngbya ectocarpi (Gomont) Anagnostidis & Komárek
Leptolyngbya foveolara (Gomont) Anagnostidis & Komárek
Leptolyngbya tenuis (Gomont) Anagnostidis & Komárek
Merismopediaceae
Synechocystis aquatilis Sauvageau
Pseudoanabaenaceae
Pseudanabaena limnetica (Lemmermann) Komárek
Prochloraceae
Prochlorococcus marinus S.W.Chisholm, S.L.Frankel, R.Goericke, R.J.Olson, B.Palenik, J.B.Waterbury, L.West-Johnsrud & E.R.Zettler
Prochloron didemni R.A.Lewin ex L.Hoffmann & W.Greuter
Prochlorothrix hollandica T.Burger-Wiersma, L.J.Stal & L.R.Mur
Synechococcaceae
Cyanobium bacillare (Butcher) Komárek, Kopeck & Cepák
Cyanobium waterburyi Komárek
Synechococcus elongatus (Nägeli) Nägeli
Thermosynechococcus elongatus Katoh, Itoh, Shen & Ikeuchi
Empire Eukaryota
Kingdom: **Chromista**
Phylum: **Cercozoa**
Chloracrachniophyceae [Chlorarachnea]
Chlorarachniales [Chlorarachniida]
Chlorarachniaceae
Bigelowiella natans Moestrup
Gymnochlora K.Ishida & Y.Hara
Lotharella globosa (K.Ishida & Y.Hara) K.Ishida & Y.Hara
Filosa
Aconchulinida
Paulinellidae

(continued)

Table 2 (continued)

- *Paulinella* Lauterborn
- Imbricatea
  - Thaumatomonadida
    - Thamatomonadidae
      - *Thaumatomastix* Lauterborn
  - Euglyphida
    - Euglyphiodae
      - *Euglypha* Dujardin

Phylum Cryptophyta

- Cryptophyceae
  - Cryptomonadales
    - Cryptomonadaceae
      - *Cryptomonas marssonii* Skuja
      - *Cryptomonas ovata* Ehrenberg
      - *Cryptomonas pyrenoidifera* Geitler
  - Pyrenomonadales
    - Geminigeraceae
      - *Guillardia theta* D.R.A.Hill & Wetherbee
      - *Proteomonas sulcata* D.R.A.Hill & Wetherbee
    - Pyrenomonadaceae
      - *Rhodomonas baltica* Karsten
      - *Rhodomonas lacustris* Pascher & Ruttner
      - *Rhodomonas maculata* Butcher ex D.R.A.Hill & Wetherbee
      - *Rhodomonas marina* (P.A.Dangeard) Lemmermann
      - *Rhodomonas salina* (Wislouch) D.R.A.Hill & Wetherbee
    - Chroomonadaceae
      - *Chroomonas placoidea* Butcher ex G.Novarino & I.A.N.Lucas

Phylum Haptophyta

- Coccolithophyceae
  - Coccolithales
    - Calcidiscaceae
      - *Calcidiscus leptoporus* (G.Murray & V.H.Blackman) Loeblich Jr. & Tappan
    - Coccolithaceae
      - *Coccolithus braarudii* (K.R.Gaarder) K.Baumann, M.Cachao, J.R.Young & M.Geisen
      - *Coccolithus pelagicus* (Wallich) J.Schiller
    - Calyptrospaeraceae
      - *Helladosphaera* Kamptner
      - *Holococcolithophora sphaeroidea* (Schiller) J.W.Jordan, L.Cros & J.R.Young
    - Hymenomonadaceae
      - *Hymenomonas roseola* Stein
  - Isochrysidales
    - Isochrysidaceae
      - *Chrysotila carterae* (T.Braarud & E.Fagerland) Andersen, Kim, Tittley & Yoon
      - *Chrysotila haptonemofera* (Inouye & Chihara) R.A. Andersen, J.I. Kim, I. Tittley & H.S.Yoon
      - *Isochrysis galbana* Parke
      - *Pseudoisochrysis paradoxa* F.D.Ott nom. inval.
      - *Ruttnera lamellosa* (Anand) R.A.Andersen, J.I.Kim, I.Tittley & H.S.Yoon
      - *Tisochrysis lutea* El M.Bendif & I.Probert
    - Neolaerhabdaceae
      - *Emiliania huxleyi* (Lohmann) W.W.Hay & H.P.Mohler

(continued)

Table 2 (continued)

