Cellular Origin, Life in Extreme Habitats and Astrobiology 26

Dinabandhu Sahoo
Joseph Seckbach *Editors*

The Algae World

Springer

Cellular Origin, Life in Extreme Habitats and Astrobiology

Volume 26

Series editor
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More information about this series at http://www.springer.com/series/5775

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ISSN 1566-0400 ISSN 2215-0048 (electronic)
Cellular Origin, Life in Extreme Habitats and Astrobiology
ISBN 978-94-017-7320-1 ISBN 978-94-017-7321-8 (eBook)
DOI 10.1007/978-94-017-7321-8

Library of Congress Control Number: 2015955246

Springer Dordrecht Heidelberg New York London

Printed on acid-free paper

Springer Science+Business Media B.V. Dordrecht is part of Springer Science+Business Media (www.springer.com)

Preface

Algae is an important group of organisms which are found in a wide range of habitats be it oceans, rivers, fresh water lakes, ponds or brackish water bodies, snow, barks of the trees, etc. Ranging from a small tiny cell to the Giant Kelp measuring upto several meters, these group of plants have some unique features which are not found in any other group of organisms. Algae have both Prokaryotic and Eukaryotic groups, large varieties of pigment systems, triphasic life cycle, a long evolutionary history, etc. Algae changed the planet's atmosphere by producing oxygen thus paving the way for evolution of life on earth. These tiny organisms not only give us oxygen to breathe, food to eat, medicines to heal, cosmetics to use but also provide a lot of information about origin of life. It has been predicted that not only in future vehicles will run on algal biofuels but also power plants will use algae for carbon dioxide sequestration. In spite of its huge importance, algae remains a much neglected subject because of its stereotype boring class room table materials as "Pond Scum". Globally, algae is already a multibillion dollar industry employing a large number of people in various industries and their value are going to increase in future. Therefore, the purpose of writing and editing this book is not to publish one more text book in the field of phycology but to give a different type of feelings and encouragement to our readers and students to understand, feel, and unravel the beauty and use of this group of organisms differently in different ways.

The Algae World has been carefully written and edited to everyone's taste starting from a layman to an undergraduate, postgraduate, doctorate, post doctorate students or professors, entrepreneurs and industries. The book has been divided into two parts: the first part "Biology of Algae" contains 10 chapters, which deals with general characters, classification and different groups of important algae. The second part "Applied Phycology" deals with more applied aspects of algae ranging from algal biotechnology, biofuel, industrial applications, nanotechnology, etc. These 22 chapters which are contributed by eminent researchers across the world will be of great interests to all. Editing this book has not been easy. It took us a lot deal of time and pursuation to prepare this book.

We hope that **The Algae World** fill up the much needed gap in the field of study of Algae (Phycology) and we thank all our authors for their timely contributions and

anonymous reviewers for their critical comments. Last but not the least, we express our gratitude to Springer's team especially Ms. Melanie van Overbeek for their kind cooperation and help in publishing this book.

Delhi, India — Dinabandhu Sahoo
Jerusalem, Israel — Joseph Seckbach
January 1, 2016

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Among his publications are books, scientific articles in the line of phytoferritin, cellular evolution, acidothermophilic algae, and life in extreme environments. He also edited and translated several popular books. Dr. Seckbach is a coauthor (with R.Ikan) of the *Chemistry Lexicon* (1991, 1999) and a coeditor of *Proceeding of Endocytobiology VII Conference* (Freiburg, Germany, 1988) and the *Proceedings of Algae and Extreme Environments meeting* (Trebon, Czech Republic, 2000). His new edited volume (with Richard Gordon) entitled *Divine Action and Natural Selection: Science, Faith and Evolution* have been published by World Scientific Publishing Company. His recent interest is in the field of enigmatic microorganisms and life in extreme environments. E-mail: seckbackh@huji.ac.il

Part I
Biology of Algae

General Characteristics of Algae

Dinabandhu Sahoo and Pooja Baweja

1 Introduction

Algae are group of plants which are known since ancient civilizations. The term algae was first introduced by Linnaeus in 1753 and it was A. L. de Jussieu (1789) who classified the plants and delimited the algae from rest of the plant world to its present status. They are autotrophic, mostly aquatic and a few are terrestrial. The plant body ranges from unicellular to multicellular structures with no vasculature and little differentiation into various tissue systems thus they are referred to as thallophytes lacking true roots, stem and leaves. There can be a single cell as small as 1 μ to large seaweeds which can grow up to more than 60 m (Fritsch 1935, 1945).

The branch of botany, dealing with the study of algae is known as **Algology/Phycology** (Gr. *Phycos* – seaweeds and *logos* – study).

2 Habitat

Algae are a group of ubiquitous organisms which are present in diverse habitats such as water (aquatic algae), land (terrestrial algae), they also grow as an epiphyte, endophyte, and as well as in extreme conditions, in other words it can be said that algae are of universal occurrence (Fig. 1).

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D. Sahoo, J. Seckbach (eds.), *The Algae World*, Cellular Origin, Life in Extreme Habitats and Astrobiology 26, DOI 10.1007/978-94-017-7321-8_1

Fig. 1 Algae growing in a pond (Courtesy: Prof. Dinabandhu Sahoo)

Fig. 2 Algal bloom in narrow channel of Dal lake (Kashmir, India) (Courtesy: Prof. Dinabandhu Sahoo)

2.1 Aquatic Algae

Predominantly the algae are aquatic and are found in **fresh water** growing in ponds, pools, lakes (Fig. 2) , rivers, tanks etc., in **brackish water** (which is unpalatable for drinking) as well as **marine water** (with moderate to very high salinity). The commonly found fresh water algae are *Nostoc*, *Oedogonium*, *Chara*, *Cladophora*, etc. Fresh water algae have been reported from both tropical (e.g., as *Cyclotella* sp., *Orthoseira roeseana*, *Cocconeis pediculus*; *Volvox* sp., *Oedogonium* sp., *Nostoc* sp., *Microcystis* sp. etc.) as well as temperate waters (*Pediastrum* sp., *Fragillaria* sp., *Nitzschia* sp.). Fresh water algae based on their habit may be free floating, or attached to the bottom and may be classified as:

2.1.1 Planktonic Algae

They float freely on the surface of water and can be further differentiated into (a) **Euplanktons**: True planktons which are free floating from the beginning and never get attached to the substratum e.g. *Volvox*, *Cosmarium*, *Microcystis*, *Chlamydomonas*, *Scenedesmus* etc. (b) **Tychoplanktons**: Initially these algae are attached to the substratum but later they detach and become free floating e.g. *Zygnema*, *Oedogonium*, *Cladophora* etc.

Fig. 3 *Ulva* (a marine green algae) and other green sp. growing on rocks (Courtesy: Prof. Dinabandhu Sahoo)

2.1.2 Benthic Algae

These algae are bottom dwellers i.e. attached to the bottom of shallow pools. Mostly they are filamentous or colonial forms and may be unicellular (Fig. 3).

The benthic algae may further be classified as **epizoic** growing on animal body surface (*Cladophora* grows on snail); **epilithic** attached to stones or rocks (*Ulothrix tenuissima*, *Tribonema minus*, *Batrachospermum monilisperme* etc.); **epipelic** attached to sand and mud (*Oedogonium sp*, *Clostarium* sp, *Cosmarium* sp. etc.) and **epiphytic** growing on surface of plants (*Vaucheria sp*., *Ulothrix* sp.).

2.1.3 Neustonic Algae

These algae grow at air water interface for e.g. *Botrydiopsis* (Xanthophyceae), *Chromatophyton* (Chlorophyceae), *Nautococcus* (Chlorococcaceae) etc.

The algae growing in sea water are commonly known as marine algae (seaweeds) and they may grow in supralittoral, sublittoral or littoral (intertidal or subtidal) zones.

2.1.4 Supralittoral

These algae grow above the water level and are found growing on the rocky shore where they are just dampened only by the splashes of high spring tide waves such as *Prasiola stipitata* (a green seaweed), *Ulothrix flacca* etc.

2.1.5 Sublittoral or Infra Littoral

These algae grow below the water level.

Some common example of algae growing in brackish water are *Oscillatoria*, *Anabaena etc.*

2.1.6 Littoral

These algae grow in the areas where there is periodic exposure of tides and is a junction between land and sea. Some of the examples of algae growing in this "subtidal zones" are *Dictyota* sp., *Rhodymenia* sp., *Grateloupia* sp., *Gracilaria* sp., *Polysiphonia* sp., *Chondrus crispus*, *Laminaria* sp. etc. Algae growing in Intertidal zones are *Porphyra* sp., *Euglena sp*., *Laminaria* sp., *Gigartina* sp., *Fucus* etc.

2.2 Aerophytes

Algae growing on the surface of leaves, bark, moist walls, flower pots, rocks, fencing wires are grouped as aerophytes. These algae have adapted for aerial mode of life and obtain their water supply from rain, dew and atmospheric humidity. Some of the examples of aerophytic algae are:

Trentepholia, *Scytonema*, *Chroococus*, *Pleurococcus*	Bark of trees (Epiphloeophytes)
Phycopeltis epiphyton, *Somatochroon*, *Cephaleuros*	Surface of leaves (Epiphyllophytes)
Cyanoderma, *Trichophilus*, *Trentopholia*, *Pleurococcus*, *Chlorococcales*	Rocks and stones

2.3 Cryophytic Algae

These algae grow in permanent or semi – permanent snow – capped mountain and polar regions of the world. These algae when grow imparts colour to the snow for e.g.

Chlamydomonas nivalis, *Scotiella*, *Gloeocapsa*	Red color
Acyclonema	Brown or purple color
Chlamydomonas yellowstonensis, *Euglena Enkistrodesmus*, *Mesotaenium*	Green color
Scitiella, *Protoderma*, *Pleurococcus*, *Nostoc*	Yellow or yellowish green
Raphidonema	Black colour of snow

2.4 Endozoic Algae

Algae growing inside the body of vertebrates or aquatic animals are called endozoic algae for e.g.

Algae	Host
Zoochlorella	*Hydra viridis*
Zooxanthella	Fresh water sponges
Oscillatoria, *Simonsiella*, *Anabaeniolum*	Several vertebrates

2.5 Epizoic Algae

Many algal forms are known to grow on the surface of other aquatic animals (epizoic algae), such as:

Oscillatoria, *Protoderma*, *Bascicladia* and *Dermatophyton*	Turtles surface
Cladophora crispata	Shells of snails and molluscs
Stigeoclonium	Gills and nose of fish

2.6 Epiphytic Algae

These grow on other aquatic plants and are not host specific (Fig. 4) for e.g.

Algae	Host
Coleochaete nitellarum	*Chara* and *Nitella*
Chaetonema	*Tetraspora* and *Batrachospermum*
Rivularia	An angiosperm *Scirpus*
Chaetophora	Leaves of *Nelumbo and Vallisnaria*
Cocconis, *Achanthus*	*Lemna*

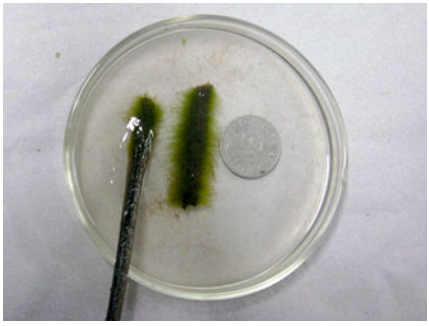

Fig. 4 *Oedogonium* filaments growing as epiphyte on another aquatic plant (Courtesy: Prof. Dinabandhu Sahoo)

Fig. 5 *Dunaliella salina* growing in salt pans of sambhar lake, Rajasthan (algae growing in extreme halophytic conditions) (Courtesy: Prof. Dinabhandhu Sahoo)

2.7 Halophytic Algae

These algae grow in waters with very high salinity (Fig. 5) may be upto 70–80 ppt for e.g. *Dunaliella*, *Stephanoptera*, *Chlamydomonas ehrenbergii*, *Oscillatoria*, *Ulothrix*.

2.8 Parasitic Algae

Members of algae are known to live as parasite and semiparasite on other algae as well as higher plants, where they cause severe damage, for e.g.

Cephaleuros virescence (Chlorophyceae) grows on Tea plants (Causes Red rust of tea)

Cephaleuros virescence (Chlorophyceae) grows on *Coffea arabica*, *Rhododendron*, *Magnolia* and *Piper nigrum* causing Red Rust.

Rhodochytrium (Chlorophyceae) grows on ragweed (*Ambrosia*) leaves

Phyllosiphon (Chlorophyceae) grows on the leaves of *Arisarum vulgare*

Polysiphonia fastigata (Rhodophyceae) grows on *Ascophyllum nodusum* as semiparasite

Ceratocolax (Rhodophyceae) grows in *Phyllophora thallus*

2.9 Symbiotic Algae

Members of cyanophycean algae grow in association with several plants such as:

- *Nostoc* grows in association with *Anthoceros*, *Notothylas*, *Blasia*
- *Anabaena cycadaceae* grows in the corolloid roots of *Cycas plants*
- *Anabaena azollae* grows in *Azolla*

2.10 Terrestrial Algae

Algae growing on soils, logs, rocks etc. are grouped under terrestrial algae. Most of the terrestrial algal genera grow on or beneath the moist soil surface, for e.g.

Vaucheria, *Botrydium*, *Fritschiella* and *Oedocladium*	Grow on soil surface
Nostoc, *Anabaena*, *Euglena*	Subterranean habit

2.11 Thermophytic Algae

These algae grow in hot springs, where the temperature may go beyond 85 °C.Almost all thermophytic algae are known from Cyanophyceae.

Cyanidium caldarium	Acidic hot springs

Other examples of thermophytic algae are *Synechococcus*, *Synechocystis*, *Phormidium*, *Scytonema etc*.

3 Thallus Organization

Range of algal thallus varies from unicellular to multicellular forms or microscopic to macroscopic structures, with their size ranging from a few microns to some meters. *Micromonas pusilla* is known to be smallest algae which is unicellular and is 1 μm (0.00004 in.), on the other hand giant kelps has longest thalli that reaches up to 60 m (200 ft) in length. The unicellular forms may remain solitary as a single unit which are capable of completing their life cycle by providing all physiological, biochemical, genetical requirements and may be motile or non-motile. When these unicellular forms are held together in a common gelatinous matrix, they constitute colonial forms, which are considered intermediate between unicellular and multicellular structures. The other intermediate stages considered in thallus organization of algae are palmella, dendroid, palmelloid, coccoid, filamentous, siphonaceous, heterotrichus, uniaxial, multiaxial etc. In colonial forms the individual cells are

independent in both structure and function. The multicellular form ranges from microscopic to macroscopic, where some of the macroscopic forms reported from phaeophyceae and rhodophyceae grow upto few meters. The multicellular forms may be parenchymatous or sometimes the thallus is differentiated (Round 1973).

3.1 Unicellular Forms

Unicellular or acellular forms can be motile or non-motile and are further grouped into three categories based on presence or absence of flagella. The motile forms are either rhizopodial or flagellates and non-motile forms are coccoid.

3.1.1 Rhizopodial

The rhizopodial forms lacks rigid cell wall and have a naked protoplast, cell envelope is periplasmic, soft and permits extensive changes in shape and size of thallus, lack flagella and instead possess cytoplasmic projections called Pseudopodia and Rhizopodia. These forms move in ameboid manner.

Examples: *Chrysamoeba* (Chrysophyceae), *Rhizochloris* (Xanthophyceae) and Dinophyceae.

3.1.2 Flagellates

The vegetative phase of many algae is a motile, flagellate unicell. Flagella may be one or two or many, equal or unequal, and tinsel or whiplash type. Motile unicells are commonly spherical, elongate, ovoid or round in cross section.

Examples: Flagellated forms are present in almost all groups of algae except Myxophyceae, Phaeophyceae, Rhodophyceae

3.1.3 Coccoid

Non motility predominates and motility is entirely absent or restricted only to reproductive stages. Occur in majority of algal classes and predominates in Xanthophyceae (70 %). Coccoid forms are provided with a rigid cell wall and are non-flagellated.

Example: *Prochloron*, *Aphanocapsa* and *Synechococcus*

3.1.4 Spiral

Unicellular, spiral filamentous.

Example: *Spirulina*

3.2 Multicellular Forms

3.2.1 Colonial Aggregation

A colony is a group of separate cells generally similar in structure and function and aggregated by a mucilaginous envelope. The colonial habit can be further differentiated into:

COENOBIUM: It has a definite number of cells arranged in a particular manner which is determined at the juvenile stage and does not increase during its subsequent growth even though the cells enlarge. It comprises aggregation of flagellate (*Volvox*) or non-motile cells (*Hydrodictyon*).

PALMELLOID: Colonial members in which "non – motile" cells remain embedded in an amorphous gelatinous or mucilaginous matrix. In this type neither the number, nor the shape and size of cells is constant. The cells are aggregated in a common mucilaginous envelope. All the cells are independent of each other and fulfil the function of an individual. Most normally flagellate or coccoid unicellular algae may enter (often temporary) palmella stage, a condition where the flagella are lost and the individuals undergo successive vegetative divisions while embedded in a common gelatinous matrix, named after the volvocalean (Chlorophyceae) genus *Palmella*. This term may be strictly applied to those algae where the cells will readily revert to a motile condition or may be expanded to include all algae where the palmelloid habit is more permanent. Example: *Palmella*, *Microcystis*.

TETRASPORAL: The motile stages (if present at all) are restricted to the reproductive cells. The regular colonies of the Cyanophyceae (e.g. *Merismopedia*, *Halopedia* as flat plates of cells and *Eucapsis*, with colonies in cubical masses) are included in the tetrasporal types. A feature of Tetrasporial form is the presence of mucilaginous pseudocilia.

DENDROID: The cells are united in a branching manner by localized production of mucilage at the base of each cell. The whole colony looks like a tree in habit. Examples: *Chrysodendron*.

RHIZOPODIAL: The cells of rhizopodial colonies are united through rhizopodia, as in *Chrysidiastrum* (Chrysophyta).

3.2.2 Filamentous Forms

Filaments are formed when vegetative divisions occur in a transverse plane. In a filament the Uniseriate row of cells are joined end to end in a transverse plane through middle lamella. In Cyanophyta, a filament includes both the trichomes (i.e. the uniseriate row of cells) and its sheath. The filament may be branched or unbranched.

Unbranched Filaments: Example: *Spirogyra*, *Zygnema*, *Oedogonium*, *Ulothrix*.
Branched Filaments: Example: *Cladophora*, *Pithophora*

The branching of the filament is either True or False.

FALSE BRANCHING: It occurs in *Scytonemataceae* (Cyanophyta), the trichome generally fragments due to the degeneration of an intercalary cell (or by the formation of biconcave separation discs) after which one or both of its ends adjacent to the dead cell grows out of the parent sheath, giving the resemblance of branching.

TRUE BRANCHING: It results from repeated transverse divisions of the lateral outgrowths produced by a few or many scattered cells of the main filament. The truly branched thalli are of four types:

- **Simple branched filament**: A branched upright filamentous thallus is attached by a simple disc derived from the basal cell Example: *Cladophora*.
- **Heterotrichous**: The thallus consists of two parts, a prostrate creeping base and an erect branched upright system. It is the most highly evolved type of filament construction. During its development, a system of branched creeping filaments is produced first and functions as the holdfast. These creeping system of filaments will further give rise to a system of erect and branched filaments Example *Stigeoclonium* (Chlorophyta) and *Ectocarpus* (Phaeophyta)
- **Parenchymatous forms**: This occurs when cells of the primary filament divide in all directions, any essentially filamentous structure is thus lost early. Example: *Porphyra*, *Ulva*, *Enteromorpha*. This type of organization is most common in Phaeophyceae (Fucales, Laminariales).
- **Pseudoparenchymatous forms**: This habit results from a close juxtaposition of the branched filaments of a single or many axial filaments. If branches from a single filament are involved as in *Batrachospermum*, the thallus construction is called as uniaxial. If branches of many axial filaments aggregates, the thallus is multiaxial e.g. *Nemalion*.