Gephyrocapsa oceanica Kamptner
Phaeocystales
Phaeocystaceae
Phaeocystis antarctica Karsten
Phaeocystis globosa Scherffel
Phaeocystis pouchetii (Hariot) Lagerheim
Prymnesiales
Chrysochromulaceae
Chrysochromulina breviturrita K.H.Nicholls
Chrysochromulina strobilus Parke & Manton
Haptolina brevifila (Parke & Manton) Edvardsen & Eikrem
Prymnesiaceae
Prymnesium kappa (Parke & Manton) Edvardsen, Eikrem & Probert
Prymnesium neolepis (M.Yoshida, M.-H.Noel, T.Nakayama, T, Naganuma & I. Inouye) Edvardsen, Eikrem & Probert
Prymnesium parvum M. Carter
Prymnesium parvum f. *patelliferum* (J.C.Green, D.J.Hibberd & R.N.Pienaar) A.Larsen
Prymnesium polylepis (Manton & Parke) Edvardsen, Eikrem & Probert
Syracospherales
Syracosphaeraceae
Syracosphaera pulchra Lohmann
Zygodiscales
Pontosphaeraceae
Scyphosphaera apsteinii Lohmann
Coccolithophyceae *incertae sedis*
Florisphaera profunda Okada & Honjo
Pavlovophyaceae
Pavlovales
Pavlovaceae
Diacronema lutheri (Droop) Bendif & Véron
Diacronema vlkianum Prauser
Pavlova pinguis J.C.Green
Rebecca salina (N.Carter) J.C.Green
Phylum: **Dinophyta**
Dinophyceae
Actiniscales
Actiniscaceae
Actiniscus pentasterias (Ehrenberg) Ehrenberg
Actiniscus pentasterias var. *arcticus* Bursa
Brachdiniales
Brachidiniaceae
Karenia brevis (C.C.Davis) G. Hansen & Ø.Moestrup
Karenia mikimotoi (Miyake & Kominami ex Oda) Gert Hansen & Ø.Moestrup
Karenia papilionacea A.J.Haywood & K.A.Steidinger
Karenia umbella de Salas, Bolch & Hallegraeff
Karlodinium micrum (B.Leadbeater & J.D.Dodge) J.Larsen
Karlodinium veneficum (D.Ballantine) J.Larsen
Takayama helix de Salas, Bolch, Botes & Hallegraeff
Takayama tasmanica de Salas, Bolch & Hallegraeff
Dinotrichales
Crypthecodiniaceae

(continued)

Table 2 (continued)

Crypthecodinium cohnii (Seligo) Javornicky
Dinotrichaceae
Gymnodinium catenatum H.W.Graham
Gymnodinium corollarium A.M.Sundström, Kremp & Daugbjerg
Gymnodinium simplex (Lohmann) Kofoid & Swezy
Kryptoperidinium foliaceum (F.Stein) Lindemann
Gonyaulacales
Ceratiaceae
Ceratium furcoides (Levander) Langhans
Ceratium hirundinella (O.F.Müller) Dujardin
Goniocaulaceae
Lingulodinium polyedra (F.Stein) J.D.Dodge
Goniodomataceae
Alexandrium catenella (Whedon & Kofoid) Balech
Alexandrium fundyense Balech
Alexandrium minutum Halim
Alexandrium ostenfeldii (Paulsen) Balech & Tangen
Alexandrium tamarense (Lebour) Balech
Coolia monotis Meunier
Gambierdiscus toxicus R.Adachi & Y.Fukuyo
Ostreopsis lenticularis Y.Fukuyo
Pyrodinium bahamense Plate
Protoceratiaceae
Protoceratium reticulatum (Claparède & Lachmann) Bütschli
Gymnodiniales
Gymnodiniaceae
Akashiwo sanguinea (K.Hirasaka) G.Hansen & Ø.Moestrup
Amphidinium carterae Hulburt
Amphidinium klebsii Kofoid & Swezy
Gymnodinium aureolum (E.M.Hulburt) G. Hansen
Gymnodinium catenatum H.W.Graham
Gyrodinium dorsum Kofoid & Swezy
Lepidodinium chlorophorum (M.Elbrächter & E.Schnepf) G. Hansen, L.Botes & M.de Salas
Woloszynskiaceae
Woloszynskia R.H.Thompson
Peridiniales
Heterocapsaceae
Heterocapsa circularisquama Horiguchi
Heterocapsa triquetra (Ehrenberg) Stein
Peridiniaceae
Parvodinium inconspicuum (Lemmermann) S.Carty
Peridinium aciculiferum Lemmermann
Peridinium cinctum (O.F.Müller) Ehrenberg
Peridinium cinctum f. *westii* (Lemmermann) Lindemann
Peridinium gatunense Nygaard
Scrippsiella hangoei (J.Schiller) J.Larsen
Scrippsiella minima Gao & Dodge
Scrippsiella sweeneyae Balech ex A.R.Loeblich III
Scrippsiella trochoidea (Stein) Balech ex Loeblich III
Prorocentrales

(continued)

Table 2 (continued)

Prorocentraceae
Prorocentrum cordatum (Ostenfeld) J.D.Dodge
Prorocentrum dentatum F. Stein
Prorocentrum donghaiense D.Lu
Prorocentrum lima (Ehrenberg) F.Stein
Prorocentrum micans Ehrenberg
Pyrocystales
Pyrocystaceae
Pyrocystis lunula (Schütt) Schütt
Pyrocystis pseudonoctiluca Wyville-Thompson
Suessiales
Suessiaceae
Biecheleria baltica Moestrup, Lindberg, & Daugbjerg
Polarella glacialis M.Montresor, G.Procaccini & D.K.Stoecker
Symbiodinium microadriaticum Freudenthal
Thoracosphaerales
Oodiniaceae
Thoracosphaera heimii (Lohmann) Kamptner
Dinophyceae *incertae sedis*
Cochlodinium polykrikoides Margalef
Levanderina fissa (Levander) Ø.Moestrup, P.Hakanen, G.Hansen, N.Daugbjerg & M.Ellegaard
Noctilucophyceae
Noctilucales
Noctilucaceae
Noctiluca scintillans (Macartney) Kofoid & Swezy
Oxyrrhida
Oxyrrhinales
Oxyrrhinaceae
Oxyrrhis marina Dujardin
Perkinsea
Perkinsorida
Perkinsidae
Perkinsus marinus (Mackin, Owen & Collier) Levine
Phylum: **Ochrophyta**
Aurearenophyceae
Aurearenales
Aurearenaceae
Aurearena cruciata Kai, Yoshii, Nakayama & Inouye
Bacillariophyceae
Achnathales
Achnathaceae
Achnanthes longipes C.Agardh
Bacillariales
Bacillariaceae
Cylindrotheca fusiformis Reimann & J.C.Lewin
Fragilariopsis curta (Van Heurck) Hustedt
Fragilariopsis cylindrus (Grunow) Krieger
Fragilariopsis kerguelensis (O'Meara) Hustedt
Nitzschia epithemoides Grunow
Nitzschia frustulum (Kützing) Grunow

(continued)

Table 2 (continued)

Nitzschia longissima (Brébisson) Ralfs
Nitzschia medioconstricta Hustedt
Nitzschia ovalis H.J.Arnott
Nitzschia palea (Kützing) W.Smith
Nitzschia pellucida Grunow
Pseudo-nitzschia delicatissima (Cleve) Heiden
Pseudo-nitzschia granii (G.R.Hasle) G.R.Hasle
Pseudo-nitzschia multiseries (Hasle) Hasle
Pseudo-nitzschia pseudodelicatissima (Hasle) Hasle
Pseudo-nitzschia pungens (Grunow ex Cleve) Hasle
Cymbellales
Anomoeoneidaceae
Dickieia subinflata (Grunow) D.G.Mann
Cymbellaceae
Cymbella cistula (Ehrenberg) O.Kirchner
Cymbella mexicana (Ehrenberg) Cleve
Encyonopsis microcephala (Grunow) Krammer
Hemiaulales
Hemiaulaceae
Eucampia Ehrenberg
Naviculales
Amphipleuraceae
Halamphora coffeaeformis (C.Agardh) Levkov
Halamphora costata (W.Smith) Levkov
Naviculaceae
Fistulifera saprophila (Lange-Bertalot & Bonik) Lange-Bertalot
Fistulifera solaris S.Mayama, M.Matsumoto, K.Nemoto & T.Tanaka
Haslea crucigera (W.Smith) Simonsen
Haslea ostrearia (Gaillon) Simonsen
Navicula arenaria Donkin
Navicula curvilineata D.G.Mann
Navicula gelida var. *antarctica* Heiden
Navicula perminuta Grunow
Navicula phyllepta Kützing
Navicula salinarum Grunow
Seminavis robusta D.B. Danielidis & D.G. Mann
Phaeodactylaceae
Phaeodactylum tricornutum Bohlin
Pinnulariaceae
Pinnularia viridis (Nitzsch) Ehrenberg
Suriellales
Entomoneidaceae
Entomoneis paludosa (W.Smith) Reimer
Thalassiophysales
Catenulaceae
Amphora Ehrenberg ex Kützing
Thalassiophysaceae
Thalassiophysa hyalina (Greville) Paddock & P.A.Sims
Bolidiophyceae
Parmales

(continued)

Table 2 (continued)

Pentalaminaceae
Pentalamina H.J.Marchant
Triparmaceae
Tetraparma pelagica B.C.Booth & H.J.Marchant
Triparma B.C.Booth & H.J.Marchant
Coscinodiscophyceae
Aulacoseirales
Aulacoseraceae
Aulacoseira granulata (Ehrenberg) Simonsen
Chaetocerotales
Chaetocerotaceae
Chaetoceros affinis Lauder
Chaetoceros brevis F.Schütt
Chaetoceros calcitrans (Paulsen) Takano
Chaetoceros curvisetus Cleve
Chaetoceros debilis Cleve
Chaetoceros decipiens Cleve
Chaetoceros dichaeta Ehrenberg
Chaetoceros didymus Ehrenberg
Chaetoceros muelleri Lemmermann
Chaetoceros neogracilis S.L.VanLandingham (as "*neogracile*")
Chaetoceros pelagicus Cleve
Chaetoceros simplex Ostenfeld
Chaetoceros socialis Lauder
Chaetoceros vixvisibilis Schiller
Coscinodiscales
Coscinodiscaceae
Coscinodiscus asteromphalus Ehrenberg
Coscinodiscus radiatus Ehrenberg
Coscinodiscus wailesii Gran & Angst
Hemidiscaceae
Actinocyclus Ehrenberg
Corethrales
Corethraceae
Corethron criophilum Castracane
Corethron hystrix Hensen
Corethron pennatum (Grunow) Ostenfeld
Ethmodiscales
Ethmodiscaceae
Ethmodiscus rex (Wallich) Hendey
Lithodesmiales
Lithdesmiaceae
Ditylum brightwellii (T.West) Grunow
Melosirales
Melosiraceae
Melosira arctica Dickie
Stephanopyxidaceae
Stephanopyxis palmeriana (Greville) Grunow
Rhizosolenales
Rhizosolenaceae