3.2.3 Siphonocladous Organization

It is restricted to members of chlorophyceae in which the unbranched (*Urospora*, *Chaetomorpha*) or branched (*Acrosiphonia*, *Cladophora*) filaments are composed of multinucleate (semi – coenocytic) cells. One of the siphonocladous algae *Valonia* occurs as a single, spherical vesicle upto 10 cm in diameter has been described as the largest plant cell.

3.2.4 Siphonous Organization

In a number of marine Chlorophyceae (order Siphonales, Bryopsidales, Dasycladales) and some Xanthophyceae (*Botrydium*, *Vaucheria*) enlargement and elaboration of the thallus proceeds in the absence of septa. Nuclear divisions are not

followed by cytokinesis (free nuclear division) and the result is a coenocytic, multinucleate thallus and a siphonous organization.

Siphonous organization ranges from Saccate (e.g. *Botrydium*) to uniaxial (e.g. *Vaucheria*, *Bryopsis*) and multiaxial (e.g. *Codium*) forms.

4 Plastids and Photosynthetic Pigments

The most prominent feature of an algal cell is the plastid, which makes an important characteristic of an algal cell for classification. Plastids which consist of chlorophyll a and chlorophyll b are called CHLOROPLASTS and the one which lacks chlorophyll b are called CHROMATOPHORES (Sharma 2011). Prescott (1969) has classified them as "the pigment containing body with chlorophyll in dominance is Chloroplast and when the pigments other than chlorophyll are dominant are called Chromatophores".

In algae, different forms and shapes of plastids are observed such as (Fig. 6):

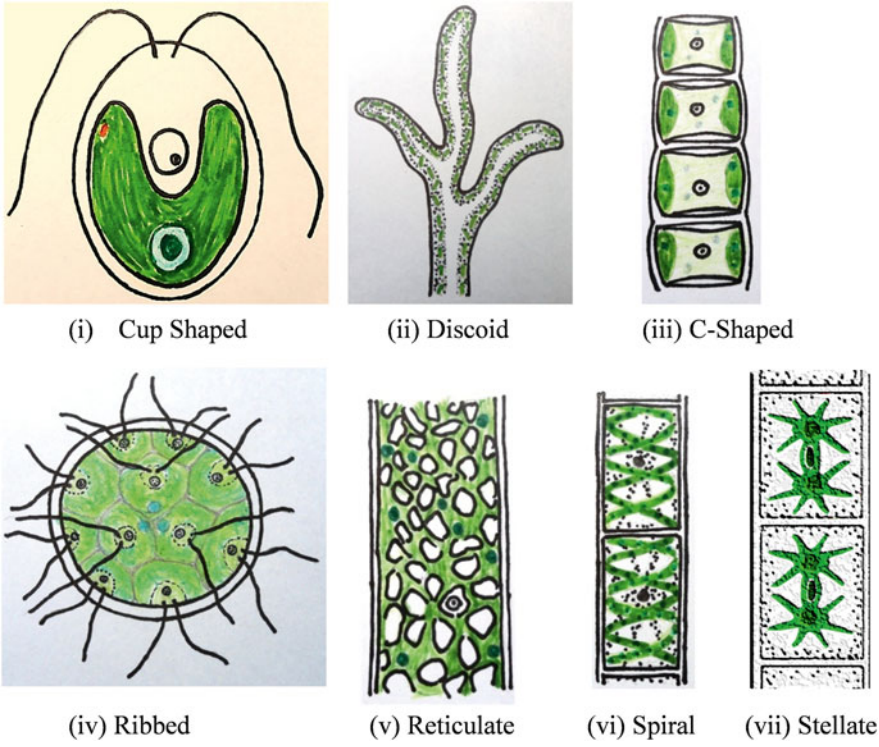

Fig. 6 Different shapes of algal chloroplast (i) *Chlamydomonas*, (ii) *Vaucheria*, (iii) *Ulothrix*, (iv) *Volvox* (v) *Cladophora*, (vi) *Spirogyra* (vii) *Zygnema*

(i) Cup shaped: *Chlamydomonas*, *Volvox*
(ii) Discoid: *Chara*, *Vaucheria*, Dinophyceae, Bryopsidophyceae and many diatoms
(iii) Girdle or C shaped: *Ulothrix*
(iv) Ribbed: *Volvocales*
(v) Reticulate: *Oedogonium*, *Hydrodictyon* and *Cladophora*
(vi) Spiral or ribbon shaped: *Spirogyra*
(vii) Stellate: *Zygnema*

According to location the plastid may be

(i) Parietal: Chaetophorales, Phaeophyta, Rhodophyta, Chrysophyceae, Pinnate Diatoms
(ii) Axial: *Porphyridium*, *Bangia* (John et al. 2002)

The basic structure of chloroplast is similar throughout the plant kingdom with an envelope, stroma and internal lamellar membranes. Only the Cyanophycean members shows the typical prokaryotic structure, where the thylakoids are not bound in any envelope and they lie freely in cytoplasm.

In general, the following types of photosynthetic pigments (Table 1) have been reported in an algal cell (Round 1973):

(i) Chlorophylls: There are five types of chlorophylls in algae a, b, c, d and e. There occurrence in different algal groups has been reported in table 1.
(ii) Carotenes: There are five types of carotenes reported in different algal groups as listed in table, and it is an accessory photosynthetic pigment. The different types of carotenes are α, β, ϒ, e, c and flavicin.
(iii) Xanthophylls: There are about 20 xanthophylls reported in algae which are characteristic of different algal groups.
(iv) Phycobilins: There are six types of phycobilins in algae.

5 Reserve Food

Polysaccharides and fats are two principle storage products in different members of algae as summarized in Table 2:

6 Cell Wall

Most of the members of algae consist of a cell wall, and cell wall consists of non-living material which are variously classified. In algae polysaccharides are chief constituent of cell wall with two major components (i) Fibrillar (Cellulose, Mannans, Xylans) and (ii) Amorphous (Alginic acid, fucoidan, galactans etc.) (Lee 2008). The fibrillar component forms the skeleton of cell wall and amorphous component

Table 1 Pigments in different algal groups

S. No.	Class	Thylakoid/Lamella	Chief pigment (chlorophyll)	Accessory pigments (carotenes)	Accessory pigments (xanthophyll)
1.	Chlorophyceae (green algae)	2 – many	a, b	α, β and c	Lutein, zeaxanthin, violaxanthin and neoxanthin
					Siphonein and siphonoxanthin (siphonales)
2.	Xanthophyceae (yellow – green)	3	a, e	β	Lutein, fucoxanthin, violaxanthin, vaucherioxanthin and neoxanthin
3.	Chrysophyceae (orange algae)	3	a, c	β	Lutein, fucoxanthin, neofucoxanthin
4.	Bacillariophyceae (diatoms/ yellow or golden brown algae)	3	a, c	α, β and e	Fucoxanthin, neofucoxanthin, diatoxanthin, diadinoxanthin
5.	Cryptophyceae (nearly brown)	2	a, c	α and e	Zeaxanthin, diadinoxanthin, cryptoxanthin
					Phycobilins – cryptomonad, phycocyanin and phycoerythrin
6.	Dinophyceae (dark yellow or brown)	3	a, c, Peridinin	β and e	Dinoxanthin, neodinoxanthin, peridinin, neoperidinin, diadinoxanthin, neodiadinoxanthin, flavoxanthin
7.	Chloromonadinae (bright green)		a, c	β	Diadinoxanthin
8.	Euglenophyceae	3	a, b	β	Lutein, zeaxanthin, neoxanthin, astaxanthin
9.	Phaeophyceae (Brown Algae)	3	a, c	β and e	Fucoxanthin, flavoxanthin, Lutein, Violaxanthin and neoxanthin
10.	Rhodophyceae (red algae)	1	a, d	α and β	r–phycocyanin, r – phycoerythrin, lutein, zeaxanthin, violaxanthin
11.	Myxophyceae (Cyanophyceae, blue green algae)	1	a	β and e, flavacene,	c–phycocyanin, c–phycoerythrin, Myxoxanthin, myxoxanthophyll

Table 2 Reserve food in different algal groups

S. no.	Class	Food reserve	Location
1.	Chlorophyceae (Green algae)	Starch	Starch grains within chloroplast envelope
2.	Xanthophyceae (Yellow – green)	Oil, fats, leucosin	With in chloroplast
3.	Chrysophyceae (Orange algae)	Oil, fats, chrysolaminarin	Cytoplasm
4.	Bacillariophyceae (Diatoms/ yellow or golden brown algae)	Fats and leucosin	Vesicles
5.	Cryptophyceae (Nearly brown)	Starch	Outside chloroplast envelope
6.	Dinophyceae (Dark yellow or brown)	Oil and Starch	Outside chloroplast
7.	Chloromonadinae (Bright green)	Oils and fats	Cytoplasm
8.	Euglenophyceae	Paramylum, fats	Cytoplasm in close proximity to chloroplast envelope
9.	Phaeophyceae (Brown algae)	Laminarin, mannitol, oil	Food is formed in pyrenoids and diffuses into the vacuoles
10.	Rhodophyceae (Red algae)	Floridean starch	Cytoplasm outside chromatophore
11.	Myxophyceae (Cyanophyceae, blue green algae)	Myxophycean starch	Chromatoplasm

forms the matrix embedding the fibrillar part. Different groups of algae has differential nature of cell wall as summarized in Table 3:

7 Flagella

A flagellum is a thread like fine structure that originates from cytoplasm. The number and position of flagella varies in all algal cell as mentioned in Table 4. Flagella are meant for locomotion in algal cells. Flagella can be equal or unequal in length, can be inserted anteriorly or posteriorly and can also be inserted laterally.

When equal in length flagella are termed Isokont and unequal condition is termed Anisokont. In heterokont condition both tinsel and whiplash type of flagella are present. The whiplash flagellum is the smooth naked flagellum and is also known as acronematic or peitchgeisel. Tinsel flagellum, which is also known as pantonematic or flimmer, is with minute hairs on its surface termed mastigonemes or flimmers.

Table 3 Cell wall composition in different algal groups

S. no.	Class	Nature of cell wall
1.	Chlorophyceae (Green algae)	Cellulose, pectin, rarely hemicellulose
2.	Xanthophyceae (Yellow – green)	Pectin
3.	Chrysophyceae (Orange algae)	Leucosin, fats, chrysolaminarin
4.	Bacillariophyceae (Diatoms/yellow or golden brown algae)	Silica, cell wall is split into two outer halve is silicified and inner is pectic
5.	Cryptophyceae (nearly brown)	Cell wall absent instead a firm periplast is present
6.	Dinophyceae (Dark yellow or brown)	Mostly cellulosic, some have a periplast
7.	Chloromonadinae (Bright green)	Cell wall absent
8.	Euglenophyceae	Absent, periplastic
9.	Phaeophyceae (Brown algae)	Cellulose, alginic acid, fucocinic acid
10.	Rhodophyceae (Red algae)	Cellulose, pectins, polysulphate esters
11.	Myxophyceae (Cyanophyceae, Blue green algae)	Pectin, hemicellulose, mucopeptide

Table 4 Nature of flagella in different groups of algae

S. no.	Class	Number of flagella	Point of insertion
1.	Chlorophyceae (Green algae)	2 or 4, equal, whiplash	Anterior
2.	Xanthophyceae (Yellow – green)	2, unequal, tinsel and whiplash	Anterior
3.	Chrysophyceae (Orange algae)	1 or 2, unequal, either both whiplash or one whiplash and other tinsel	Anterior
4.	Bacillariophyceae (Diatoms/ Yellow or golden brown algae)	1, tinsel	Anterior
5.	Cryptophyceae (Nearly brown)	2, equal or unequal, flimmergeissel i.e. both flagella bears lateral hairs, arranged in two opposite rows on longer flagellum and in one row on the shorter flagellum	Apical or lateral
6.	Dinophyceae (Dark yellow or brown)	2, unequal (heterokont)	Latero-ventral
7.	Chloromonadinae (Bright green)	2, equal (isokont)	Apical or lateral
8.	Euglenophyceae	1 rarely 2 or 3, tinsel	Anterior
9.	Phaeophyceae (Brown algae)	2, unequal, whiplash and tisel	Lateral
10.	Rhodophyceae (Red algae)	Absent	
11.	Myxophyceae (Cyanophyceae, Blue green algae)	Absent	

8 Reproduction

Algae reproduces both asexually as well as sexually. The asexual method of reproduction includes reproduction by vegetative methods and reproduction by spores. The different methods of reproduction in algae are discussed below:

8.1 Vegetative Methods

The vegetative reproduction is a type of reproduction where a part of thallus becomes specialized and gets detached from the parent to form a new offspring. The new individual thus formed in this way is genetically identical to parent and no variation is observed. The different ways by which algae reproduces vegetatively are:

8.1.1 Budding

Some vesicles which get detached from the parent plant by the formation of a septum, and are cable of giving rise to a new thallus are called buds. For example: *Protosiphon* (Fig. 7).

8.1.2 Cell Division

Simplest method of reproduction, where, the unicellular algae divides into two by fission. For example: *Chlamydomonas* (Fig. 8), Diatoms.

Fig. 7 Budding in *Protosiphon*

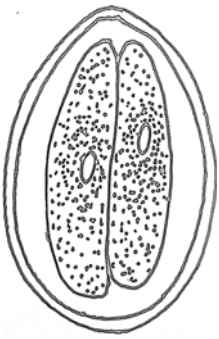

Fig. 8 Cell division in *Chlamydomonas*

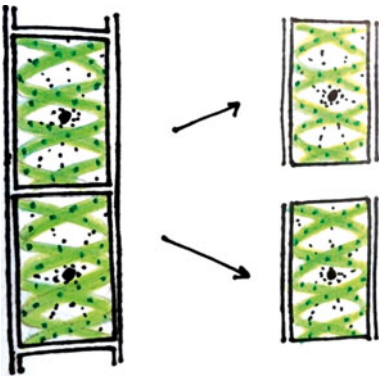

Fig. 9 Fragmentation in *Spirogyra*

8.1.3 Fragmentation

During this process the filamentous thalli breaks into two or many fragments (Fig. 9). Each fragment is capable of giving rise to a new filament. The fragmentation may result due to accidental breakage or formation of separation of disc. For example *Spirogyra*, *Nostoc*, *Oscillatoria*.

8.1.4 Bulbils

These are tuber like outgrowths developed mostly at the rhizoidal tips. In *Chara* they develop at the nodes. These bulbils when detach from the parent plant give rise to new thallus. For example: *Chara*.

8.1.5 Hormogones

These are short segments of filaments with in the sheath of parent filament. Hormogones are many celled segments and are commonly found in members of cyanophyceae.

8.1.6 Amylum Stars

Present on lower nodes in *Chara*. These are star shaped aggregation of starch.

8.2 Asexual Reproduction or Reproduction by Spores

8.2.1 Akinetes

These are thick walled spores with abundance of food reserve. They withstand the unfavourable conditions and germinate on the onset of favorable conditions. Example: *Nostoc*, *Gloeotrichia*, *Ulothrix*, *Cladophora*, *Pithophora*.

8.2.2 Autospores

During unfavourable conditions protoplast inside the sporangium divides and forms spores which are identical to parent plant are termed autospores. The autospores are non-motile, thick walled and abundant in food reserve. Example: *Scenedesmus*, *Chlorella* etc.

8.2.3 Aplanospores

They may be formed singly or by the repeated division of the sporangium of parent plant during drought (unfavourable conditions). Example: *Ulothrix*.

8.2.4 Bispores

When two spores are formed in a sporangium they are called bispores and the sporangium is termed as bisporangium (Fig. 12) as reported in *Grateloupia filicina* (Baweja and Sahoo 2002); *Porphyra* and in *Lithophylum littorale* (West and Hommersand 1981).

8.2.5 Carpospores

These are formed in carposporangium during triphasic life cycle of rhodophycean members. They are formed from zygote and are diploid in nature. Example: *Polysiphonia*, *Gracilaria*, and *Grateloupia* (Baweja 2006).

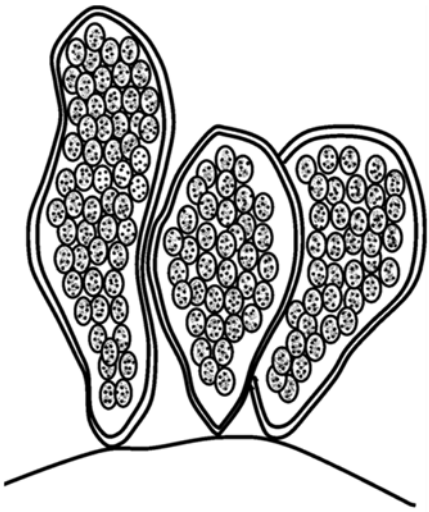

Fig. 10 Endospores of *Dermocarpa*

8.2.6 Endospores

These are formed in the sporangium by successive repeated divisions of cell contents. All spores are formed first, then the sporangium opens to liberate the motile spores (Fig. 10). e.g. *Dermocarpa clavata*.

8.2.7 Exospores

During exospore formation in Cyanophycean members, the sporangium gets burst at the apex and is exposed to the external environment and further by successive repeated divisions of cell contents the spherical spores are formed which are termed exospores. All spores get liberated one by one. For example *Chamaesiphon*, *Stichosiphon* (Fig. 11).

8.2.8 Hypnospores

Aplanospores with thick walled and abundance of food reserve are known as hypnospores. They may germinate directly or their protoplast may divide further to form zoospores which germinate to new plant. Example: *Pediastrum*, *Sphaerella* etc.

Fig. 11 Exospores in *Chamaesiphon*

8.2.9 Monospores

Single spores formed in a sporangium are termed as monospores. Commonly monospores are found in Brown and red algae and are considered to be the commonest asexual spores of red algae (Dixon 1963; South and Whittick 1987).

8.2.10 Neutral Spores

These are common in red algae for example *Bangia* where, the vegetative cells directly gets transformed into spores and such spores are termed neutral spores.

8.2.11 Paraspores

When more than four spores are formed because of reduction division in a sporangium in red algae, such spores are called as paraspores or polyspores for example: *Palmaria elegans*, *Ceramium* sp. (Whittick 1977; Rueness 1973; Sharma 2011).

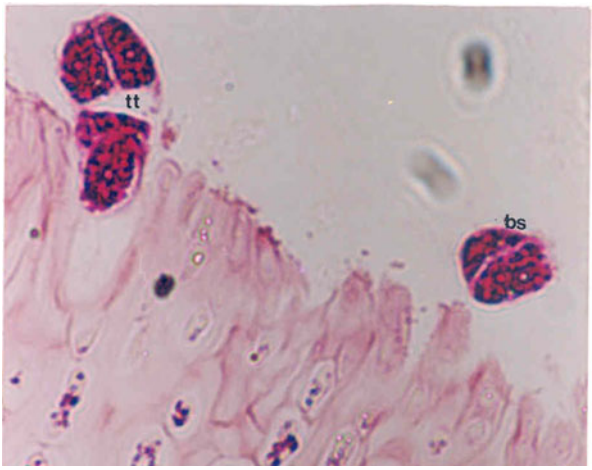

Fig. 12 A bispore (bs) and Tetraspore (tt) of *Grateloupia filicina* (Courtesy: Dr. Pooja Baweja)

8.2.12 Statopsores

Thick and ornamented smooth walled spores of bacillariophyceae are termed as Statospores. Example: *Chaetoceros* (Bacillariophyceae), Chrysophyceae, Xanthophyceae etc (Pienaar 1980; Sandgren 1983; Sharma 2011).