(continued)

Table 2 (continued)

Proboscia inermis (F.Castracane) R.W.Jordan & R.Ligowski
Thalassiosirales
Stephanodiscaceae
Cyclotella cryptica Reimann, Lewin & Guillard
Cyclotella meneghiniana Kützing
Stephanodiscus hantzschii var. *pusillus* Grunow
Stephanodiscus neoastraea Håkansson & Hickel
Skeletonemataceae
Skeletonema costatum (Greville) Cleve
Skeletonema marinoi Sarno & Zingone
Thalassiosiraceae
Conticribra weissflogii (Grunow) K.Stachura-Suchoples & D.M.Williams
Porosira pseudodenticulata (Hustedt) Jousé
Thalassiosira aestivalis Gran
Thalassiosira antarctica Karsten
Thalassiosira baltica (Grunow) Ostenfeld
Thalassiosira gravida Cleve
Thalassiosira oceanica Hasle
Thalassiosira pseudonana Hasle & Heimdal
Thalassiosira tumida (Janisch) Hasle
Triceratales
Triceratataceae
Odontella aurita (Lyngbye) C.Agardh
Odontella sinensis (Greville) Grunow
Triceratium dubium Brightwell
Chrysophyceae
Chromulinales
Chromulinaceaeae
Ochromonas danica E.G.Pringsheim
Ochromonas ovalis Doflein
Dinobryaceae
Poterioochromonas malhamensis (Pringsheim) Péterfi
Paraphysomonadaceae
Chrysosphaerella Lauterborn
Paraphysomonas De Saedeleer
Spiniferomonas E.Takahashi
Chrysophyceae *ordo incertae sedis*
Chrysophyceae familia incertae sedis
Chrysoderma C.Billard
Chrysomerophyceae
Chrysomeridales
Chrysomeridaceae
Chrysomeris ramosa N.Carter
Chrysowaernella hieroglyphica (Waern) Gayral & Lepailleur
Giraudyopsis stellifer P.J.L.Dangeard
Dictyochophyceae
Dictyochales
Dictyochaceae
Dictyocha fibula Ehrenberg
Dictyocha speculum Ehrenberg

(continued)

Table 2 (continued)

- Florenciellales
  - Florecielllales *incertae sedis*
    - *Pseudochattonella farcimen* (W. Eikrem, B. Edvardsen, & J. Throndsen) W. Eichrem
    - *Pseudochattonella verruculosa* (Y.Hara & M.Chihara) S.Tanabe-Hosoi, D.Honda, S.Fukaya, Y.Inagaki & Y.Sako
- Eustigmatophyceae
  - Eustigmatales
    - Eustigmataceae
      - *Eustigmatos vischeri* D.J.Hibberd
      - *Vischeria helvetica* (Vischer & Pascher) D.J.Hibberd
      - *Vischeria punctata* Vischer
    - Monodopsidaceae
      - *Monodopsis subterranea* (J.B.Petersen) D.J.Hibberd
      - *Nannochloropsis gaditana* L.M.Lubián
      - *Nannochloropsis granulata* B.Karlson & D.Potter
      - *Nannochloropsis oceanica* Suda & Miyashita
      - *Nannochloropsis oculata* (Droop) D.J.Hibberd
      - *Nannochloropsis salina* D.J.Hibberd
- Fragilariophyceae
  - Fragilariales
    - Fragilariaceae
      - *Asterionella formosa* Hassall
      - *Asterionellopsis glacialis* (Castracane) Round
      - *Ceratoneis closterium* Ehrenberg
      - *Pseudostaurosira trainorii* E.A. Morales
  - Raphoneidales
    - Rhaphoneidaceae
      - *Delphineis* G.W.Andrews
- Pelagophyceae
  - Pelagomonadales
    - Pelagomonadaceae
      - *Aureococcus anophagefferens* Hargraves & Sieburth
      - *Pelagomonas calceolata* R.A.Andersen & G.Saunders
  - Sarcinochrysales
    - Sarcinochrysidae
      - *Nematochrysopsis marina* (J.Feldmann) C.Billard
      - *Sarcinochrysis marina* Geitler
- Phaeophyceae
  - Desmarestiales
    - Desmarestiaceae
      - *Desmarestia* J.V.Lamouroux
      - *Himantothallus grandifolius* (A.Gepp & E.S.Gepp) Zinova
  - Dictyotales
    - Dictyotaceae
      - *Dictyota bartayresiana* J.V.Lamouroux
      - *Padina gymnospora* (Kützing) Sonder
  - Ectocarpales
    - Acinetosporaceae
      - *Pylaiella littoralis* (Linnaeus) Kjellman
    - Chordiariaceae
      - *Dictyosiphon foeniculaceus* (Hudson) Greville

(continued)

Table 2 (continued)