8.2.13 Tetraspores

Commonly found in red algae. These are formed in four in number in a tetrasporangium (Fig. 12). E.g. *Porphyra*, *Gracilaria*, *Grateloupia*.

8.2.14 Zoospores

The motile and naked spores are known as zoospores. They may be haploid or diploid and are formed in zoosporangium. The zoospores of *Vaucheria* are known as synzoospores as they are multinucleate and multiflagellate. Zoospores may have two, four or many flagella (Fig. 13) for example:

Biflagellate zoospores:	*Chlamydomonas*, *Ulothrix*
Quadriflagellate:	*Ulothrix*
Multiflagellate	*Oedogonium*

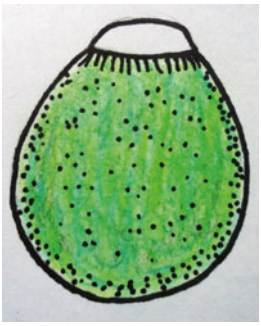

Fig. 13 Zoospores of *Oedogonium*

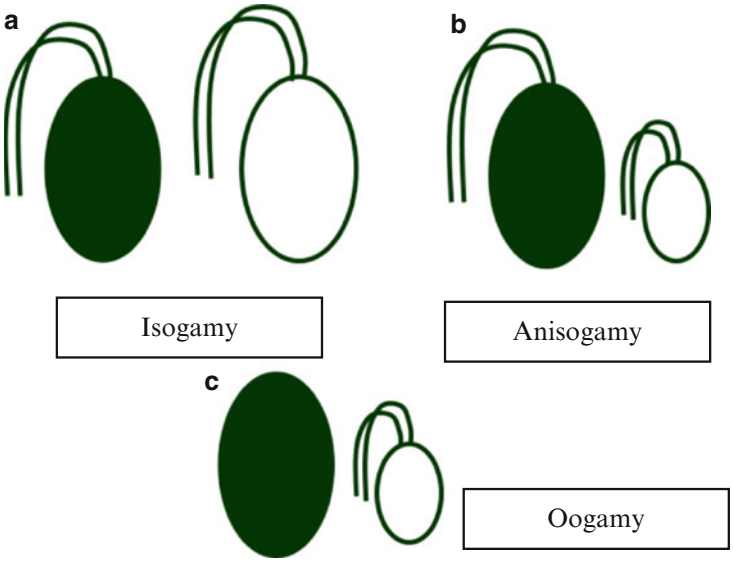

Fig. 14 Sexual reproduction in algae (**a**) Isogamy: Fusing gametes are morphologically identical; (**b**) Anisogamy: Fusing gametes are morphologically dissimilar; (**c**) Oogamy: Female gamete becomes immobile, larger in size and male gamete is mobile and smaller in size

8.3 Sexual Reproduction

Sexual reproduction has been reported from all members of algae except cyanophyceae. In sexual reproduction two opposite mating types (gametes) fuse to form a zygote. Depending on the structure and behaviour of fusing gametes (Fig. 14) it can be classified as:

8.3.1 Isogamy

In it the fusing gametes are iso gametes (iso – similar) i.e. they are morphologically and physiologically similar. Example: *Chlamydomonas*, *Ulothrix* etc.

8.3.2 Anisogamy

In it the fusing gametes are aniso gametes (aniso – dissimilar) i.e. they are morphologically and physiologically not similar and are different. In some algae physiological anisogamy has been reported where the gametes are morphologically similar but physiologically different (e.g. *Spirogyra*). Example: *Chlamydomonas braunii* etc.

8.3.3 Oogamy

It is an advanced type of sexual reproduction and has been observed in higher plants and animals too. In algae, usually the male partner is motile and female partner is non-motile with exception in Rhodophyceae where male partners are too non-motile and reaches female by the water currents. In algae the male antherozooids are formed in Antheridium and female gametes are formed in oogonium. Example: *Chlamydomonas*, *Oedogonium*, *Chara*, *Polysiphonia*, *Porphyra* etc.

8.3.4 Autogamy

It is commonly reported in Diatoms, where the fusing gametes are formed from the same mother cell and are haploid. After fusion they form a diploid zygote with no genetic variation.

9 Perennation or Survival Strategies in Algae

The freshwater and sub aerial algae are unprotected and are frequently exposed to the risk of desiccation and extremes of temperature, on the other hand, marine algae live in conditions where variations in external environmental conditions of life are rare but still they are exposed to tidal fluctuations. To combat this, algae has developed certain methods to carry over the period of severe conditions till the next growing season. Thus, perennation can also be defined as a temporary rest for algae, where all the metabolic activities are ceased till the onset of favourable season. However, fresh water and sub aerial algae undergo perennation by producing asexual thick walled spores, the marine algae avoids the tidal fluctuations by secreting a lot of mucilage which keeps them dehydrated during the low tides (Vashishtha et al. 2007).

Main methods of perennation is fresh water and sub aerial algae are (a) Akinetes (2) Aplanospores (3) Palmella Stage (4) Cysts (5) Oospore or Zygote (6) Hormospores (7) Hypnospores (8) Tubers.

9.1 Akinetes

Akinetes are specially modified vegetative cells with thick, resistant cell wall and abundance of food reserve. These are formed during conditions of drought, when the whole thalli or filament dries and die but akinetes remains to overcome the unfavourable conditions. As the favourable conditions returns, they develop into new plants. Example: *Ulothrix*, *Cladophora*, *Pithophora*, and *Nostoc*.

9.2 Aplanospores

These are thick walled non motile spores which are formed during unfavourable conditions. They are formed by the rounding off cytoplasmic contents and development of thick wall. Aplanospores are liberated by the separation of parent wall. After a period of rest and on the approach of favourable conditions they develop into new plants. Example: *Chlamydononas*, *Ulothrix*, *Coleochaete*, *Oedogonium*.

9.3 Palmella Stage

In *Chlamydomonas* during adverse conditions the vegetative cell divides and form new daughter cell. Which further increase in number by repeated cell divisions. All daughter cells thus formed remain clustered together inside a mucilaginous envelop. This envelope is formed by the gelatinisation of parent cell wall of the successive generations. This is called Palmella stage, which is of short duration and represents milder method of perennation.

9.4 Cysts

During unfavourable conditions the plant body of certain algae like *Euglena*, *Vaucheria*, *Protosiphon* produces cysts, which separates. Each cyst germinates directly into a new plant on the approach of favourable conditions. In *Vaucheria* and *Protosiphon*, the cysts are termed Coenocysts as in these algae cysts are formed of multinucleate segment of thallus.

9.5 Oospore or Zygospore

In algae zygote or zygospore do not germinate directly and undergo a resting period before the onset of favourable conditions, therefore formation of zygotes is considered a method of perennation in green algae. The zygote which is formed as a result of sexual fusion are thick walled structure with plenty of food material.

9.6 Hormospores

During unfavourable conditions, hormogones in blue green algae gets separated from the tips of trichomes. These hormogones gets rounded off, secretes a thick wall and are termed hormopsores.

9.7 Hypnospores

When aplanospores secretes a thick wall around them to withstand extreme stressful conditions such as winter and draught, are termed as hypnospores. These spores remain in quiescent condition for some time, and as the favourable conditions approaches they germinate into a new plant for e. g. *Westiella* (Cyanophyceae).

9.8 Tubers

These are commonly reported in *Chara* and *Cladophora* and are outgrowth of rhizoids. They are formed from specialised rhizoidal cell which divides a number of time, gets thick walled and store abundance of food reserve material. As these are produced from the subterranean part of the plant, these are able to withstand the extremes of drought and temperature.

Acknowledgment The authors are grateful for the support provided by Mrs. Meena Malhotra, Department of Botany. Maitreyi College, University of Delhi for the hand diagrams.

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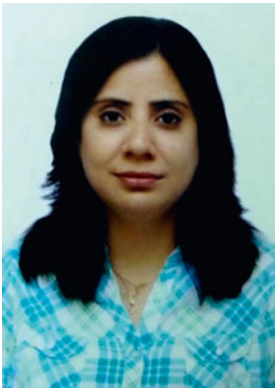

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Classification of Algae

Pooja Baweja and Dinabandhu Sahoo

1 Introduction

Classification is the systematic grouping of organisms into categories on the basis of relationships between them, where the relationship can be either evolutionary or structural. The hierarchy for the classification of plants is Division, Class, Order, Family, Genus and Species as per International code of botanical nomenclature (ICBN) (Sharma 2011). Different categories of algae as recommended by ICBN are:

Division:	Phyta	E.g. Chlorophyta
Sub – division:	Phytina	
Class:	Phyceae	E.g. Chlorophyceae
Sub – class:	Phycidae	
Order:	Ales	
Sub – order:	Inales	
Family:	Aceae	
Sub – family:	Oideae	
Tribe:	Ease	
Genus:		
Species:		
Variety:		
Form:		

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D. Sahoo, J. Seckbach (eds.), *The Algae World*, Cellular Origin, Life in Extreme Habitats and Astrobiology 26, DOI 10.1007/978-94-017-7321-8_2

2 Evolution of Algal Classification

The history of classification dates back to Carolous Linnaeous, who first classified plants into 25 classes based on "sexual system" considering the number of stamens and carpels in their flowers. Out of his 25 classes, in "Cryptogamia" which contains plants with "concealed reproductive organs" Linnaeus, proposed 14 algal genera of which only 4, Conferva, *Ulva*, *Fucus* and *Chara* are now considered as algae (Dixon 1973).

W. H. Harvey is considered as one of the first algologist who proposed the first descriptive algal classification. Since W. H. Harvey several classifications have been proposed based on a variety of characters including morphological, physiological, biochemical and more recently the molecular characters have also been considered. The main characters which are being widely used for algal classification are:

I. Photosynthetic Pigments: Chlorophylls, Carotenoids (Carotenes and Xanthophylls), Phycobilins
II. Biochemical nature of food reserve.
III. Cell wall composition
IV. Flagella

The major classification proposed by different algologists for algae are:

2.1 Classification Proposed by W. H. Harvey (1836)

William Henry Harvey (1811–1866), was a pioneer algologist. He classified algae for the first time in 1836 into four groups based on the colour of thallus:

S. No.	Group	Colour
1.	Chlorospermae	Green
2.	Melanospermae	Brown
3.	Rhodospermae	Red
4.	Diatomacea	Diatoms

2.2 Classification Proposed by A. W. Eichler (1883)

Eichler (1883), created a new division Thallophyta while classifying the plants and grouped Algae and Fungi together in this. Further Eichler classified algae into five groups:

S. No.	Group	Colour
1.	Cyanophyceae	Blue green algae
2.	Diatomeae	Diatoms
3.	Chlorophyceae	Green algae
4.	Phaeophyceae	Brown algae
5.	Rhodophyceae	Red algae

2.3 Classification Proposed by Engler and Prantle (1887–1915)

In first edition of *Die Naturlichen Pflanzenfamilien* algae and fungi were grouped together under Euthallophyta and different groups of algae were identified as:

1.	Schizophyta	Cyanobacteria	
2.	Flagellatae		
3.	Dinoflagellata	Flagellate protists	
4.	Bacillariales	Diatoms	
5.	Euphyceae	Algae	Conjugatae
			Chlorophyceae
			Charophyta
			Phaeophyta
			Rhodophyceae
6.	Eumycetes	Fungi	

2.4 Classification Proposed by West (1916)

Based on reproductive structures and presence or absence of flagella West divided algae into four categories:

1.	Isokontae	Flagella of equal size
2.	Akontae	Flagella absent
3.	Stephanokontae	Flagella crowned
4.	Heterokontae	Flagella of unequal size

2.5 Classification Proposed by A. Pascher (1931)

A. Pascher, proposed an evolutionary classification based on phylogeny and inter-relationships among various groups. In his classification algae has been placed above the rank of a division. He classified algae into eight divisions which were further subdivided into different classes as:

S. No.	Division	Classes
1.	Chrysophyta	(a) Chrysophyceae
		(b) Diatomeae
		(c) Heterokontae
2.	Phaeophyta	(a) Phaeophyceae
3.	Pyrrophyta	(a) Cryptophyceae
		(b) Desmokontae
		(c) Dinophyceae

(continued)

S. No.	Division	Classes
4.	Euglenophyta	(a) Euglenophyceae
5.	Chlorophyta	(a) Chlorophyceae
		(b) Conjugatae
6.	Charophyta	(a) Characeae
7.	Rhodophyta	(a) Bangineae
		(b) Floridineae
8.	Cyanophyta	(a) Myxophyceae

2.6 Classification Proposed by J. E. Tilden (1933)

J. E. Tilden (1933), based on reserve food material, pigmentation and flagellation classified algae into five classes. According to Tilden, Pigments are of vital importance in the development and advancement of algal members and thus supported the retention of names of algae based on colours.

S. No.	Class	Colour
1.	Myxophyceae	Blue green
2.	Rhodophyceae	Red
3.	Phaeophyceae	Brown
4.	Chrysophyceae	Yellow green
5.	Chlorophyceae	Green

Characteristic Features

1. Myxophyceae: True cell wall absent, true nucleus absent, central protoplast is transparent surrounded by peripheral pigmented protoplast. In peripheral protoplast pigments like phycocyanin (blue coloured pigment) and chlorophylls present thus imparting blue green colour to cell. Carotenes present. In rare forms (e.g. *Oscillatoria, Phormidium*) phycoerythrin is present. True starch absent. Flagella absent.
2. Rhodophyceae: Principal pigment is phycoerythrin which masks other pigments, chlorophyll, carotene, xanthophyll and phycocyanin are also present. Floridean starch present. Flagella absent.
3. Phaeophyceae: Principal pigment is fucoxanthin, other pigments are chlorophyll a, b, Carotene, xanthophyll. Flagella present, one or two, unequal.
4. Chrysophyceae: Golden brown chromatophores present, assimilation product fatty oil, flagella present.
5. Chlorophyceae: Chlorophylls dominate, flagella present, reserve food material starch.

2.7 *Classification Proposed by F. E. Fritsch (1935)*

F. E. Fritsch (1935), also known as Father of Phycology, proposed the most acceptable and comprehensive algal classification. Fritsch published two volumes of "Structure and Reproduction of the Algae". His classification is based on different characteristics as pigmentation, chemical nature of reserve food material, flagellar arrangement (kind, number and point of insertion), presence or absence of organized nucleus in cell and mode of reproduction. He emphasized the account of living forms of algae as compared to fossil forms, all of which have been grouped in one class. He classified algae into 11 classes as:

S. No.	Class	Orders
1.	Chlorophyceae	**Nine**
	(Green algae)	Volvocales, chlorococcales, ulotrichales, cladophorales
		Chaetophorales, oedogoniales, conjugales, sipohonales, charales
2.	Xanthophyceae	**Four**
	(Yellow – green)	Heterochloridales, heterococcales
		Heterotrichales, heterosiphonales
3.	Chrysophyceae	**Three**
	(Orange algae)	Chrysomonadales, chrysophaerales
		Chrysotrichales
4.	Bacillariophyceae	**Two**
	(Diatoms/yellow or golden brown algae)	Centrales, pennales
5.	Cryptophyceae	**Two**
	(Nearly brown)	Cryptomonadales, cryptococcales
6.	Dinophyceae	**Six**
	(Dark yellow or brown)	Desmomonadales, thecatales
		Dinophysiales, dinoflagellata, dinococcales, dinotrichales
7.	Chloromonadineae	**One**
	(Bright green)	Chloromonadales
8.	Euglenophyceae	The class has been divided into **three families**:
		Euglenaceae, astasiaceae, peranemaceae
9.	Phaeophyceae	**Nine**
	(Brown algae)	Ectocarpales, tilopteridales, cutariales, sporochnales, desmarestiales, laminariales, sphacelariales, dictyotales, fucales
10.	Rhodophyceae	**Seven**
	(Red algae)	Bangiales, nemalionales, gelidiales, cryptonemiales
		Gigartinales, rhodymeniales, ceramiales
11.	Myxophyceae	**Five**
	(Cyanophyceae, blue green algae)	Chroococcales, chamaesiphonales, pleurocapsales nostocales, stigonemales

The characteristics features of different classes as proposed by Fritsch are:

2.7.1 Class I: Chlorophyceae (Green Algae)

(a) Occurrence: Aquatic (mostly freshwater and few are marine) as well as terrestrial.
(b) Pigments: Chlorophyll a and b; Carotenoids and Xanthophylls.
(c) Pyrenoids: Present.
(d) Reserve food material: Starch.
(e) Cell wall: Cellulosic.
(f) Structure: Unicellular motile to multicellular, heterotrichous filamentous.
(g) Flagella: Present, equal length (isokont), situated anteriorly, one whiplash and another one is tinsel.
(h) Reproduction: Vegetative, Asexual and Sexual reproduction (isogamous, anisogamous and oogamous).

Orders (9):	1. Volvocales	2. Chlorococcales	3. Ulotrichales
4. Cladophorales	5. Chaetophorales	6. Oedogoniales	7. Conjugales
8. Sipohonales	9. Charales		

2.7.2 Class II: Xanthophyceae (Yellow: Green)

(a) Occurrence: Mostly freshwater and a few are marine.
(b) Pigments: Chlorophyll a, e, β carotene and xanthophylls.
(c) Pyrenoids: Absent.
(d) Reserve food material: Oil.
(e) Cell wall: Rich in pectic compounds and composed of two equal pieces overlapping at the edges. Flagella unequal.
(f) Structure: Unicellular motile to simple filamentous.
(g) Flagella: Present, two unequal, situated anteriorly. Longer one tinsel and shorter one whiplash.
(h) Reproduction: Vegetative, Asexual and Sexual (Mainly Isogamous, Anisogamy is rare, Oogamous in *Vaucheria*).

Orders (4):	1. Heterochloridales	2. Heterococcales	3. Heterotrichales
	4. Heterosiphonales		

2.7.3 Class III: Chrysophyceae (Orange Algae)

(a) Occurrence: Mostly fresh water a few are marine.
(b) Pigments: Chlorophyll a, Dominant pigment is Phycocrysin.
(c) Reserve food material: Leucosin, fats, Chrysolaminarin.
(d) Cell wall: Silicified or Calcified, Cellulose absent.

(e) Structure: Unicellular motile to branched filamentous.
(f) Flagella: Present, Two in number, equal or may be unequal, inserted anteriorly.
(g) Reproduction: Vegetative and Sexual (normally absent, but if present isogamous).

Orders (3): 1. Chrysomonadales 2. Chrysophaerales 3. Chrysotrichales

2.7.4 Class IV: Bacillariophyceae (Diatoms/Yellow or Golden Brown Algae)

(a) Occurrence: Cosmopolitan in nature, found everywhere in fresh water, marine water, soil and terrestrial habitats.
(b) Pigments: Chlorophyll c, β carotene, Fucoxanthin, Diatoxanthin, Didinoxanthin.
(c) Pyrenoids: Present.
(d) Reserve food material: Fats, Volutin.
(e) Cell wall: Composed of silica as well as pectic substances. Divided in two halves outer half is hydrated silica and inner half is composed of pectic substances.
(f) Structure: Unicellular or Colonial.
(g) Flagella: Single, pantonematic in motile stages.
(h) Reproduction: Cell division and auxospore formation.