Ectocarpaceae
Ectocarpus siliculosus (Dillwyn) Lyngbye
Scytosiphonaceae
Hydroclathrus clathratus (C.Agardh) M.A.Howe
Fucales
Sargassaceae
Sargassum oligocystum Montagne
Laminariales
Laminariaceae
Macrocystis pyrifera (Linnaeus) C.Agardh
Saccharina japonica (Areschoug) C.E.Lane, C.Mayes, Druehl & G.W.Saunders
Saccharina latissima (Linnaeus) C.E.Lane, C.Mayes, Druehl & G.W.Saunders
Sphacelariales
Stypocaulaceae
Halopteris scoparia (Linnaeus) Sauvageau
Tilopterales
Halosiphonaceae
Halosiphon tomentosus (Lyngbye) Jaasund
Raphidophyceae
Chattonellales
Chattonellaceae
Chattonella marina (Subrahmanyan) Hara & Chihara
Chattonella marina var. *antiqua* (Hada) Demura & Kawachi
Chattonella subsalsa B.Biecheler
Fibrocapsa japonica S.Toriumi & H.Takano
Heterosigma akashiwo (Y.Hada) Y.Hada ex Y.Hara & M.Chihara
Vacuolariaceae
Gonyostomum semen (Ehrenberg) Diesing
Raphidophyceae *incertae sedis*
Olisthodiscus luteus N.Carter
Synurophyceae
Synurales
Mallomonadaceae
Mallomonas Perty
Synura petersenii Korshikov
Synuraceae
Tessellaria volvocina (Playfair) Playfair
Xanthophyceae
Botrydiales
Botrydiaceae
Botrydium granulatum (Linnaeus) Greville
Mischococcales
Centritactaceae
Bumilleriopsis filiformis Vischer
Pleurochloridaceae
Acanthochloris bacillifera Pascher
Pleurochloris meiringensis Vischer
Monodopsis subterranea (J.B.Petersen) D.J.Hibberd
Trachydiscus minutus (P.Bourrelly) H.Ettl
Tribonematales

(continued)

Table 2 (continued)

Tribonemataceae
Tribonema aequale Pascher
Phylum: **Chromista *phylum incertae sedis***
Chromera velia R.B.Moore et al.
Kingdom: **Plantae**
Phylum: **Charophyta**
Charophyceae
Charales
Characeae
Chara canescens J.L.A.Loiseleur-Deslongschamps
Chara corallina C.L.Willdenow
Conjugatophyceae
Desmidales
Closteriaceae
Closterium acerosum Ehrenberg ex Ralfs
Closterium aciculare T.West
Closterium ehrenbergii Meneghini ex Ralfs
Desmidaceae
Cosmarium bioculatum Brébisson ex Ralfs
Cosmocladium constrictum (W.Archer) W.Archer
Micrasterias americana Ehrenberg ex Ralfs
Pleurotaenium trabecula Nägeli
Staurastrum cingulum (West & G.S.West) G.M.Smith
Staurastrum crenulatum (Nägeli) Delponte
Staurastrum inversenii var. *americanum* Scott & Grönblad
Staurastrum inversenii Nygaard
Staurastrum paradoxum Meyen ex Ralfs
Tetmemorus brebissonii Ralfs
Xanthidium subhastiferum West
Peniaceae
Penium cylindrus Brébisson ex Ralfs
Penium margaritaceum Brébisson
Penium spirostriolatum J.Barker
Zygnematales
Mesotaeniaceae
Netrium digitus (Brébisson ex Ralfs) Itzigsohn & Rothe
Netrium oblongum (De Bary) Lütkemüller
Planotaenium interruptum (Brébisson ex Ralfs) O.V.Petlovany & Palamar-Mordvintseva
Mesostigmatophyceae
Mesostigmatales
Mesostigmataceae
Mesostigma viride Lauterborn
Phylum: **Chlorophyta**
Chlorophyceae
Chaetopherales
Chaetopheraceae
Stigeoclonium helveticum Vischer
Uronema confervicola Lagerheim
Chlamydomonadales
Chlamydomonadaceae

(continued)

Table 2 (continued)

Chlamydomonas allensworthii Starr, Marner & Jaenicke
Chlamydomonas debaryana Goroschankin [Gorozhankin]
Chlamydomonas globosa J.W.Snow
Chlamydomonas microsphaera Pascher & Jahoda
Chlamydomonas moewusii Gerloff
Chlamydomonas nivalis (F.A.Bauer) Wille
Chlamydomonas noctigama Korschikov
Chlamydomonas oblonga Pringsheim
Chlamydomonas reinhardtii P.A. Danegard
Chloromonas augustae (Skuja) T.Pröschold, B.Marin, U.W.Schlösser & M.Melkonian
Chloromonas rosae H.Ettl
Gloeomonas kupfferi (Skuja) Gerloff
Lobochlamys segnis (H.Ettl) T.Pröschold, B.Marin, U.W.Schlösser & M.Melkonian
Chlorococcaceae
Chlorococcum ellipsoideum Deason & H.C.Bold
Chlorococcum littorale M.Chihara, T.Nakayama & I.Inouye
Chlorococcum submarinum Ålvik
Nautococcus pyriformis Korshikov
Coccomyxaceae
Coccomyxa subellipsoidea E.Acton
Dunaliellaceae
Dunaliella acidophila (Kalina) Massyuk
Dunaliella bioculata Butcher
Dunaliella minuta W.Lerche
Dunaliella parva W.Lerche
Dunaliella primolecta Butcher
Dunaliella tertiolecta Butcher
Dunaliella salina (Dunal) Teodoresco
Pallmellaceae
Palmella mucosa Kützing
Palmellopsidaceae
Palmellopsis Korshikov
Phacotaceae
Hemitoma Skuja
Rhopalosolenaceae
Kentrosphaera Borzì
Scotiellocystoidaceae
Graesiella emersonii (Shihara & R.W.Krauss) H.Nozaki, M.Katagiri, M.Nakagawa, K.Aizawa & M.M.Watanabe
Spondylomoraceae
Pascherina P.C.Silva
Spondylomorum Ehrenberg
Volvocaceae
Didymochloris Pascher in Fott
Eudorina elegans Ehrenberg
Eudorina unicocca G.M.Smith
Pandorina charkowiensis Korschikov
Pandorina morum (O.F.Müller) Bory de Saint-Vincent
Platydorina caudata Kofoid
Pleodorina californica W.R. Shaw
Volvox aureus Ehrenberg

(continued)

Table 2 (continued)

Volvox carteri F. Stein
Volvulina pringsheimii Starr
Chlamydomonadales *Incertae sedis*
Ettlia oleoabundans (S.Chantanachat & H.C.Bold) J.Komárek
Haematococcales
Haematococcaceae
Haematococcus pluvialis Flotow
Oedogoniales
Oedogoniaceae
Oedogonium Link ex Hirn
Sphaeropleales
Chromochloridaceae
Chromochloris zofingiensis (Dönz) Fucíková & L.A.Lewis
Hydrodictyaceae
Hydrodictyon reticulatum (Linnaeus) Bory de Saint-Vincent
Monactinus simplex (Meyen) Corda
Pediastrum duplex Meyen
Sorastrum Kützing
Tetraëdron caudatum (Corda) Hansgirg
Tetraëdron minimum (A.Braun) Hansgirg
Microsporaceae
Microspora Thuret
Scenedesmaceae
Acutodesmus obliquus (Turpin) Hegewald & Hanagata
Coelastrella striolata var. *multistriata* (Trenkwalder) Kalina & Puncochárová
Coelastrum microporum Nägeli
Desmodesmus subspicatus (Chodat) E.Hegewald & A.Schmidt
Scenedesmus armatus (R. Chodat) R. Chodat
Scenedesmus communis Hegewald
Scenedesmus fuscus (Shihara & R.W. Krauss) Hegewald
Scenedesmus quadricauda (Turpin) Brébisson
Scenedesmus quadrispina Chodat
Selenastraceae
Ankistrodesmus falcatus (Corda) Ralfs
Monoraphidium contortum (Thuret) Komárková-Legnerová
Monoraphidium convolutum (Corda) Komárková-Legnerová
Monoraphidium griffithii (Berkeley) Komárková-Legnerová
Monoraphidium neglectum Heynig & Krienitz
Raphidocelis subcapitata (Korshikov) Nygaard, Komárek, J.Kristiansen & O.M.Skulberg
Chlorodendrophyceae
Chlorodendrales
Clorodendraceae
Scherffelia dubia (Perty) Pascher
Tetraselmis chui Butcher
Tetraselmis gracilis (Kylin) Butcher
Tetraselmis striata Butcher
Tetraselmis subcordiformis (Wille) Butcher
Tetraselmis suecica (Kylin) Butcher
Tetraselmis tetrathele (West) Butcher
Tetraselmis viridis (Rouchijajnen) R.E.Norris, Hori & Chihara

(continued)

Table 2 (continued)