Orders (2): 1. Centrales 2. Pennales

2.7.5 Class V: Cryptophyceae (Nearly Brown)

(a) Occurrence: Found in both freshwater and marine waters.
(b) Pigments: Chlorophyll a, c, Xanthophylls – diatoxanthin, phycocyanin and phycoerythrin.
(c) Pyrenoids: Pyrenoid like bodies present but independent of chromatophores.
(d) Reserve food material: Starch and/or oil.
(e) Cell wall: Absent
(f) Structure: Unicellular with anterior groove or pocket.
(g) Flagella: Biflagellate, both flagella apical or lateral, hairy, may be equal or unequal.
(h) Reproduction: Mostly binary fission, Sexual reproduction is rare but only of isogamous type.

Orders (2): 1. Cryptomonadales 2. Cryptococcales

2.7.6 Class VI: Dinophyceae (Dark Yellow or Brown)

(a) Occurrence: Mostly marine and a few are freshwater forms.
(b) Pigments: Chlorophyll a, c 2, β carotene, peridinin, neoperidinin, dominant pigments are xanthophylls.
(c) Chromatophores: Present, Discoid.
(d) Reserve food material: Starch and Fat.
(e) Cell wall: Cellulosic.
(f) Structure: Mostly unicellular, branched filamentous and motile.
(g) Flagella: Present, two, equal.
(h) Reproduction: Sexual reproduction isogamous type (rare).

Orders (6): 1. Desmomonadales 2. Thecatales 3. Dinophysiales 4. Dinoflagellata 5. Dinococcales 6. Dinotrichales

2.7.7 Class VII: Chloromonadinae (Bright Green)

(a) Occurrence: Fresh water forms.
(b) Pigments: Xanthophylls in excess.
(c) Pyrenoids: Absent
(d) Reserve food material: Fat and Oil
(e) Cell wall: Absent.
(f) Structure: Motile unicells.
(g) Flagella: Two, Equal.
(h) Reproduction: By cell division, Sexual reproduction absent.

Orders (1): 1. Chloromonadales

2.7.8 Class VIII: Euglenophyceae

(a) Occurrence: Freshwater forms are known only.
(b) Pigments: Chlorophyll a, b, β carotene, astaxanthin, antheraxanthin, diadinoxanthin, neoxanthin.
(c) Pyrenoids: Pyrenoid like bodies are present in some.
(d) Reserve food material: Paramylon and some polysaccharides.
(e) Cell wall: Proteinaceous.
(f) Structure: Unicellular.
(g) Flagella: Present (one or two).
(h) Reproduction: By cell division, Sexual reproduction if present is of isogamous type.

The class has been divided into **three families**:

1. Euglenaceae 2. Astasiaceae 3. Peranemaceae

2.7.9 Class IX: Phaeophyceae (Brown Algae)

(a) Occurrence: Mostly marine.
(b) Pigments: Fucoxanthin is dominant, Chlorophyll a, c and carotene.
(c) Pyrenoids: Stalked pyrenoids present outside the chloroplast envelope..
(d) Reserve food material: Laminarin, mannitol and fats.
(e) Cell wall: Cellulose, alginic acid and fucinic acid.
(f) Structure: Microscopic to branched, filamentous macroscopic parenchymatous plants.
(g) Flagella: Zoospores flagellated, flagella unequal, one is tinsel type.
(h) Reproduction: Sexual reproduction (isogamous, anisogamous and oogamous).

Orders (9):

1. Ectocarpales 2. Tilopteridales 3. Cutariales 4. Sporochnales
5. Desmarestiales 6. Laminariales 7. Sphacelariales
8. Dictyotales 9. Fucales

2.7.10 Class X: Rhodophyceae (Red Algae)

(a) Occurrence: Mostly marine.
(b) Pigments: r- phycoerythrin and r – phycocyanin, chlorophyll a, d, carotene and xanthophylls.
(c) Pyrenoids: Chromatophores present and pyrenoid like bodies are present in lower forms.
(d) Reserve food material: Floridean starch.
(e) Cell wall: Outer pectic and inner cellulosic.
(f) Structure: Multicellular (uniaxial or multiaxial).
(g) Flagella: Absent (cell non – motile).
(h) Reproduction: Sexual and oogamous type.

Orders (7):

1. Bangiales 2. Nemalionales 3. Gelidiales 4. Cryptonemiales
5. Gigartinales 6. Rhodymeniales 7. Ceramiales

2.7.11 Class XI: Myxophyceae (Cyanophyceae, Blue Green Algae)

(a) Occurrence: Mostly fresh water.
(b) Pigments: c – phycocyanin, chlorophyll a, β carotene and c- phycoerythrin.
(c) Pyrenoids: Absent.
(d) Reserve food material: Myxophycean starch and cyanophycean granules (proteins).
(e) Cell wall: Mucopeptides, amino acids, fatty acids and carbohydrates.
(f) Structure: Unicellular or Multicellular. Cells are prokaryotic in nature.
(g) Flagella: Absent (cell non – motile).
(h) Reproduction: Vegetative and asexual, sexual reproduction absent (genetic recombination is reported in some members)

Orders (5):
1. Chroococcales 2. Chamaesiphonales 3. Pleurocapsales
4. Nostocales 5. Stigonemales

Some important suggestions proposed by Fritsch in his classification can be summarized as below (Sharma 2011):

1. According to Fritsch, algae as a group must be considered as Division, therefore it cannot be further divided into "phyta" and he thus classified algae in 11 classes.
2. Class Conjugatae of Pascher's classification should be treated as an order (= conjugates) of class Chlorophyceae.
3. Division Charophyta (Pascher 1914) should be treated only as an order Charales in class Chlorophyceae.
4. Euglenophyta was further separated into two separate classes i.e. Euglenineae and Chlromonadineae.
5. Inclusion of Xanthophyceae, Bacillariophyceae and Chrysophyceae were separated because of dissimilarities between them.

2.8 Classification Proposed by G. M. Smith (1955)

G.M. Smith supported the classification proposed by Pascher (1914, 1931) and proposed a new classification with certain modifications. He divided algae into divisions and further into classes. The seven divisions of algae as proposed are:

S. No.	Divisions	Classes
1.	Chlorophyta	Chlorophyceae e.g. *Volvox*
		Charophyceae e.g. *Chara*
2.	Euglenophyta	Euglenophyceae e.g. *Euglena*
3.	Pyrrophyta	Desmophyceae e.g. *Desmarestia*
		Dinophyceae e.g. *Dinophysis*

S. No.	Divisions	Classes
4.	Chrysophyta	Chrysophyceae e.g. *Chromolina*
		Xanthophyceae e.g. *Botrydium*
		Bacillariophyceae e.g. *Pinnularia*
5.	Phaeophyta	Isogenerateae e.g. *Ectocarpus*
		Heterogenerateae e.g. *Mynomena*
		Cyclosporeae e.g. *Sargassum*
6.	Cyanophyta	Myxophyceae e.g. *Nostoc, Anabaena*
7.	Rhodophyta	Rhodophyceae e.g. *Polysiphonia, Gracilaria*, *Batrachospermum*

Smith also recognized algae of uncertain systematic position and placed them under chloromonadales and cryptophyceae.

2.9 Classification Proposed by G. F. Papenfuss (1955)

G. E. Papenfuss proposed algal classification based on phylogenetic relationship. He recognized 7 division and 12 classes as described in the following table. The blue green algae were kept together in a separate phylum Schizophya along with bacteria.

S. No.	Divisions	Class
1.	Chlorophycophyta	Chlorophyceae
2.	Charophycophyta	Charophyceae
3.	Euglenophycophyta	Euglenophyceae
4.	Chrysophycophyta	(a) Xanthophyceae
		(b) Chrysophyceae
		(c) Bacillariophyceae
5.	Pyrrophycophyta	(a) Dinophyceae
		(b) Cryptophyceae
		(c) Chloromonadophyceae
6.	Phaeophycophyta	Phaeophyceae
7.	Rhodophycophyta	Rhodophyceae

2.10 Classification Proposed by V. J. Chapman (1962)

Chapman, considered pigments, morphological characters, biochemical differences and also phylogenetic relationships with in different algae for its classification. He divided algae into four different divisions and further into classes as given in table:

S. No.	Divisions	Class
1.	Euphycophyta	(a) Chlorophyceae
		(b) Phaeophyceae
		(c) Rhodophyceae
2.	Myxophycophyta	(a) Myxophyceae
3.	Chrysophycophyta	(a) Chrysophyceae
		(b) Xanthophyceae
		(c) Bacillariophyceae
4.	Pyrrophycophyta	(a) Cryptophyceae
		(b) Dinophyceae

2.11 Classification Proposed by Christensen (1964)

Christensen (1964), divided algae on the basis of prokaryotic and eukaryotic features of cell into Prokaryota and Eukaryota.

			Division	
Algae	Prokaryota		(a) Cyanophyta	
	Eukaryota	(i) Aconta (Motile stages absent)	(a) Rhodophyta	
		(ii) Contophora (Motile stages present)	(a) Chlorophyta	Chlorophyll a and b predominates
			(b) Charophyta	
			(c) Euglenophyta	
			(d) Xanthophyta	Carotenoids predominates, chlorophyll a present, chlorophyll b absent
			(e) Chrysophyta	
			(f) Bacillariophyta	
			(g) Pyrrophyta	
			(h) Cryptophyta	
			(i) Phaeophyta	

2.12 Classification Proposed by G. W. Prescott (1969)

The basis for algal classification by Prescott (1969) was presence or absence of true nucleus, pigmentation, biochemical nature of cell wall, reserve food material, life history and reproduction. Based on these criteria he classified algae into nine phyla with different classes:

S. No.	Phyla	Classes	No. of orders
1.	Chlorophyta	(a) Chlorophyceae	17
		(b) Charophyceae	01
2.	Euglenophyta		02

(continued)

S. No.	Phyla	Classes	No. of orders
3.	Chrysophyta	(a) Chrysophyceae	05
		(b) Bacillariophyceae	02
		(c) Heterokontae (Xanthophyceae)	05
4.	Pyrrophyta	(a) Desmokontae (Desmophyceae)	01
		(b) Dinokontae (Dinophyceae)	05
5.	Phaeophyta	(a) Isogeneratae	05
		(b) Heterogeneratae	05
		(c) Cyclosporae	05
6.	Rhodophyta	(a) Bangioideae	04
		(b) Florideae	06
7.	Cyanophyta	(a) Coccogoneae	03
		(b) Hormogoneae	05
8.	Cryptophyta		02
9.	Chloromonadophyta		01

2.12.1 Phylum I: Chlorophyta (Green Algae): Chlorophyll a, b; Food Reserve Material: Starch

Class: (A) Chlorophyceae:

Orders:
1. Volvocales: Motile cells, reproduction isogamy to oogamy
2. Tetrasporales: Unicellular. Gelatinous, reproductive cells motile but vegetative cells may assume motility, reproduction isogamous
3. Ulotrichales: Filamentous forms, filaments unbranched, basal cell and distal cell may or may not differentiate, chloroplast parietal, cell wall constructed in one section, reproduction isogamous or anisogamous
4. Microsporales: Filaments unbranched, parietal chloroplast, cell wall peculiarly constructed in two sections, reproduction isogamous or anisogamous
5. Cylindrocapsales: Filaments unbranched, chloroplast massive chloroplast, cells enclosed in a lamellar mucilage, reproduction oogamous
6. Sphaeropleales: Filamentous, unbranched, no differentiation into basal and distal cell, filaments laterally adjoined, reproduction isogamous or anisogamous
7. Ulvales: Thallus foliose, hollow tubes, differentiation in basal and distal cells, filaments laterally adjoined, reproduction isogamous or anisogamous

8. Schizogonales (Prasiolales): Filaments uniseriate or multiseriate (sometimes with false branching), may become foliose or frond like, chloroplast stellate, sexual and asexual reproduction absent
9. Chaetophorales: Uniseriate, branched, differentiation in basal and distal cells, branches taper apically, parietal chloroplast, sexual reproduction isogamous or anisogamous
10. Coleochaetales: Filaments branched, erect or prostrate on a substrate, cells frequently with walls bearing hairs, sexual reproduction oogamous
11. Trentepholiales: Filaments branched, erect or both erect and prostrate, without tapering, branches without hairs, sexual reproduction isogamous
12. Cladophorales: Branched or unbranched, uniseriate, differentiation in basal and distal cells, cell walls thick and lamellate, cells coenocytic, chloroplast parietal net, zoospores for asexual reproduction, sexual reproduction isogamous or anisogamous
13. Siphonocladales: Semi – siphonous, coenocytic filaments, differentiation in basal and distal cells, chloroplast a reticulum, sexual reproduction isogamous or anisogamous
14. Siphonales: Non segmented, coenocytic filaments, sexual reproduction isogamous or rarely oogamous
15. Oedogoniales: Filaments branched or unbranched, differentiation in basal and distal cells, cells larger at the anterior end, cell division peculiar, parietal chloroplast, zoospores for asexual reproduction, sexual reproduction oogamous
16. Zygnematales (Conjugales): Unicellular or filamentous without basal – distal differentiation, chloroplast large, axial or parietal, asexual reproduction by parthenospore, sexual reproduction by the metamorphosis of the vegetative cell protoplast to form isogamous or anisogametes, united by conjugation
17. Chlorococcales: Coenocytic, unicellular or colonial, vegetative reproduction present through autospores or autocolonies, asexual reproduction by zoospores, sexual reproduction isogamous, anisogamous (rare), oogamous

Class: (B) Charophyceae (Stoneworts):

Order: 1. Charales: Macroscopic, differentiation in basal and distal cells, thallus differentiated into nodes and internodes, nodes bear whorl of branches of limited growth, sexual reproduction oogamous

2.12.2 Phylum II: Euglenophyta (Euglenoid Algae)

Chloroplast oval, diffuse or star shaped, chlorophyll a, b (some members colourless), motile flagellated, flagella one, two or three, unicellular, food reserve paramylum starch.

Orders:
1. Euglenales: Unicellular, zoospores and gametes absent
2. Colaciales: Colonial, cells attached on stalks, vegetative cell metamorphos into a uni-flagellate zoospore, anterior end downward in a mucilaginous capsule

2.12.3 Phylum III: Chrysophyta (Yellow: Green Algae)

Carotenoids or xanthophylls predominates, chloroplast variously shaped, food reserve oil and leucosin, flagella present, two, unequal in length and morphology, sexual reproduction isogamous or oogamous (rare)

Class (A) Chrysophyceae:
Orders:
1. Chrysomonadales: uniflagellate, temporary amoeboid stage
2. Rhizochrysidales: amoeboid, temporary flagellate stage
3. Chrysocapsales: non – motiles
4. Chrysosphaerales: unicellular or colonial, non – motile
5. Chrysotrichales: filamentous, branched, differentiation in basal and distal cell

Class: (B) Bacillariophyceae (diatoms):
Orders:
1. Centrales: isodiametric or circular (top/valve view), ornamented (pits, strie etc.), radial, cell walls without a raphe
2. Pennales: elongated cells, rectangular, crescent, valves with a raphe or a pseudoraphe

Class: (C) Heterokontae (xanthophyceae):

Unicellular, colonial, filamentous, siphonaceous, carotenes predominate, flagellated, two in number, unequal in length (i.e. heterokontae), reproduction isogamous, anisogamous, oogamous

Orders:
1. Rhizochloridales: unicellular, rhizopodial
2. Heterocapsales: non – motile, colonial, gelatinous
3. Hetercoccales: unicellular or colonial, autospores and internal cell divisions for reproduction
4. Heterotrichales: branched or unbranched, differentiation of basal and distal cells
5. Heterosiphonales: coenocytic

2.12.4 Phylum IV: *Pyrrophyta* (Dinoflagellates)

Dorsiventrally differentiated, motile, flagella two, carotenes, xanthophylls and peridinin predominates as pigments, food reserve starch or oil, reproduction by cell division, cysts or rarely by conjugation (Ceratium)

Class: (a) Desmokontae (Desmophyceae): Cells without a transverse furrow (girdle) and with apical flagella

Class: (b) Dinokontae (Dinophyceae): Cells with transverse and a longitudinal furrow

Orders:
1. Gymnodiniales: Dinoflagellates without cell wall, periplast present which may be spirally striated
2. Peridiniales: Dinoflagellates with a wall, wall contains definite plates, specifically variable in number and arrangement
3. Dinocapsales: dinoflagellates, palmelloid, non – motile, enclosed in a mucilagenous envelope, dinoflagellate type of zoospores present
4. Dinococcales: Dinoflagellate without plates in wall, stationary, occurring as attached or free floating cysts, dinoflagellate type of zoospores present but do not carry an ordinary cell division
5. Dinotrichales: Simple or sparingly branched, filaments, creeping and prostrate, dinoflagellate type of zoospores present

2.12.5 Phylum V: Phaeophyta (Brown Algae, Brown Seaweeds)

Thalli may be erect or prostrate, branched, tufted, fronds or foliose, pigment fucoxanthin, food storage as soluble carbohydrate, reproductive cells motile, reproduction by zoospores, isogamous, anisogamous or heterogamous.

Sub – phylum (1): Phaeosporeae

Class: (a) Isogeneratae: alternation of similar generations. Orders: 05

Orders:
1. Ectocarpales: branched, uniseriate, forms bushy tufts, differentiation of basal and distal cells, microscopic to macroscopic, reproduction isogamous and anisogamous
2. Sphacelariales: branched, multiseriate filaments, apical cell present, reproduction isogamous
3. Cutleriales: branched, flat, ribbon like or flat, hairs present at the tips of branches, isomorphic alternation of generation
4. Dictyotales: frond like, branched ribbon, holdfast present, growth by apical cell
5. Tilopteridales: branched, uniseriate filaments, differentiation into basal and distal cell, asexual reproduction by non – motile monospores

Class: (B) Heterogeneratae: alternation of dissimilar generations. Orders: 05

Orders:
1. Chordariales: consist of erect or crustose like thalli
2. Sporochnales: thallus branched, sporophytic, hairs present at the apices, grouped sporangia with in a swollen receptacle, growth intercalary
3. Desmarestiales: thallus frond – like, trichothallic growth, heteromorphic alternation of generation
4. Dictyosiphonales: tubular sporophyte, saccate or foliaceous thallus, heteromorphic alternation of generation
5. Laminariales: large frond, holdfast present, heteromorphic alternation of generation

Sub – phylum (2): Cyclosporae:

Plants frond like with distinct holdfast, reproduction only by spores, no gametophytic generations, reproduction oogamous, conceptacles present.

2.12.6 Phylum VI: Rhodophyta (Red Algae)

Dominant pigment Phycoerythrin, floridean starch as food storage product, no motile cells.

Sub – phylum (1): Bangiodeae:

Orders:
1. Porphyridiales: unicellular forms
2. Goniotrichales: filaments uniseriate or multiseriate, reproduction by cell division and monospore
3. Bangiales: filaments or fronds uniseriate or multiseriate, reproduction by monospores or carpogonia
4. Compsopogonales: filamentous, branched, arbuscular, monopsores present, carpogonia absent

Sub – phylum (2): Florideae:

Orders:
1. Nemalionales: mostly multiaxial, reproduction by carpogonia, tetrasporophyte present in some
2. Gelidiales: uniaxial, branched filaments, carpogonial branch simple, auxiliary cell absent, tetrasporophyte present
3. Cryptonemiales: erect, encrusted with calcium, uni or multiaxial, specialized carpogonial filaments present which may be clumped, auxiliary cells present, tetrasporophyte present

4. Gigartinales: branched, erect or prostrate, filamentous, membranous, carpogonial branch short and unspecialized, auxiliary cells present and is formed from a vegetative cell in an unspecialized cell, tetrasporophyte present
5. Rhodymeniales: multiaxial, carpogonial branch short, auxiliary cells present and grows from supporting cell, tetrasporophyte present
6. Ceremiales: auxiliary cell present which grows from supporting cell following fertilization, carpogonium is formed from supporting cell, tetrasporophyte present

2.12.7 Phylum VII: Cyanophyta (Blue – Green Algae)

Unicellular, colonial, filamentous, phycocyanin predominates as pigment, cells without true nucleus, motile cells absent, sexual reproduction absent.

Sub – phylum (1): Coccogoneae:

Orders:
1. Chroococcales: spherical cells, unicellular or colonial, reproduction only by fission
2. Chamaesiphonales: elongated or club shaped cells, solitary, attached, reproduction by spores
3. Pleurocapsales: cells arranged to form prostrate clumps or strands, sometimes pseudofilamentous, reproduction by endospores

Sub – phylum (2): Hormogoneae:

Orders:
1. Oscillatoriales: filamentous, unbranched, with or without sheath, no differentiation of cells
2. Nostocales: filamentous, unbranched, differentiation into heterocysts
3. Scytonematales: filamentous, false branching, often forms tufts
4. Stigonematales: filamentous, branched, heterocyst present, branching true
5. Rivulariales: filamentous, tapers from base to tip, sometimes false branching present, heterocysts and akinetes present

2.12.8 Phylum VIII: Cryptophyta (Blue and Red Flagellates)

Compressed, unsymmetrical or flagellated slipper shaped cells present, xanthophylls or chlrophylls present, food reserves starch like.

Orders:
1. Cryptomonadales: motile cells
2. Cryptococcales: non – motile cells, zoospores present

2.12.9 Phylum IX: Chloromonadophyta (Chloromonads)

Unicellular, biflagellate, chlorophyll predominates, food reserve oil, trichocysts present.

Orders: 1. Chloromonadales

2.13 Classification Proposed by F. E. Round (1973)

F. E. round divided algae again on the basis of presence or absence of true nucleus, membrane bound organelles and phylogenetic relationship etc. He classified algae into 12 phyla and further into classes.

The classification proposed by Round is

S. No.	Group	Phylum
1.	Prokaryota	Cyanophyta
2.	Eukaryota	Euglenophyta
		Chlorophyta
		Charophyta
		Prasinophyta
		Xanthophyta
		Haptophyta
		Dinophyta
		Bacillariophyta
		Chrysophyta
		Phaeophyta
		Rhodophyta

2.14 Classification Proposed by V. J. Chapman and D. J. Chapman (1973)

They classified algae into Prokaryota and Eukaryota which were further divided into divisions and classes as:

S. No.		Division	Class
1.	Prokaryota	I. Cyanophyta	(a) Cyanophyceae
2.	Eukaryota	II. Rhodophyta	(a) Rhodophyceae
		III. Chlorophyta	(a) Chlorophyceae
			(b) Prasinophyceae
			(c) Charophyceae
		IV. Euglenophyta	(a) Euglenophyceae
		V. Chloromonadophyta	(a) Chloromonadophyceae
		VI. Xanthophyta	(a) Xanthophyceae
		VII. Bacillariophyta	(a) Bacillariophyceae
		VIII. Chrysophyta	(a) Chrysophyceae
			(b) Haptophyceae
		IX. Phaeophyta	(a) Phaeophyceae
		X. Pyrrophyta	(a) Dinophyceae
			(b) Desmophyceae
		XI. Cryptophyta	Cryptophyceae

2.15 *Classification Proposed by H. C. Bold and M. J. Wynne (1978)*

Bold and Wynne followed the classification proposed by Papenfuss and they accepted the use of "phyco" before "phyta" in algal divisions. They divided algae into nine divisions as:

S. No.	Divisions
1.	Cyanochloronta
2.	Chlorophycophyta
3.	Charophyta
4.	Euglenophycophyta
5.	Phaeophycophyta
6.	Chrysophycophyta
7.	Pyrrophycophyta
8.	Cryptophycophyta
9.	Rhodophycophyta

2.16 *Classification Proposed by S. P. Parker (1982)*

Parker classified algae into Prokaryota and Eukaryota based on presence or absence of membrane bound organelles. His classification is proposed as:

S. No.	Group	Division	Class
	Prokaryota	Cyanophycota	(a) Cyanophyceae
		Prochlorophycota	(a) Prochlorophyceae
	Eukaryota	Rhodophycota	(a) Rhodophyceae
		Chromophycota	(a) Chrysophyceae
			(b) Prymnesiophyceae
			(c) Xanthophyceae
			(d) Eustigmatophyceae
			(e) Bacillariophyceae
			(f) Dinophyceae
			(g) Phaeophyceae
			(h) Raphidophyceae
			(i) Cryptophyceae
		Euglenophycota	(a) Euglenophyceae
		Chlorophycota	(a) Chlorophyceae
			(b) Charophyceae
			(c) Prasinophyceae

2.17 Classification Proposed by R. E. Lee (2008)

Lee classified algae in two groups Prokaryota and Eukaryota which were further divided into divisions. Prokaryota has just one division Cyanophyta, whereas Eukaryota were further divided on the basis of nature of chloroplast membrane.

S. No.		Groups	Divisions	Class
	Prokaryota	I	(i) Cyanophyta	Cyanophyceae
	Eukaryota	II. Chloroplast surrounded by the two membranes of the chloroplast envelope	(b) Glaucophyta	
			(c) Rhodophyta	
			(d) Chlorophyta	
		III. Chloroplast surrounded by one membrane of chloroplast endoplasmic reticulum	(a) Euglenophyta	
			(b) (Euglenoids)	
			(c) Dinophyta	
			(d) (Dinoflagellates)	
		IV. Chloroplast surrounded by two membranes of chloroplast endoplasmic reticulum envelope	(a) Cryptophyta (cryptophytes)	
			(b) Prymnesiophyta (haptophytes)	Prymnesiophyceae
			(c) Heterokontophyta (heterokonts)	Chrysophyceae
				Synurophyceae
				Dictyophyceae
				Pelagophyceae
				Bacillariophyceae
				Raphidophyceae
				Xanthophyceae
				Eustigmatophyceae
				Phaeophyceae

2.17.1 Group I: Prokaryota

Division I Cyanophyta
Class I Cyanophyceae

Pigments: Chlorophyll a; phycobilliproteins

2.17.2 Group II: Eukaryota (Chloroplast Surrounded by the Two Membranes of the Chloroplast Envelope)

Division I Glaucophyta
Represent intermediate position in the evolution of chloroplast; endosymbiotic cyanobacteria carries out photosynthesis

Division II Rhodophyta
Pigments: chlorophyll a, phycobilliproteins; no flagellated cells; storage product is floridean starch

Division III Chlorophyta
Chlorophylla a and b, storage product: starch inside chloroplast

2.17.3 Group III: Eukaryota (Chloroplast Surrounded by One Membrane of Chloroplast Endoplasmic Reticulum Envelope)

Division I Euglenophyta (euglenoids)
Pigments: chlorophyll a and b, one flagellum with a spiraled row

Division II Dinophyta (dinoflagellates)

2.17.4 Group IV: Eukaryota (Chloroplast Surrounded by Two Membrane of Chloroplast Endoplasmic Reticulum Envelope)

Division I Cryptophyta (cryptophytes)
Nucleomorph is present between inner and outer membranes of chloroplast endoplasmic reticulum; pigments: chlorophyll a and c, phycobiliproteins

Division II Prymnesiophyta (haptophytes)
Class I Prymnesiophyceae
Pigments: chlorophyll a, c 1, c 2, fucoxanthin; flagella: two, whiplash; storage product: chrysolaminarin found in vesicles in cytoplasm; cells are commonly covered with scales

Division III Heterokontophyta (heterokonts)
Class I Chrysophyceae
Chlorophyll a and c 1 and sometimes c 2; fucoxanthin; flagella: two, anterior tinsel and posterior whiplash; storage product: chrysolaminarin
Class II Synurophyceae
Plant body bilaterally symmetrical; flagella: two; chloroplast endoplasmic reticulum absent; chlorophyll C 1 absent
Class III Dictyophyceae
Tentacles or rhizopodia present; chlorophyll C 1
Class IV Pelagophyceae
Unicellular, cells small and appears as indistinct protoplasm
Class VI Bacillariophyceae
Cell wall silicified; pigments: chlorophyll a, c 1 and c 2; fucoxanthin; flagella: absent
Storage product: chrysolaminarin
Class VII Raphidophyceae
Pigments: chlorophyll a and c; flagella: two, anterior tinsel, posterior whiplash
Class VIII Xanthophyceae
Chloroplast has an eye spot; chlorophyll a and e present; flagella: anterior tinsel, posterior whiplash
Class IX Eustigmatophyceae
Unicellular; fresh water and terrestrial; pigments: chlorophyll a, β carotene, violaxanthin, vaucherioxanthin; pyrenoids: present polygonal, absent in zoospores
Class X Phaeophyceae
Mostly marine; pigments: fucoxanthin, chlorophyll a, c and carotene; cell wall: alginic acid, fucinic acid, cellulose; reserve food material: laminarin (polysaccharide), manitol (alcohol), fat; pyrenoids: present; flagella: presentin zoospores, one is tinsel type; sexual reproduction: isogamy to oogamy

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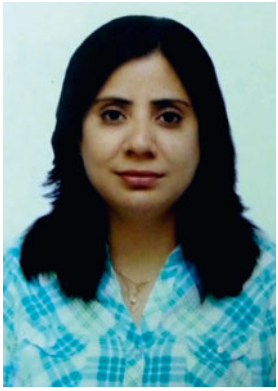

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Cyanobacteria

Sunaina Zutshi and Tasneem Fatma

1 Introduction

Cyanobacteria are the oldest oxygenic photoautotrophs on earth i.e. they utilize energy from sunlight accomplish numerous upregulation of cellular metabolic activity. Like green plants and true algae, they produce oxygen and also get their food in an identical manner. They have the capacity to colonize almost all conceivable habitats due to their supplementary capability to synthesis of compound with the help of radiant energy resourcefully (Van Liere and Walsby 1982). Few species have anoxygenic photosynthesis probability (Cohen et al. 1986). Many species have capacity to grow heterotrophically in the dark (Smith 1982; Bastia et al. 1993). All cyanobacteria contain prokaryotic cell organization, the photosynthetic pigment chlorophyll a, plus accessory pigments phycobiliprotein. Cyanobacteria are commonly known as "blue green algae" as their principal pigment is a bluish green C-phycocyanin. They may be unicellular or multicellular, free living or colonial unbranched filamentous or branched filamentous, heterocystous or non heterocystous, aquatic or terrestrial in nature. In fresh planktonic forms gas vacuole are present which increase nutrient availability because of diurnal vertical mixing in water column (Gibson and Smith 1982). Polysaccharide sheath protect trichome against desiccation (Whitton 1987). They differ from other algae as they don't have well organized cell organelle and their pigments are not restricted to definite chromatophores, but are distributed throughout the peripheral cytoplasm. Their nucleus is of primitive type which lack nuclear membrane and nucleolus. They do not have flagella and their movement is

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D. Sahoo, J. Seckbach (eds.), *The Algae World*, Cellular Origin, Life in Extreme Habitats and Astrobiology 26, DOI 10.1007/978-94-017-7321-8_3

brought about by gliding action that allows them to hunt for optimal conditions. They lack sexual reproduction. Cyanobacterial biomass is economical than bacteria and also secret restriction enzymes which are commercially available, can be promoted at low cost like various *Anabaena*, *Microcoleus*, Nostoc sp. (Elhai and Wolk 1988). Cyanobacteria has drawn worldwide attention for possible use in photoproduction of biofuels, NH_3 scrubbing of excess atmospheric greenhouse gases including CO_2, production of various secondary metabolites, vitamins, toxins, cosmetics, dairy products, food grade dyes and other therapeutic substances.

2 Cell Structure

The cyanobacteria have prokaryotic organization. They lack membrane bound organelles. The internal membranes which separate DNA from cytoplasm, photosynthetic and respiratory organelles are absent (Fig. 1).

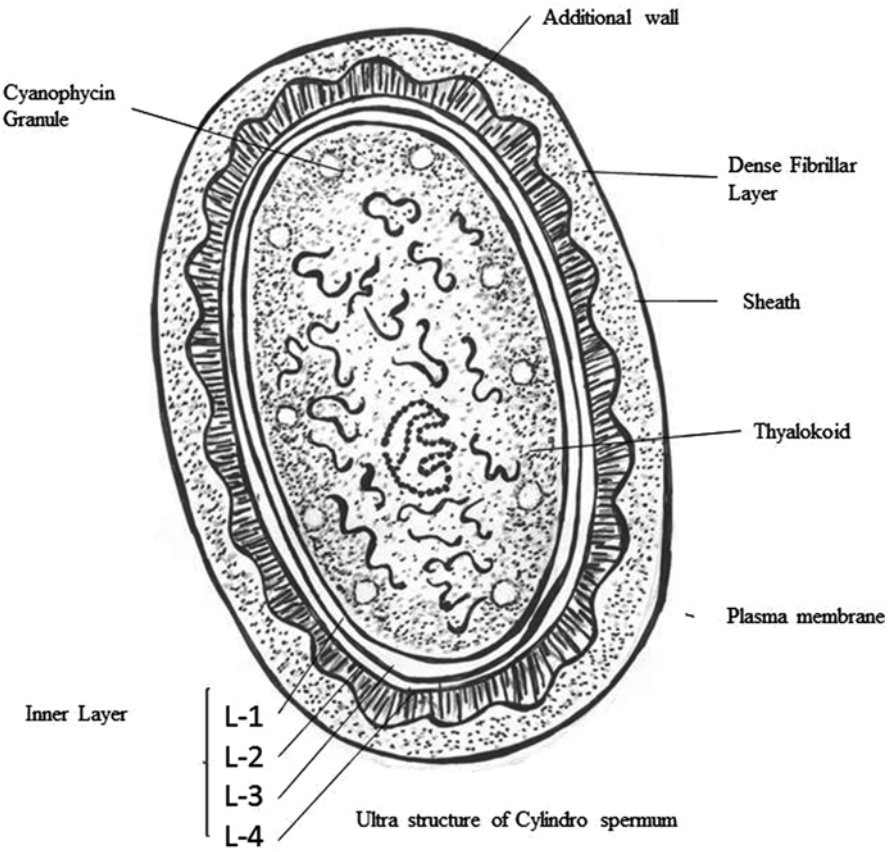

Fig. 1 Cyanobacterial cell (Source: http://www.youarticlelibrary.com)

2.1 Sheath

Outside the cell wall is present mucilaginous sheath which consist of three layers of microfibrils reticulately arranged within an amorphous matrix. The sheath retains absorbed water which is useful during the period of desiccation and for sloughing off parasite.

2.2 Cell Wall

Inside the sheath is present a double layered cell wall, a very rigid structure that gives shape to the cell. The inner layer is made of mucopeptide and muramic acid. Electron microscopy studies showed that the wall consist of four layers. Each layer is 10 nm thick. The second (L-II) layer has mainly peptidoglycan and first (L-I) lie next to plasmalemma. In structure and function the cell wall is similar to the gram negative bacteria. The cell wall in both is composed of mucopeptide together with carbohydrates, amino acids and fatty acids.

2.3 Plasma Membrane

Beneath the cell wall is present plasmalemma. This membrane invaginates inside the cells, which are considered sites for biochemical reactions and functions normally associated with mitochondria, endoplasmic reticulum and golgi bodies in Eukaryotic cells.

2.4 Photosynthetic Apparatus

Membrane bound plastids are absent, instead pigments are found embedded within lamellae composed of two membranes joined at the ends. The structures are known as thylakoids. They appear as elongated, flattened sacs consisting of two unit membranes, each about 75 A^0 thick. Adjacent thylakoids are separated from each other by a space of 50 nm, occupied by contiguous rows of discoidal phycobilisomes that transfer light energy to phytosystem II reaction centers like chlorophyll b, c, d. They are able to dissolve in water and balanced in solution and poor light sensitive. The basic subunit of a phycobilisome consists of apoproteins α and β, each of which is attached to a chromophore (Anderson and Toole 1998; Samsonoff and MacColl 2001). In the core of the phycobilisome α and β are attached to allophycocyanins, which are adjacent to chlorophyll in the energy

transmission way. In the outer rods, α and β are attached to phycocyanin. The α and β molecules are assembled into hexamers (α_1 and β_1) cylindrical in shape and are joined together by linker polypeptides. The hexamers that make up the core of the phycobilisomes are assembled in pairs, wit-h the hexamers of the rods radiating from the core. The linker polypeptides are basic whereas the hexamers are acidic; this proposes that electrostatic interactions are important in assembling phycobiliproteins. There are high-molecular-weight polypeptides that anchor the phycobilisomes to the area of the thylakoid membrane that contains the reaction centre and associated chlorophylls (Fig. 2).

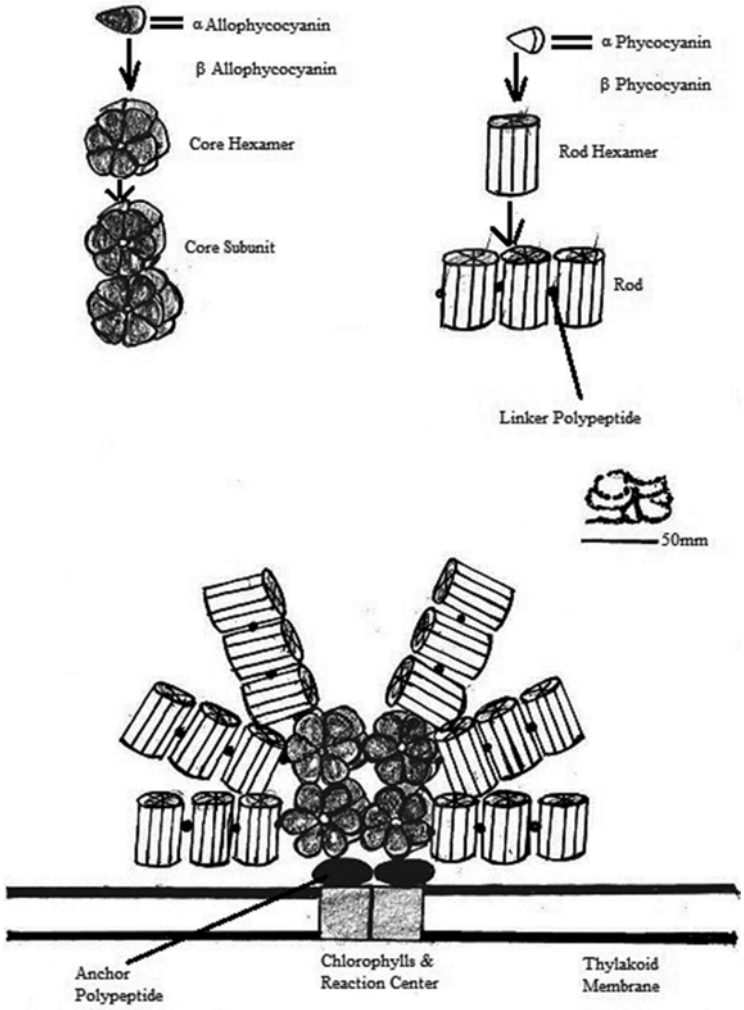

Fig. 2 Cyanobacterium (*Synechococcus*) phycobilisome (Adapted from Grossman et al. 1993)

Structurally phycobilin, are chromoproteins in which the prosthetic group (non-protein part of the moleculeor chromophores is a tetrapyrole bile pigment) known as phycobilins and is tightly bound to the apoprotein (protein part of the molecule) moiety by covalent linkage and it's difficult to separate the pigments from apoprotein. The cyanophyceae have four phycobiliproteins: c-phycocyanin (λ_{max} 620 nm) and allophycocyanin (λ_{max} 650 nm), c-phycoerythrin (λ_{max} at 565 nm), and phycoerythrocyanin (λ_{max} at 568 nm). All cyanobacteria contain the first two, whereas c-phycoerythrin and phycoerythrocyanin occur in few species.