- Mamiellophyceae
  - Mamiellales
    - Bathycoccaceae
      - *Bathycoccus prasinos* W.Eikrem & J.Throndsen
      - *Ostreococcus lucimarinus* [species appears not to be described, strain CCE9901]
      - *Ostreococcus tauri* C.Courties & M.-J.Chrétiennot-Dinet
    - Mamiellaceae
      - *Micromonas pusilla* (Butcher) I.Manton & M.Parke
- Pedinomonadophyceae
  - Pedinomonadales
    - Pedinomonadaceae
      - *Pedinomonas minor* Korshikov
      - *Pedinomonas tuberculata* (Vischer) Gams
- Pyramomonadophyceae
  - Prasinococcales
    - Prasoinococcaceae
      - *Prasinococcus capsulatus* H.Miyashita & M.Chihara
  - Pseudoscourfeldiales
    - Pynococcaceae
      - *Pseudoscourfieldia marina* (J.Throndsen) Manton
      - *Pycnococcus provasolii* R.R.L.Guillard
  - Pyramimonadales
    - Pyramomonadaceae
      - *Pyramimonas cordata* G.I.McFadden
      - *Pyramimonas grossii* Parke
      - *Pyramimonas propulsa* Moestrup & Hill
- Trebouxiophyceae
  - Chlorellales
    - Chlorellaceae
      - *Chlorella lobophora* V.M. Andreyeva
      - *Chlorella minutissima* Fott & Nováková
      - *Chlorella pyrenoidosa* H. Chick
      - *Chlorella sorokiniana* Shihira & R.W.Krauss
      - *Chlorella variabilis* Shihira & R.W.Krauss
      - *Chlorella vulgaris* Beyreink [Beijerink]
      - *Chlorella vulgaris* var. *autotrophica* (Shihira & Krauss) Fott & Nováková
      - *Micractinium pusillum* Fresenius
      - *Mucidosphaerium pulchellum* (H.C.Wood) C.Bock, Proschold & Krienitz
      - *Parachlorella kessleri* (Fott & Nováková) Krienitz, E.H.Hegewald, Hepperle, V.Huss, T.Rohr & M.Wolf
      - *Prototheca wickerhamii* Tubaki & Soneda
    - Oocystaceae
      - *Chloroidium ellipsoideum* (Gerneck) Darienko, Gustavs, Mudimu, Menendez, Schumann, Karsten, Friedl & Proschold
      - *Chloroidium saccharophilum* (W.Krüger) Darienko, Gustavs, Mudimu, Menendez, Schumann, Karsten, Friedl & Proschold
      - *Oocystis marssonii* Lemmermann
      - *Pseudochlorococcum* P.A.Archibald
    - Chlorellales *incertae sedis*
      - *Picochlorum atomus* (Butcher) Henley, Hironaka, Guillou, M.Buchheim, J.Buchheim, M.Fawley & K.Fawley
      - *Picochlorum eukaryotum* W.J.Henley, J.L.Hironaka, L.Guillou, M.A.Buchheim, J.A.Buchheim, M.W.Fawley & K.P.Fawley
      - *Picochlorum maculatum* (Butcher) Henley, Hironaka, Guillou, M.Buchheim, J.Buchheim, M.Fawley & K.Fawley
  - Prasiolales

(continued)

Table 2 (continued)

Koliellaceae
Koliella antarctica C.Andreoli, G.M.Lokhorst, A.M.Mani, L.Scarabel, I.Moro, N.La Rocca & L.Tognetto
Prasiolaceae
Desmococcus olivaceus (Persoon ex Acharius) J.R.Laundon
Prasiola crispa (Lightfoot) Kützing
Prasiola stipitata Suhr ex Jessen
Stichococcus bacillaris Nägeli
Stichococcus minor Nägeli
Trebouxiales
Botryococcaceae
Botryococcus braunii Kützing
Trebouxiaceae
Lobosphaera incisa (Reisigl) Karsten, Friedl, Schumannn, Hoyer & Lembcke
Ulvophyceae
Bryopsidales
Caulerpaceae
Caulerpa racemosa (Forsskål) J.Agardh
Codiaceae
Codium fragile (Suringar) Hariot
Ostriobiaceae
Ostreobium Bornet & Flahault
Cladophorales
Boodleaceae
Boodlea composita (Harvey) F.Brand
Cladophoraceae
Chaetomorpha basiretrorsa Setchell
Chaetomorpha tortuosa (Dillwyn) Kleen
Pithophoraceae
Dictyosphaeria cavernosa (Forsskål) Børgesen
Valoniaceae
Valonia aegagropila C.Agardh
Dasycladales
Dasycladaceae
Dasycladus vermicularis (Scopoli) Krasser
Trentepohliales
Trentepohliaceae
Trentepohlia arborum (C.Agardh) Hariot
Trentepohlia aurea (Linnaeus) C.F.P.Martius
Ulotrichales
Gloeotilaceae
Binuclearia lauterbornii (Schmidle) Proschkina-Lavrenko
Gomontiaceae
Monostroma hariotii Gain
Ulotrichaceae
Acrosiphonia J.Agardh
Ulothrix zonata (Weber & Mohr) Kützing
Ulvales
Ulvaceae
Enteromorpha bulbosa (Suhr) Montagne
Ulva intestinalis Linnaeus

(continued)

Table 2 (continued)

Ulva lactuca Linnaeus
Chlorophyta *incertae sedis*
Picocystis salinarum R.A.Lewin
Phylum:**Glaucophyta**
Glaucophyceae
Glaucocystales
Glaucocystaceae
Cyanophora paradoxa Korshikov
Glaucocystis nostochinearum Itzigsohn
Gloeochaete wittrockiana Lagerheim
Phylum:**Rhodophyta**
Bangiophyceae
Bangiales
Bangiaceae
Bangia atropurpurea (Mertens ex Roth) C.Agardh
Bangia fuscopurpurea (Dillwyn) Lyngbye
Porphyra umbilicalis Kützing
Pyropia columbina (Montagne) W.A.Nelson
Pyropia haitanensis (T.J.Chang & B.F.Zheng) N.Kikuchi & M.Miyata
Pyropia leucosticta (Thuret) Neefus & J.Brodie
Pyropia perforata (J.Agardh) S.C.Lindstrom
Pyropia yezoensis (Ueda) M.S.Hwang & H.G.Choi
Compsopogonophyceae
Erythropeltidales
Erythrotrichiaceae
Sahlingia subintegra (Rosenvinge) Kornmann
Cyanidiophyceae
Cyanidiales
Cyanidiaceae
Cyanidioschyzon merolae P.De Luca, R.Taddei & L.Varano
Cyanidium caldarium (Tilden) Geitler
Galdieriaceae
Galdieria sulphuraria (Galdieri) Merola
Florideophyceae
Batrachospermales
Lemaneaceae
Lemanea fluviatilis (Linnaeus) C.Agardh
Bonnemaisoniales
Bonnemaisoniaceae
Asparagopsis armata Harvey
Asparagopsis taxiformis (Delile) Trevisan de Saint-Léon
Delisea pulchra (Greville) Montagne
Ptilonia okadae Yamada
Ceramiales
Callithamniaceae
Callithamnion gaudichaudii C.Agardh
Ceramiaceae
Ceramium virgatum Roth
Delesseriaceae
Caloglossa leprieurii (Montagne) G.Martens
Radicilingua Papenfuss

(continued)

Table 2 (continued)

Rhodomelaceae
Acanthophora spicifera (M.Vahl) Børgesen
Bostrychia Montagne
Chondria armata (Kützing) Okamura
Laurencia J.V.Lamouroux
Polysiphonia Greville
Rhodomela confervoides (Hudson) P.C.Silva
Stictosiphonia J.D.Hooker & Harvey
Wrangeliaceae
Georgiella confluens (Reinsch) Kylin
Corallinales
Corallinaceae
Amphiroa J.V.Lamouroux
Corallina officinalis Linnaeus
Ellisolandia elongata (J.Ellis & Solander) K.R.Hind & G.W.Saunders
Jania rubens (Linnaeus) J.V.Lamouroux
Gelidiales
Gelidiellaceae
Gelidiella acerosa (Forsskål) Feldmann & G.Hamel
Gelidium J.V.Lamouroux
Gigartinales
Caulacanthaceae
Catenella Greville
Cystocloniaceae
Cystoclonium purpureum (Hudson) Batters
Gigartinaceae
Chondrus crispus Stackhouse
Gigartina skottsbergii Setchell & N.L.Gardner
Iridaea cordata (Turner) Bory de Saint-Vincent
Phyllophoraceae
Gymnogongrus Martius
Mastocarpus stellatus (Stackhouse) Guiry
Phyllophora Greville
Solieriaceae
Eucheuma isiforme (C.Agardh) J.Agardh
Gracilariales
Gracilariaceae
Curdiea racovitzae Hariot
Gracilaria birdiae E.M.Plastino & E.C.Oliveira
Gracilaria chilensis C.J.Bird, McLachlan & E.C.Oliveira
Gracilaria damaecornis J.Agardh
Gracilaria domingensis (Kützing) Sonder ex Dickie
Gracilaria tenuistipitata C.F.Chang & B.M.Xia
Gracilaria vermiculophylla (Ohmi) Papenfuss
Gracilariopsis lemaneiformis (Bory de Saint-Vincent) E.Y.Dawson, Acleto & Foldvik
Hydropuntia cornea (J.Agardh) M.J.Wynne
Nemaliales
Galaxauraceae
Actinotrichia fragilis (Forsskål) Børgesen
Palmariales
Palmariaceae
Devaleraea ramentacea (Linnaeus) Guiry

(continued)

Table 2 (continued)

Palmaria decipiens (Reinsch) R.W.Ricker
Palmaria palmata (Linnaeus) Weber & Mohr
Plocamiales
Sarcodiaceae
Trematocarpus antarcticus (Hariot) Fredericq & R.L.Moe
Rhodymeniales
Lomentariaceae
Lomentaria articulata (Hudson) Lyngbye
Porphyridiophyceae
Porphyridiales
Porphyridiaceae
Porphyridium aerugineum Geitler
Porphyridium cruentum (S.F. Gray) Nägeli
Porphyridium purpureum (Bory de Saint-Vincent) K.M.Drew & R.Ross
Rhodellophyceae
Dixonieellales
Dixoniellaceae
Dixoniella grisea (Geitler) J.L.Scott, S.T.Broadwater, B.D.Saunders, J.P.Thomas & P.W.Gabrielson
Rhodellales
Rhodellaceae
Rhodella violacea (Kornmann) Wehrmeyer
Stylonematophyceae
Stylonematales
Stylonemataceae
Rhodosorus marinus Geitler
Stylonema alsidii (Zanardini) K.M.Drew
Kingdom: Protozoa
Phylum: **Euglenozoa**
Euglenophyceae
Euglenales
Euglenaceae
Euglena gracilis Klebs
Euglena longa (Pringsheim) Marin & Melkonian
Euglena pisciformis Klebs
Peranemataceae
Urceolus sabulosus Stokes
Eutreptiales
Eutreptiaceae
Eutreptiella gymnastica Throndsen
Eutreptia viridis Perty
Phylum: **Picozoa**
Picomonadea
Picomonadida
Picomonadidae
Picomonas judraskeda R.Seenivasan, S.Sausen, L.K.Medlin & M.Melkonian
Protozoa *incertae sedis*
Ebriophyceae
Ebriales
Ebriopsidaceae
Hermesinum adriaticum O.Zacharias

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