2.5 Intra Cytoplasmic Inclusions

Inside the plasma membrane various kinds of inclusion have been found in cyanobacteria like.

I. *Cyanophycin granules* – Are enormous and frequently found near the cross-walls of filamentous forms and are composed of a polymer (arginine and asparagine) which utilize as nitrogen replacement.
II. *Polyhedral bodies* (now called carboxysomes) – Contain important enzymes, ribulose diphosphate carboxylase.
III. *Polyphosphate bodies* (volutin) – Sources of phosphate for the production of nucleic acids, phospho lipids and ATP.
IV. *Polyglucoside bodies* – Similar to α granule which are known to store carbon and energy in the form of carbohydrate. They areknown to function in blasting the cell and play a role in buoyancy regulation.
V. *Poly-beta-hydroxybutyric acid* – is biopolymer found exclusively in prokaryotes in which it act as a storage and is also used as proplastic e.g., *Spirulina* and *Nostoc muscorum*

2.6 Gas Vacuoles

Cyanobacterial gas vacuoles are not tonoplast bound, instead they are made of protein cylinders which are hollow packets. These vacuoles contain metabolic gases and function as gas vesicles that help cells move upward, towards the light, where photosynthesis can occur more readily. When many vesicles are present, cyanobacterial cells tend to float. If photosynthesis increases cell contain higher concentration of sugars that increases the turgidity and collapsing of the gas vesicle. Collapse of gas vesicle causes cyanobacteria to sink. In deeper water respiration is performed by cellular carbohydrates allowing gas vesicle to re-form as a result buoyancy cycle continues and this can be interrupted by cyanobacterial blooms at the surface (Fig. 3). These thick blooms may be unable to get sufficient resources to produces sugars, which allow gas vesicles to remain intact.

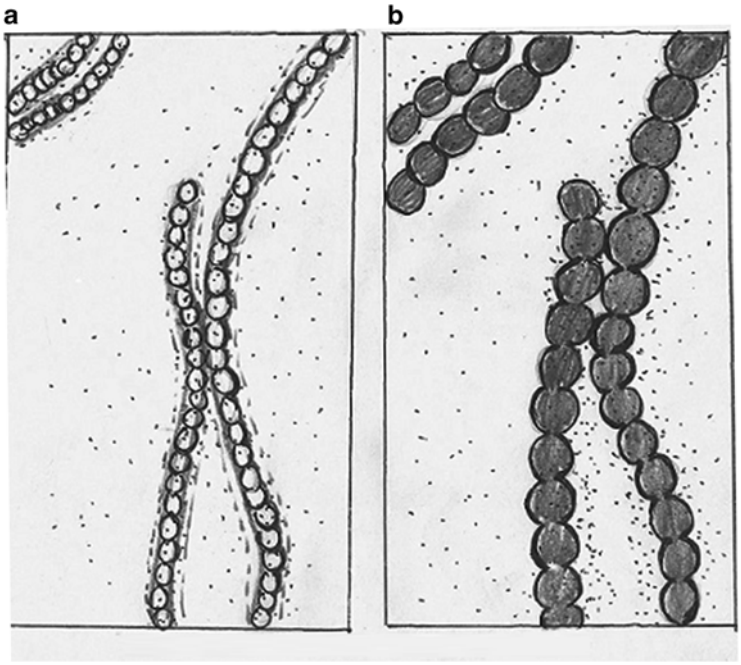

Fig. 3 (**a**) Filaments of *Anabaena flos-aquae* before and (**b**) after collapsing of their gas vacuoles by pressure (Soure: Walsby 1992)

2.7 Nucleoplasm

Nucleolus and nuclear envelope are absent (no true nucleus), instead the DNA present in the cytosol (cytosol = liquid component of the cytoplasm). Since DNA is not associated with protein material (histone or protamines), organized chromosomes are not found as in eukaryotic cells. RNA is present in addition to DNA.

3 Habitat

Cyanobacteria are genetically highly diverse and are found in a wide range of habitats including ponds, lakes, rivers, oceans, temperate soils, geothermal waters, desert soils, rocks, polar regions and hypersaline waters (Whitton and Potts 2000). They form microbial mats, biofilms and benthic communities in relationship with other entities. Occasionally they are found in certain life-threatening habitats like thermal springs, hot deserts and Antarctica. Many fresh water cyanobacterial species can tolerate and grow from 0 °C under ice to 26–35 °C in tropical zone. The optimum temperature for thermophilic cyanobacteria may be 45 °C or more (Darley 1982). The soil is the best terrestrial cyanobacterial habitat as light, humidity,

temperature; nutrient and pH are easily available for their growth. They are often subjected to intense solar radiations. They counteract the harmful effects of ionizing radiations in the ultraviolet region by synthesizing scytonemin and mycosporinin like protein (Mushir and Fatma 2011). In the desert *Microcoleus* form crust during favourable conditions. The filaments of *Microcoleus* are pushed out of their common sheath leaving behind polysaccharide material which on drying forms a cementing layer which protects the soil against erosion. Cyanobacteria are capable of independent existence in various environments but they also form symbiotic association with an extensive variety of plants and animals e.g., with algae (diatoms), fungi (lichens), bryophytes (*Anthroceros*), pteridophytes (*Azolla fonds*), gymnosperms (Cycas corolloid roots), angiosperms (*Gunnera*), non-photosynthetic protists (*Glaucophyta*), protozoons (*Euglena*), marine sponges (*Chondrilla nucula*), echiuroid (*Bonellia fuliginosa*) and corel reef (*Prochlorococcus*) (Carpenter 2002; Bergman et al. 2007).

4 Classification

Taxonomy brings a stage to classify a specific entity to the levels of species. The representation of ordering is termed as classification and phylogeny which aids to hold patterns of descent of organism among the entities categorize. Due to algal-like feature, they have a control of chlorophyll rather than bacteriophyll and they do not have photosystem like algae and higher plants, they were called blue green algae. Fritsch (1945) and Papenfuss (1955) recognized five orders of blue green algae. These five orders have been distinguished on the basis of morphological features, cell form, type of cell division and type of resulting colony, presence of inclusions, polar granules, gas vacuoles specialized cells (akinetes and heterocysts).

I. ORDER: CHROOCOCCALES
They are fresh water, marine or terrestrial, unicellular or colonial forms; colonies are without polarity; reproduction by fission or endospore; heterocyst and hormogonia absent (e.g., *Chroococcus*, *Gloeocapsa*, *Micocystis*, *Aphanocapsa*) (Fig. 4(i)).

II. ORDER: PLEUROCAPSALES
Fresh water or marine, filamentous; reproduction by endospores; heterocysts not formed (e.g., *Pleurocapsa*) (Fig. 4(ii)).

III. ORDER: CHAMAESIPHONALES
Mostly marine, few freshwater, unicellular or colonial; colonies with distinct polarity; reproduction by endospores or exospores; heterocyst and hormogonia are absent (e.g., *Chamaesiphon*, *Democarpa*) (Fig. 4(iii)).

IV. ORDER: NOSTOCALES
Mostly fresh water, a few marine, filamentous algae; filaments not differentiated into prostrate and upright system; multiplication by hormogonia; may or may not have heterocyst; spore and akinetes present (e.g., *Scytonema*, *Nostoc*, *Oscillatoria*, *Gloeotrichia*, *Rivularia*, *Spirulina*, *Anabaena*, *Lyngbya*) (Fig. 4(iv)).

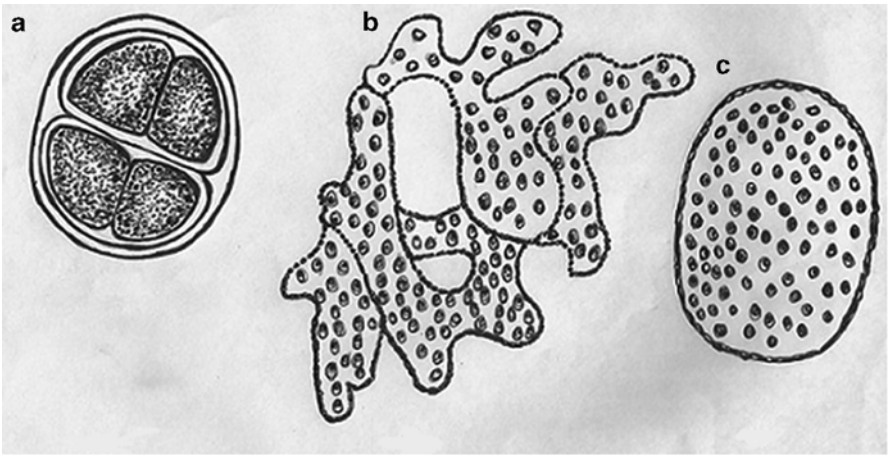

Fig. 4(i) (**a**) Chroococcus (**b**) *Microcystis* (**c**) *Aphanocapsa* (Source: Mur et al. 1999)

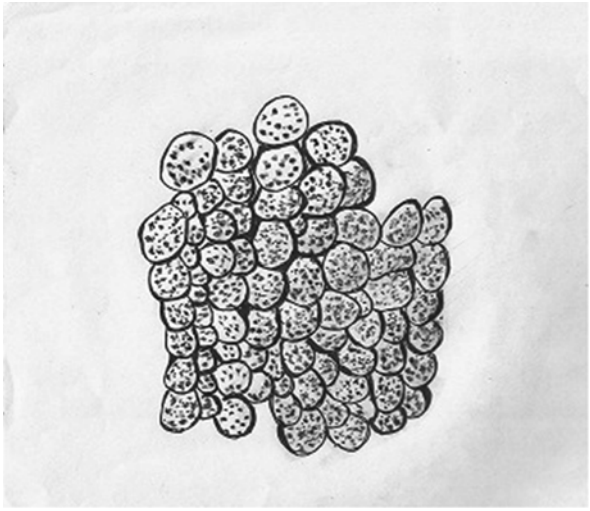

Fig. 4(ii) Filamentous *Pleurocapsa* (Source: Mur et al. 1999)

V. ORDER: STIGONEMATALES
Freshwater, filamentous algae with distinct prostrate and upright filaments, showing true branching, reproduce by hormogonia or rarely by akinetes, heterocyst present (e.g., *Stigonema*, *Mastigocladus*, *Nostochopsis*) (Fig. 4(v)).

Boone and Castenholz (2001) in Bergeys manual of systematic Bacteriology showed Blue green algae are single celled (gram-negative eubacteria); i.e., absence of nuclei and other components and contain a distinctive cell wall made of peptidoglycan and have numerous structures which distant them from other bacteria, particularly their photosynthetic organelles and manufacture of free oxygen by the light driven system. Hoffmann et al. (2005) proposed a ranking

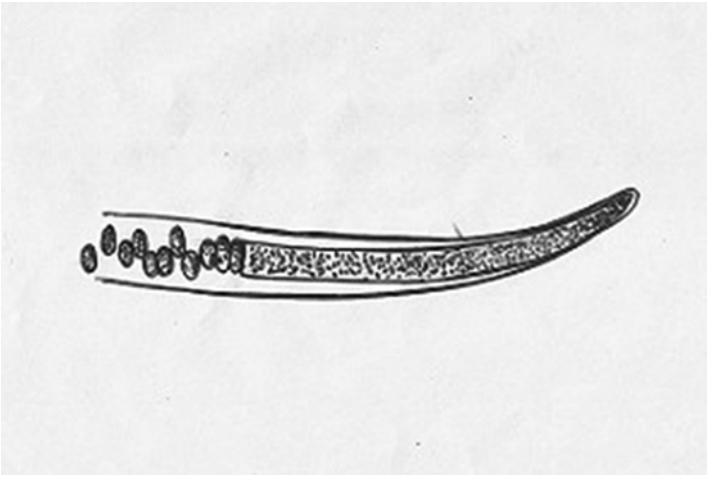

Fig. 4(iii) Unicellular Heteropolar *Chamaesiphon* (Source: Mur et al. 1999)

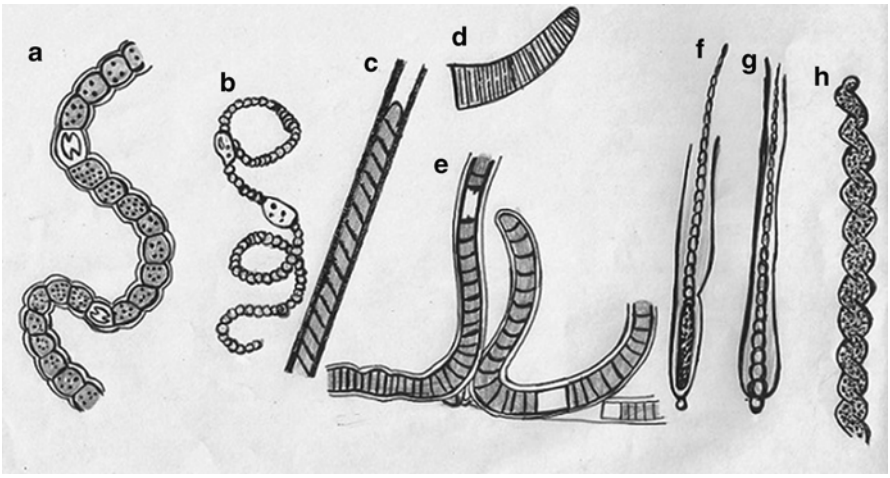

Fig. 4(iv) (**a**) *Nostoc*, (**b**) *Anabaena*, (**c**) *Lyngbya*, (**d**) *Oscillatoria*, (**e**) *Scytonema*, (**f**) *Gloeotrichia*, (**g**) *Rivularia*, (**h**) *Spirulina* (After Smith 1950)

pattern and purse the International code of botanical nomenclature and taking all features into consideration from phonological to gene level. They are classified and entitled as cyanoprokaryota with three sub class Synechoccineae, Oscillatorineae and Nostocineae. A group of light dependent microorganism were kept in distinctive procaryotic class, the prochlorophyta, because of lack of biliprotein and presence of chlorophyll (a &b), but on the support of genetic facts former group is currently holding a specified position within the Cyanobacteria and contains the species like *Prochloroccus* marinus (sea) and *Prochlorothrix* (fresh water).

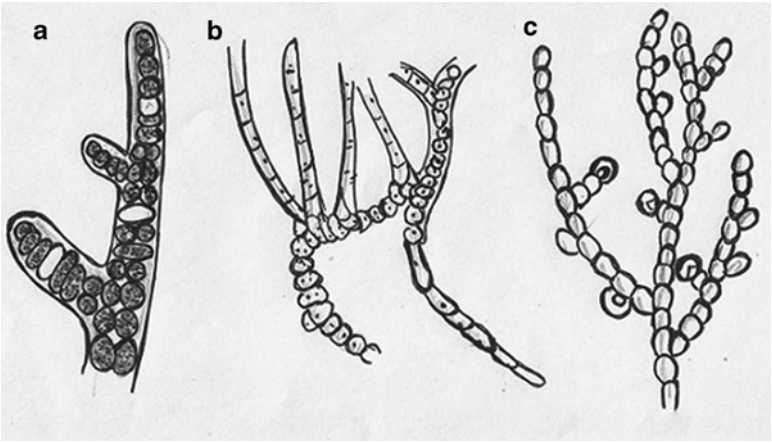

Fig. 4(v) (**a**) *Stigonema*, (**b**) *Mastigocladus*, (**c**) *Nostochopsis* (After Smith 1950)

5 Range of Thallus

Cyanobacteria exist in various morphological forms ranging from non-filamentous, coccoid and palmelloid form to filamentous ones. But, in all the forms the cellular structure and the functioning of the vegetative cells are basically the same. The non-filamentous forms are mostly coccoids being either single *cells* (*Chroococcus*, *Synechococcus*, *Anacystis*, *Gleocapsa*) or grouped in palmelloid colonies (*Gloeothece*, *Aphanocapsa*). The coccoid forms are mainly spherical or sometimes cylindrical or fusiform, multiplying in one/two/three directions. During division, they form daughter cells which may readily separate out or may remain aggregated forming characteristic colonies. These colonies are either microscopic or macroscopic in nature. They may be cubical/spherical/square/irregular depending upon the planes and directions in which the cell divides. *Merismopedia* and *Holopedia* colonies, where the cells are either tubular or cubical, form distinct pattern of arrangement. *Chamaesiphon* represents a series of forms, where the organism has got base and apex differentiation although it is unicellular in nature. *Pleurocapsaa* and *Hyella* represent a group of non-filamentous forms with heterotrichous organization very similar to that found in most advanced cyanobacteria. The filamentous form is the consequences of frequent cell divisions in one plane and in a single direction producing a large number of uniseriate cells, forming a chain or a thread like structure. The cells in the trichome are held tighter by separation walls or a common gelatinous sheath. The trichome may be straight as in *Oscillatoria*, but in *Spirulina* it is more or less permanently spirally coiled. In *Rivularia* it is whip-like with upper-end tapering into a colourless multicellular hair. In *Aphanizomenon*, the trichome tapers towards both the ends. In some genera, such as *Oscillatoria* and *Lyngbya*, the filament may contain a single trichome, while in *Microcoleus* and *Hydrocoleus* several trichomes are present in a single filament. *Tolypothrix*,

Scytonema, *Stigonema*, *Hapalosiphon*, *Westiellopis*, *Schizothrix* exhibit different types of branching of their filaments. In general, very little branching occurs in cyanobacteria. The cyanobacteria belonging nostocaccae, rivulariaceae and stigonemataceae develop one or more special thick-walled cells, called heterocyst as observed in *Nostoc*, *Anabaena*, *Rivularia*, *Stigonema* etc.

6 Special Cells

6.1 Akinetes

Akinetes (from the Greek "akinetes", meaning motionless) are thought to function as an asexual resting state that are capable of resisting harsh environments (winter) and can germinate to form new cells when conditions improve. Akinetes are produced only by cyanobacteria that are also capable of producing heterocysts. They are larger than vegetative cells and have thickened extra cellular envelope that enlarge and fill with food reserves e.g., *Nostoc*, *Stigonematales* and *Cylindrospermum* (Fig. 5). Akinetes and heterocyst contain greater quantities of storage compounds glycogen & proteins (cyanophycin) and uphold low level of metabolic activities but not vegetative cells. Akinetes are typically distinguished from heterocysts, by absence of nitrogen-fixation and large size.

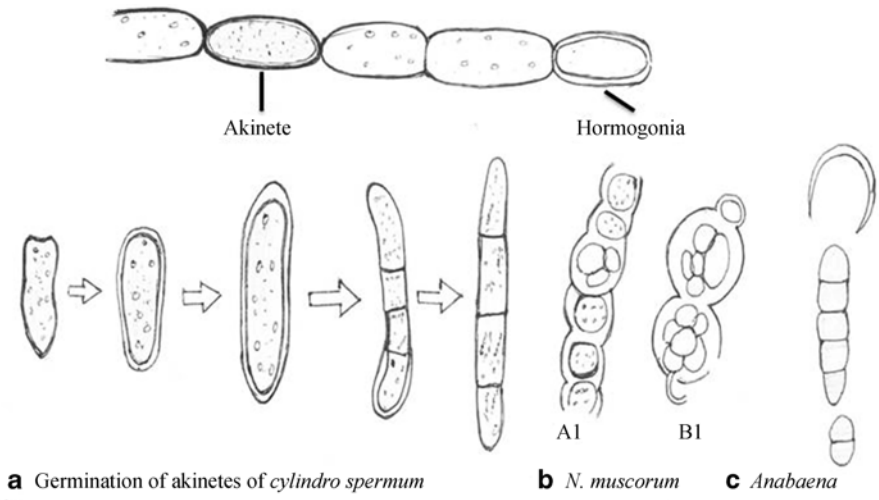

Fig. 5 (**a**) The germination of an akinetes of *Cylindrospermum* (Modified from Moore et al. 2004). (**b**) *Nostc*: A_1–B_1 germination of akinetes *N. muscorum* (**c**) germination of akinetes in *Anabaena*

6.2 *Heterocysts*

These are the special cells designed for nitrogen fixation and found in the members of *Nostocales* and *Stigonematales*. The heterocysts have thick outer wall. Heterocyst develops from vegetative cells before it enlarges in size. It forms a multilayered envelop outside the cell wall and is also differentiated. The outer fibrous layer is uniformly thick, whereas the middle (homogenous) and inner (thin lamellar) layer are thicker at the polar ends. The thickness of walls restricts oxygen dispersal into the cell, while enzymes neutralize O_2 that enter the cell. They arise from enlarged vegetative cells and are characterised by the presence of conspicuous pore at one or both poles through these pores cytoplasmic continuity is maintained with adjacent vegetative cells. In a mature heterocyst the polar pores become plugged with a refractive material (polar nodules), due to which their cytoplasmic connections with their cells are broken. In terminal heterocyst the pore is usually present only at one end e.g., *Gloeotricha* whereas intercalary heterocyst have pores at both ends e.g., *Nostoc* and *Scytonema* and rarely lateral as in *Nostochopsis*. They may be solitary or in pairs (*Anabaena*). Heterocyst contains dense and homogenous cytoplasm (Fig. 6).

They contain mostly carotenoids hence yellowish in colour. Like cyanobacterial cell they show Occurrence of Chl a, but nonappearance of phycobiliprotein. Some mechanisms of the photosystem are down-regulated. Absence of rubisco and inactivation of PS II leads them to neither do carbon dioxide fixation nor oxygen

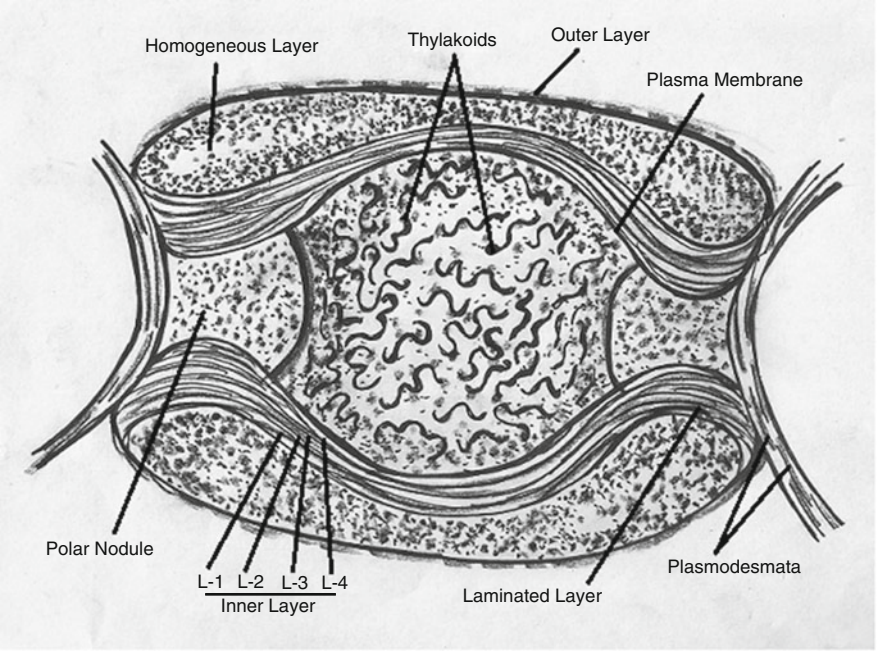

Fig. 6 The ultrastructure of a heterocyst in L.S. (diagrammatic) (After Waterbury and Stainer 1977)

production. Thylakoids (lamellae) are tightly packed and become concentrated near the periphery in a complex reticulate manner. These lamellae contain two lipids, glycolipids and acyl lipids which are not found in vegetative cells. There is gradual dissolution of storage granules and breakdown of photosynthetic thylakoids and sometimes these lamellae may form swollen vesicles and they become concentrated along the periphery and at the poles.

6.2.1 Function of Heterocyst

Early workers considered heterocyst as dead cell, thus having no functions. According to (Brand 1903) if heterocyst is removed from the trichome, sporulation is inhibited. The vegetative cells adjacent to heterocyst sporulate earlier than those away from it. Canabaeus (1929) found a sort of connection between the formation of gas vacuole and heterocyst. According to Fogg (1944) these are the sites for salt accumulation. Fritsch (1951) suggested that it also stimulates growth and cell division.

Fay et al. (1968) considered heterocyst as N_2-fixing organ particularly under aerobic conditions. Its thick wall restricted O_2 entry in to the heterocysts and protect nitrogenase inaction in presence of oxygen. Important heterocystous filamentous nitrogen fixing forms are *Anabaena*, *Aulosira*, *Nostoc*, *Tolypothrix*, *Cylindrospermum*, *Mastigocladus*, *Anabaenopsis*, *Calothrix and Scytonema*. Whereas few non heterocystous forms underneath anaerobic conditions also fix atmospheric nitrogen (e.g., *Plectonema boryanum*).

Nitrogenase reduces nitrogen gas into ammonia which in turn is metabolized to glutamine in the presence of enzyme glutamine synthetase (GS). Now this glutamine is then transported from heterocyst to the adjacent vegetative cell, where it is converted into glutamate by the enzyme glutamine oxoglutarate aminotransferase (GOGAT). Both glutamine and glutamate, by various transamination reactions, form other amino acids. The fixed nitrogen may be utilized in number of ways like it is assimilated by the cyanobacteria themselves. Soluble nitrogenous compounds are liberated from healthy cells into the culture medium and after death the nitrogenous compounds are broken down into ammonia, which is eventually converted into nitrate by nitrifying bacteria. During development of heterocyst three DNA excision events take place that allows expression of nitrogen fixing genes.

6.3 Hormogonia

Hormogonia are formed by a broad range of cyanobacteria and consist of short, non-heterocystous filaments of cells which are often smaller than the cells of the vegetative trichome from which they are derived (Rippka et al. 1981). They are commonly found in *Nostoc*, *Oscillatoria* and *Cylindrospermum*. Hormogonia possess gliding motility unlike the vegetative trichome (Rippka et al. 1981). Many hormogonia also produce gas vacuole giving them buoyancy and this together with

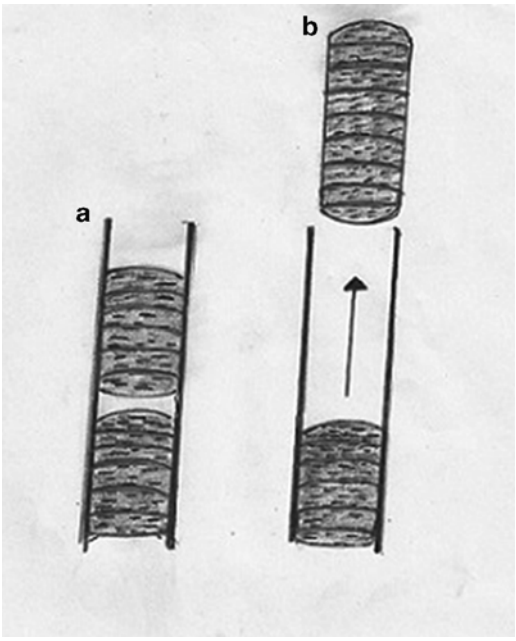

Fig. 7 (**a**, **b**) Hormogonia formation in *Oscillatoria* (After Smith 1950)

their motility makes them effective agent of dispersal (Mohan et al. 1992). Hormogonia are relatively transient morphological form. After a short time motility is lost and under nitrogen deficient conditions, heterocysts develop at positions which are specific to the genus involved. (Fig. 7).

7 Reproduction

Vegetative and asexual mode of reproduction is present. Vegetative reproduction accomplish by cell division, fragmentation and hormogonia formation. Unicellular forms exhibit binary fission, while filamentous multicellular forms and colonial forms exhibit disintegration, in which sections of the organism become separated from the parent, drift away, and mature into new individuals. Exospores and endospores are products of successive bipartition and are characteristic of the chamaesiphonales and pleurocapsales. The liberated endospores germinate immediately without a resting periods. Besides, a variety of asexual bodies such as hormogonia, hormocysts, nannocytes and akinetes also facilitate their vegetative propagation (Waterbury and Stainer 1978). Sexual reproduction is absent (Sex organs, gametes and flagellated zoospores are altogether absent), however, in some species (e.g., *Anabaena*, *Nostoc*, *Synechoccus* and *Cylindrospermum*) genetic recombination has been reported. Conjugation has not been observed in cyanobacteria.

8 Economic Importance of Cyanobacteria

It is important to exploit aquatic resources for the growing food and industrial requirement of the world. The possible use of aquatic blue-green algae for the productivity of natural resources like vit. Antibiotics, enzymes, pigments etc. has been documented and exploited for various commercial purposes.

8.1 Cyanobacteria as Food Supplement

Among cyanobacteria *Spirulina* has immense profitable significance because of high nutritive value, protein content (65–71 % of dry weight), low fat, high vitamin (particularly B_{12}) as well as essential fatty acids, γ- linolenic acid which are easily converted into arachidonic acid, prostaglandin E_2 in human body that has blood pressure lowering action and smooth muscle contraction and take essential part in fat absorption (Kulshreshtha et al. 2008). It also contains trace elements and a number of uncharted bioactive compounds (Fig. 8). In China, the *Spirulina* industry is supported by the State Science and Technology Commission as a natural strategic program (Li and Qi 1997). Iron is well absorbed in *Spirulina* than any other supplements. Subsequently, it thus signifies a sufficient source of iron in anemic pregnant women (Pyufoulhoux et al. 2001). *Spirulina platensis* contains about 13.6 % carbohydrate (Shekharam et al. 1987). Lack of cellulose in its wall, makes it an suitable and vital foodstuff for individuals with problems of poor intestinal absorption and

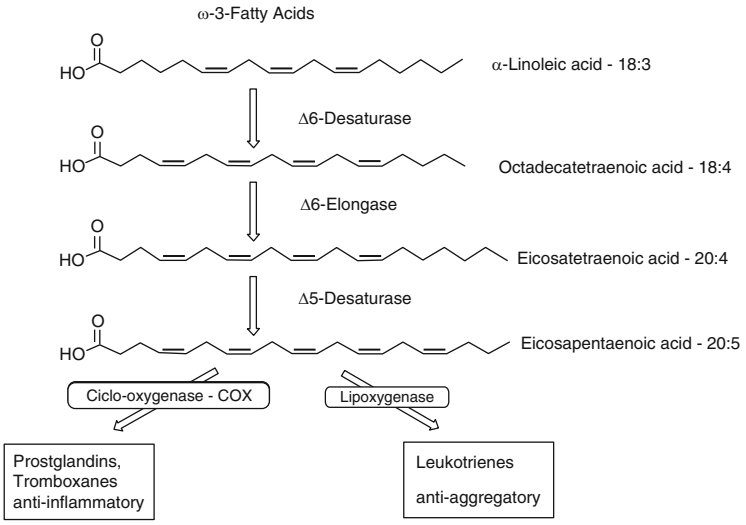

Fig. 8 A streamlined biosynthesis outline of fatty acid (Adapted from Sayanova and Napier 2004)

elderly patients (Richmond 1992). A novel polysaccharide "*Immulina*" is highly water soluble, high molecular weight, and immuno stimulatory. It has been isolated from *Spirulina* (Pugh et al. 2001). It is a basic foodstuff in parts of Africa and Mexico. Besides, *Spirulina* pills and capsules, there are also pastries, and *Spirulina-filled* chocolate blocks. Since ancient times in many countries cyanobacteria are served as a side dish. Most of the companies which are associated with blue green produces a variety of products from which are commercially available like medicines, fries, macaroni, liquescent extracts etc. and Cyanotech Company (Hawaii, USA) established a method in desiccating a biological material in the interest of evading the oxidation process of carotene and fatty acid. They also sell the products in powder form named *Spirulina pacifica*.

Aphanizomenon flos-aquae is sold as food in California as health supplement since the early 1980s. In 1998, 10 -10_6 kg (dry weight) was marketed with a value of $100 million (Carmichael et al. 2000). The colonies of *Nostoc commune* are boiled and are used as food in China and Java (Gao and Ye 2003). In Mexico City *N. commune* and *Brachytrichia* "are sold as small cakes that are used for making breads with cheese flavour." *Anabaena* is also consumed as human food in Santiago, South America, North United States and south china.

8.2 As Feed

Blue green algae especially *Arthospira* is used as feedstuff for extensive diversity of wildlife like felines, canines, aquarium fishes, cattle, intimidates and breeding bulls. Cyanobacteria affect the morphology and physiology of the their dependent organism. In fact, 50 % of the present world production of *Arthrospira* is used as food supplement (Yamaguchi 1997). In 1999, the output of cyanobacteria for aqua farming reached 1000 t (62 % for molluscs, 21 % for shrimps, and 16 % for fish) cyanobacteria (*Spirulina*) can be used up to a level of 5–10 % as incomplete replacement for conventional feed (Muller-feuga 2004). *Phormidium valderianum* BDU 30501 is used as aquaculture feed source having nutritional qualities (Thajuddin and Subramanian 2005).

8.3 Therapeutic and Chemopreventive Applications

Cyanobacterial pigments, especially phycocyanin, allophycocyanin, and phycoerthrin show strong antioxidant, anti-inflammatory, antitumour action. They show stimulation of immune system and ability to treat ulcer and haemorrhoidal bleeding. It lowers the risk of heart disease and defends against old age-linked disease (Subhashini et al. 2004; Bhaskar et al. 2005). Cyanobacterial phycocyanin selectively stops cytochrome oxidase 2 (COX-2), which is over expressed in mammary

glands sarcoma; having not any effect on COX_1 (Reddy et al. 2000). Alzheimer's and Parkinson's illness can be healed by CPC (Rimbau et al. 2001), and prevent mouth and skin tumours. Phycocyanin of *Spirulina* plays a major role in treatment of renal failure caused by mercury and pharmaceutical drug. The antioxidant property is enhanced by β- carotene, lycopene, and luteint that are abundant in cyanobacteria. Besides it also exhibit neuroprotective, hepatoprotective and anticancer property (Reddy et al. 2000, Becker 2004).

Presence of eicosapentaenoic acid and linolenic acid in cyanobacteria offer good scope for the study of these organisms. They have a steroid decrease impact in creatures and humans being due to the presence of enzyme lipoprotein lipase. The level of the total sterol was decreased experimented on rat serum when cyanobacterial biomass was added with foodstuff having high cholesterin content (Iwata et al. 1990). Chlorophyll in *Apbanizomenon flos-aquae* show hypocholesterolemic effect, which the liver function and reduces blood cholesterol level (Vlad et al. 1995). It also constrains the activity of a maltase and sucrase in the digestive tract of rats (Kushak et al. 1999) and indicates faster retrieval from minor shocking brain injury (Valencia and Walker 1999). It shows robust toxicity towards leukemic cell lines and hinders tubulin polymerization by binding at colchicine site (Lai and Yu 1996). Administration of *Spirulina* brought about a significant decrease in blood sugar in non-insulin dependent diabetes (Khrusheed et al. 2012; Anwer et al. 2012). Clinical and biochemical evaluation of *Spirulina* showed successful use in treatment of obesity, of acute anaemia and improvement of haemoglobin in humans administration of *Spirulina* at 2–5 g/day. Patient suffering from rheumatoid arthritis-fed with *Spirulina fusiformis* showed marked improvement and decrease in pain and swelling (Mohan 1992). *Spirulina* promotes the development of *Lactobacillusacidophilus* and other probiotics. It also showed antiviral activity against Herpes, Influenza and Cytomeglo- virus (Blinkova et al. 2001). *Spirulina* can prevent HIV- I duplication in humanoid derived T- cell and in humanoid marginal blood mononuclear cells (Ayehunie et al. 1998). Cyanobacterial extracts of *Hapalosiphon* show antimycobaterial activity, antibacterial activity (Rao et al. 2007; Bhaleya et al. 2006). They also have photodynamic therapy and uses in other clinical applications (Panday et al. 2009; Kyadari et al. 2014).

8.4 Cyanobacteria Produce Secondary Metabolites

They are distinguishing the utmost favorable cluster of entities which produces new and biochemically active usual goods are separated. Cyanobacteria produce various bioactive compounds and extensive range of toxins (Fig. 9). Apart from cytotoxicity they show antitumor, antiviral, antibiotics, antimalarial, multi-drug resistance reversers and immuno suppressive properties (Burja et al. 2001). Besides the immune effects, blue green algae recovers metabolic rate.

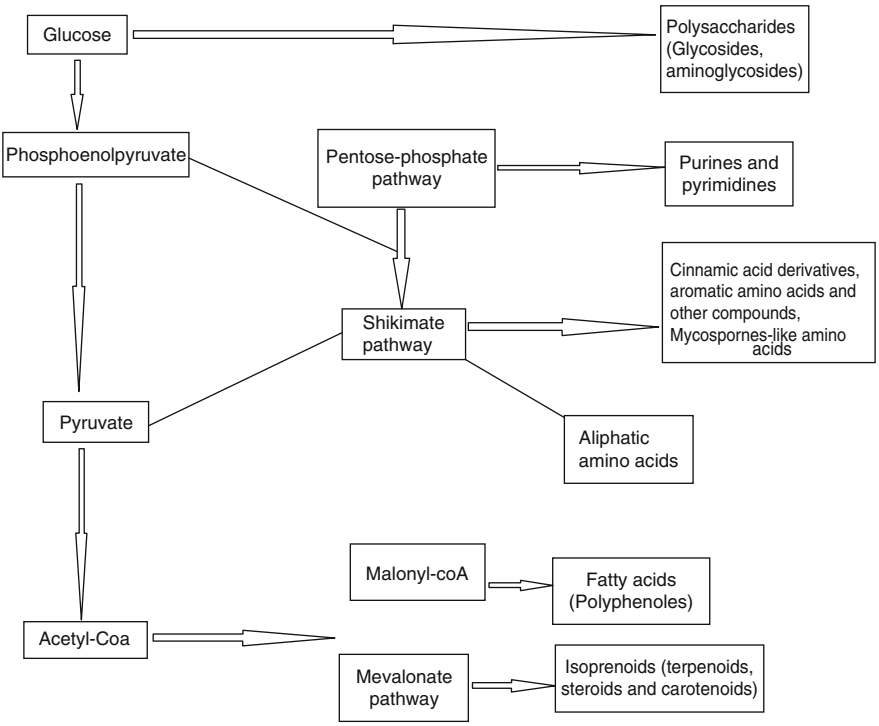

Fig. 9 Systematic representation of metabolites biosynthesis (Modified from Burja et al. 2001)

8.5 Agriculture Purpose

Cyanobacterial biofertilizer are excellent renewable natural nitrogen resources for many crops (Mahmoud et al. 2007; Ali Laila and Mostafa Soha 2009). Cyanobacteria (e.g., *Anabaena*, *Nostoc*, *Clothrix*, *Oscillatoria*, *Tolypothrix*) enhanced seed growth and development. Both quality and quantity of grains/crop were enriched. By using suggested amount 50 % inorganic N_2 fertilizer might be protected by means of cyanobacterial biofertilizer (Mahmoud et al. 2007; Ali Laila and Mostafa Soha 2009; Amal et al. 2010). *Azolla – Nostoc* association has been used as green manure in rice fields of South China, Vietnam, and India (Lumpkin and Plucknett 1980). Blue-green algae also help in reclamation of saline and alkaline soils. The growth of cyanobacteria in saline and alkaline water-logged fields results in decline in pH and increase in P, N and organic contents and thus converting it into fertile and cultivable land. It can also enhance minerals (P and K) contents in soil which promote indirectly the development of plant (Selvarani 1983; Brijvir 1992) various *Anabaena*

sp., produces ammonia, while *Aphanocapsa and Porphyri-diumcruentum* produces polysaccharide (Hameed and Ebrahim 2007). Cyanobacterial isolates capable of mineralizing organic phosphorus have phosphatase activity (Giraudet et al. 1997).

Cyanobacterial extracts include an abundant amount of bioactive compounds that effect plant growth and development. The most common growth regulators are gibberellins, auxin, cytokinin, ethylene and abscisic acid that improve growth and production of plants like *Daucus* carota (Wake et al. 1992), *Santalum* album (Bapat et al. 1996), *Oryzae sativa* (Storni et al. 2003), *Lilium alexandrae* hort (Zaccaro et al. 2006) and *Beta vulgaris* L. (Aly et al. 2008).

Cyanobacteria *Nostoc muscorum* has been shown to influence antifungal action on soil fungi and particularly those produce diseases (De Caire et al. 1976, 1987, 1990; Mule et al. 1977), other fungi producing wood blue stain (Zulpa et al. 2003), lettuce white mould caused by *Sclerotinis sclerotiorum* (Tassare et al. 2008). *N. muscorum* can also be used for biocontrol of insects. These penetrate and scratch waxy covering on the body of the insects leading to its death due to dehydration. *Synechocystis* PCC 6803, *Agmenellum quadruplicatum* PR-6 and *Anabaena* PCC 7120 are used in mosquito biocontrol (Ngalo et al. 2008; Khasdan et al. 2003).

8.6 Biological Remeditaion

Throughout the world's ever growing population and industrial advancement results in environmental pollution problem e.g., heavy metal, pesticides that are not easily biodegraded and persist in the environment. The removal of such pollutants from wastes prior to disposal, are absolutely essential. The success of bioremediation is dependent on the selection of appropriate micro-organism with precise ecological state. Cyanobacteria (especially those capable of diazotrophic growth) offer distinct advantages as potential biodegradation organism, since their survival is not dependent on the presence of high concentration of organic compounds.

8.6.1 Heavy Metals Degradation

Microorganisms and microbial products can be extremely effective as bio accumulators of soluable and particulate forms of metals, exclusively from dilute external medium. It is of two types: non metabolic and metabolic which operate in isolation or together. Survival of organisms in metalliferous environment may be by compartmentalization of metals in vacuole, cell wall, polyphosphate bodies (Jensen et al. 1982), metal tolerant enzymes/antioxidant enzymes and substrates (Chadd et al. 1996). Production of metal binding proteins (Reddy and Prasad 1992; Mallick et al. 1994), adenylated nucleotides (Palfi et al. 1991) and sidophores (Uma and

Subramanian 1994). The mechanism of metal removal by microbes is generally active process and occurs in two phases: (i) A rapid binding of caution to the negatively charged groups of cell walls and (ii) followed by a subsequent metabolism dependent intracellular uptake (Gipps and Coller 1980; Norris and Kelly 1977). The nonmetabolic (biosorption) is rapid and reversible accounting for about 90 % of total heavy metal uptake. This process is more promising for commercial development as the organism can be used in multiple cycles (Darnell et al. 1986) and have an edge over conventional waste water treatment facilities (Modak and Natrajan 1995). One of the commercial products available is Alga Sorb™, Biorecovery Inc New Mexico (*Spirulina* and *Chlorella*) which is based on biosorption phenomenon. Immobilized cyanobacteria have more potential in metal removal than their free living counterpart e.g., *Anabaena doliolum* (Singh et al. 1989; Gijzen et al. 1988; Rai and Mallick 1992).

8.6.2 Pesticides Degradation

The bioaccumulation and biomagnifications of residual insecticides in phytoplankton's which constitute the prime makers in the food cycle are biologically and toxicologically significant. It is well established that cyanobacterial biomass have larger surface area attracting biophilic pesticide molecules thus helping in predicting the impact of pollution in aquatic system, removal of toxicants from waste water and as bioassay organisms. Depending on the type, biological property and concentration of pesticides and the algal strains, their effect could be inhibitory, selective or even stimulator. It has been observed that cyanobacterial forms used in biofertilizers are capable of tolerating pesticides levels recommended for fields applications. Cyanobacteria have been reported to accumulate very high concentration of insecticides. *Synechococcus elongates*, *Anacystics nidulans* and *Microcystes aeruginosa* degrade many organophosphorus and organochlorine insecticides from the aquatic system (Kruitz 1999; Vijayakumar 2012).

Cyanobacteria have the ability to damage naturally occurring aromatic compounds, including naphthalene (Cerniglia et al. 1980a, b), phenanthrene (Narro et al. 1992) phenol and naphthalene (Ellis 1977). Methyl parathion degradation by cyanobacteria has been demonstrated by (Megharej et al. 1994).

8.6.3 Dye Degradation

Synthetic dye released in the environs by the industries have complex aromatic structure, this feature makes it constant and hard to biodegrade. In an aquatic environment the dye not only affects the aquatic plants but also create toxic and mutagenic products. Decolourization of these dyes by physical or chemical methods has commercial and organizational disadvantage. Biologic degradation of synthetic dyes takes place due to discharge of laccase, lignin peroxidase, manganese peroxidase (Wong and Jian 1999; Lopez et al. 2004). Laccases, benzenediol, oxygen

oxidorectase are multicopper containing enzymes, often extracellular in nature. They utilize molecular O_2 to oxidize an extensive range of aromatic and nonaromatic mixtures by a fundamental catalyzed mechanism (Polaina and Mac Cabe 2007; Couto 2007). Due to its low specificity its marketable and biotechnological impact is great (Viviane et al. 2009). Cyanobacterial laccase can be used for decolourization of synthetic dye (Afreen and Fatma 2013).

Biofeul

Global warming, exhaustion of fossil fuels and cumulative rate of petroleum had enforced us to hunt for alternate, ecological, renewable eco-friendly well-organized and profitable energy sources. Biomass can function as an exceptional substitute to fulfil the current and upcoming fuel demands. Any form of fuel produced from biological material is called biofuel. Cyanobacteria are used for the manufacture of biofuels in a productive, reasonable and ecofriendly manner. Few biofuel producing cyanobacteria are *Nostoc* sp., *Phormidium angustissimum TISTR 8979* (Rodjaroen et al. 2007), *Spirulina fusiformis* (Rafiqul et al. 2003), *Synechococcus* sp., *Lyngbya* sp., (Karpanai et al. 2013).

The climate change as well as greenhouse effect is resulting in skinning of cultivable land area and an increase in water covered areas. Such waters can be exploited for production of cyanobacterial biomass laden with carbohydrate/lipids that can be utilized for production of biofuel (bioethanol and biodiesel).

Ethanol is extensively used around the world as biofuel which is less toxic and biodegradable manufactured from plentiful supplies of starch/cellulose. It can also be used as a fuel for electric power generation, in fuel cells (thermo-chemical action) (Petrou and Pappis 2009). The most important bioethanol production countries in the world are Brazil, US and Canada (Chiaramonti 2007). Being photosynthetic in nature cyanobacteria produce high carbohydrate content which can be utilized as feedstock for bioethanol yield (*Synechococcus* sp. PCC 7002).

Hydrogen is used as sources of renewable fuel as it cause no pollution. In view of this H_2 production is important area in biotechnology. In achieving this goal photosynthetic machinery is employed. Water is subjected to photolysis, which lead to splitting of water molecules into oxygen, electrons and hydrogen ions (H^+). Hydrogen ions (H^+) are converted into hydrogen gas (H_2) which can be collected and used as a fuel (Hameed and Ebrahim 2007).

Bioplastic

Bioplastic can be defined as a plastic derived from renewable materials of biological origin, that exclude biomass embedded in geological formation or transformed into fossil fuels. Bioplastics produced from renewable carbon resources add to our effort to conserve fossil resources which are directly or indirectly used for plastic production. Degradation of bioplastics takes 5–6 weeks as compared to thousands of years

for petroleum based plastics. The degradation products of bioplastics are carbon dioxide and water (Brandl et al. 1990; Steinbuchel and Valentin 1995) which can be used during photosynthesis by autotrophs, thus bioplastics are useful in their ultimate 'waste state' also. Their biocompatibility is making them also innovative product in the medical field (Katti et al. 2002; Philip et al. 2007). Some promising bioplastic applications contain biodegradable carriers like bone tissue replacement, surgical needles, structure materials etc. that exhibit the capacity to provide remedies for an assumed time within the individual's body, (Zinn et al. 2001; Chen and Wu 2005).

PHB is one of the common biopolymer that appears as a substitute to the common plastics due to its hydrophobicity, complete biodegradability and biocompatibility with visual clarity. Cyanobacteria are however the exclusive oxygen producing photosynthetic prokaryotes that accumulate PHB (Miyake et al. 1996).

The first cyanobacterial species which reported the presence of PHB is *Chlorogloea fritschii* in the year 1966. Existence of PHB has been confirmed in *Spirulina* sp., *Aphanothece* spp., *Gloeothece* sp. and *Synechococcus* sp. (Campbell et al. 1982; Fernandez-Nava et al. 2008), *Synechocystis* sp. (Quillaguaman et al. 2010), *Gleocapsa* spp. and *Nostoc* spp. (Shrivastav et al. 2010), *Microcystis aeruginosa*, *Trichodesmium thiebautti* in *Oscillatoria limosa* sp. and *Gloeothece* sp. (Sabirova et al. 2005).

Cyanobacterial Toxins

Cyanotoxins belong to a rather divers group of chemical substance each of which shows precise toxic mechanism in vertebrates. Cyanotoxins mainly affect three organs: livers, nervous system and skin (Sivonen and Jones 1999). The neurotoxins are alkaloids (nitrogen-containing compounds of low molecular weight) that have no effect on penetrability of potassium and constrains nerve condition by obstructing sodium channels (Adelman et al. 1982). Symptoms include shocking, muscle jolting, breathless and fit. The neuro-toxins can be fatal at high concentrations due to respiratory arrest caused by failure of the muscular diaphragm. The two neurotoxins produced by cyanobacteria are **anatoxin** and **saxitoxin**. Anatoxins are synthesized by *Anabaena*, *Aphanizomenon*, *Oscillatoria* and *Trichodesmium* (Negri et al. 1997).

Hepatotoxins are low molecular weight peptide and are interception of protein phosphatases (Arment and Carmichael 1996). The sign of poisoning include weakness, vomiting, diarrhoea, and cold extremities. Death occur within a short period of time after initial exposure which occur due to intra hepatic haemorrhage, reflected by increase in the liver mass as a portion of body mass (Jackson et al. 1984). Cyanobacteria produce two types of hepatotoxins, the **microcystins** and **nodularins** (Rinehart et al. 1994). The Microcystin is produced by *Microcystis*, *Anabaena*, *Nostoc*, *Nodularia*, and *Oscillatoria* while the nodularins are produced by *Nodularia* (Kotak et al. 1995; Bolch et al. 1999).

Cyanotoxins can also inhibit the growth of other algae. This is called an **allelopathic interaction** where one organism affects the growth of second organism. An

example of this is the inhibition of the freshwater dinoflagellate *Peridinium gatunense* by the microcystin produced by the cyanobacterium *Microcystis* lake Kinneret (Sea of Galilee), Israel. The *Microcystis* cyanotoxins abolish carbonic anhydrase activity in the dinoflagellate and inhibit growth (Sukenik et al. 2002).

In order to aquent students with cyanobacteria occurring in their surrounding some example are discussed below.

Microcystis (Fig. 4(i)b)

Microcystis are commonly found in nutrient rich freshwater bodies. The colonies may be round or irregular. There are hundreds or thousands of small, cells in each colony. The spherical or elongated cells in a colony float freely in a colorless, homogeneous mucilaginous matrix. They lack individual sheath and exhibit typical cyanophycean cell structure. Numerous pseudovacuoles are frequently present in a cell. In tropical countries it multiplies so prolifically that impart a bluish-green tinge to the pond which seems like "Pea soup." They reproduce only vegetatively. The vegetative propagation takes place either by fission or by fragmentation of the colony. The fission is simple cell division which takes place in all planes. In *M. flosaquae* the modified endospores are formed by repeated division of cell contents. Some species like *M. aeruginosa* cause water blooms in lakes that often results in severe reduction of oxygen. This leads to mass mortality of fishes and other aquatic animals due to suffocation. *Microcystis* contain toxins known as microcystins which is highly toxic to fishes and aquatic birds. There are several other cyanobacterial species that produce microcystin including: (*Anabaena*, *Oscillatoria*, *Nostoc*, *Hapalosiphon and Anabaenopsis*) Microcystins are hepatotoxins.

Acute toxicity is the main concern with microcystins. The compound attacks the liver causing hemorrhaging and liver failure. The threat to human health is of great concern since long-term exposure to relatively low doses could encourage the development of liver tumors. The toxin is also fatal to domesticated animals that drink contaminated water. *M. toxica* is highly poisonous and is responsible for the death of thousands of sheep and cattles. In humans, some species cause gastric trouble, if they are ingested along with drinking water or during swimming. There are several species of *Microcystis* which inhibit the growth of bacteria such as *staphylococcus*, *clostridium*, zooplanktons and several other algae.

Spirulina (Fig. 4(iv)h)

Spirulina are aquatic organisms, typically inhabiting in a wide variety of fresh water, often found densely intertwined with the *Oscillatoria. Spirulina* occur in tropical and subtropical fresh water bodies rich in carbonates/bicarbonates and high pH. *Spirulina* are blue-green non heterocyst solitary and free floating filaments, composed of cylindrical vegetative cells wounded into a loose or close helix. The motion is usually creeping or gliding in the direction of a longitudinal axis and may take place both backward and forward. In some species, gliding movement go along with the revolution of the trichome, taking place in the direction of coiling. The

protoplast is differentiated into a peripheral pigmented chromoplasm and a central centroplasm. Cyanophycin granules are, however, absent and are capable to persist over a wide range of temperature, some species are even found in hot springs (*S. plantensis and S. maxima*). Species of *Spirulina* are rich in proteins and iron content, essential vitamins and unsaturated fatty acids. They are receiving considerable attention for their use as supplement to diet. *Spirulina* reproduces vegetatively by the process of fission.

Nostoc (Fig. 4(iv)a)

Species of *Nostoc* occur in fresh water as well as in terrestrial habitats. The fresh water species form macroscopic colonies on the surface of water. Each colony is bounded externally by a pellicle like membrane. *N. Commune*, a terrestrial species of alpine regions, forms leathery or rubbery sheet on the moist soil. Some species of *Nostoc* are endophytic and they occur in symbiotic association within other plants. For example *N. punctiforme* is found in the coralloid roots of *Cycas* and *Anthoceros* thallus, and *N. sphaericum* and *N. colleme* are the components of lichens and occur in symbiotic association with fungi. Species like *N. amplissimum*, attains the diameter of 30 cm or almost the size of hen's egg. Some species of *Nostoc* are grown in rice fields. They are able to fix atmospheric nitrogen, thus helping in increasing fertility of the soil. The trichomes of *Nostoc* are uniseriate, usually contorted and twisted in various ways. An important feature is the presence of prominent constrictions between the adjacent cells which give a characteristic moniliform appearance to the trichome. Each trichome is enveloped in a gelatinous sheath which is transparent, hyaline or colored. Usually many trichomes aggregate together, and their gelatinous envelopes dissolve to form colonies of various shapes and sizes. The trichomes of *Nostoc* are characterized by the presence of heterocysts which are intercalary or occasionally terminal, usually solitary and of much the same size and shape as the vegetative cell. The intercalary heterocysts have two polar nodules and the terminal ones have only one (basal) polar nodule. Trichome mostly breaks near heterocyst and forms hormogonia and thus they help in its multiplication.

Nostoc reproduces only vegetatively. Sexual reproduction is completely absent. The vegetative propagation takes place by the following methods.

Fragmentation: The colony may break into small fragments due to mechanical, physiological or other factors. Each fragment has the capability to develop into a new colony.

Hormogonia: The trichome breaks into small segments or pieces by the death and decay of vegetative cells. Multicellular fragments so formed are called hormogonia. They come out of the gelatinous sheath of the colony, grow rapidly and form new colonies. Very often, the hormogonia fail to come out of the parent colony and divide inside the gelatinous sheath of the parent colony. This result in a large number of trichomes inside the parent colony, the trichome breaks near heterocyst.

Akinetes: During unfavorable periods, some vegetative cells enlarge and transformed into thick walled resting spores or akinetes which accumulates food material. They have an additional three layered coat outside the normal cell wall. The outer layer is variously sculptured and its protoplasm becomes highly granular.

Development of akinetes normally takes place in a mature colony. It is always the vegetative cell adjacent to the heterocyst which is the first to metamorphose into akinetes. Subsequently, many vegetative cells are transformed into akinetes. It is not unusual to find all the cells between the two successive heterocysts transformed into akinetes. Metamorphosis of the vegetative cell into akinetes is probably simulated by certain chemical substances secreted by heterocysts. The akinetes have tremendous resistance for cold and drought. During favorable conditions, the protoplasm become active and breaks the thick outer wall and forms a new trichome.

Hetrocysts: In some species of *Nostoc* (e.g., *N. commune*) heterocysts act as resisting pores. The protoplasm of the heterocyst of such species becomes functional and germinates to form a new trichome. At the time of germination the protoplast divides by a traverse wall to form two cells. Both these cells divide again to form a 4-celled germling. The thick wall of the heterocyst ruptures at this stage and the germling comes out and develops into a new trichome.

Endospore: In some species of *Nostoc* (*N. commune*, *N. microscopicum*) the protoplasm of the heterocyst divides successively to form endospores. Unlike akinetes, the endospores are thin walled and hence cannot be regarded as resting spores acquaintance. Disintegration of the capsule wall results in their liberation, and the rounded spores later germinate to form new trichomes.

Fusion of Filaments: Although true sexual reproduction and sex organs are absent in *Nostoc* as in other blue-greens; yet fusion and anastomosis of trichomes have been observed in *N. muscorum*. This phenomenon can be compared with somatogamy.

Anabaena (Fig. 4(iv)b)

Anabaena is an autotrophic freshwater colonial blue green alga found throughout the world. The long chain of cells is known as trichome it is a filamentous cyanobacterium that exists as plankton. It is not specific to freshwater but it can inhabit a various range of temperatures and salinities. Numerous species of *Anabaena* have been found in soil and aid to add nutrients to the soil. It also acts as an effective natural fertilizer. It grows in long filaments of vegetative cells. The *Anabaena* genus is known for its intercalary heterocysts and isolated or amorphous filaments.

Heterocyst: Specialized cell for fixing nitrogen (nitrogen to ammonia for amino groups and other uses). In order to avoid diffusion of oxygen in heterocyst, have three thick extra layers outside which keeps nitrogenase separated from oxygen and thus reduces the amount of oxygen in heterocysts than of vegetative cells.

Gelatinous sheath: Holds cells in filament, protects filament from predator or harmful environmental factors.

Thylakoids: Membranes increase surface area for photosynthetic pigments (Fig. 10).

Anabaena is somewhat motile and having a sliding type of movement, and this feature make it distinct from a nearby relative like *Nostoc*. It is supposed that this drive is produced by waves of momentum through the whole trichome and also its slime emission is used to help in movement. Related to other forms of cyanobacteria it has a discrete antiquity. *Anabaena* typically reproduces via fragmentation. Hormogonia and dead cells help in fragmentation.

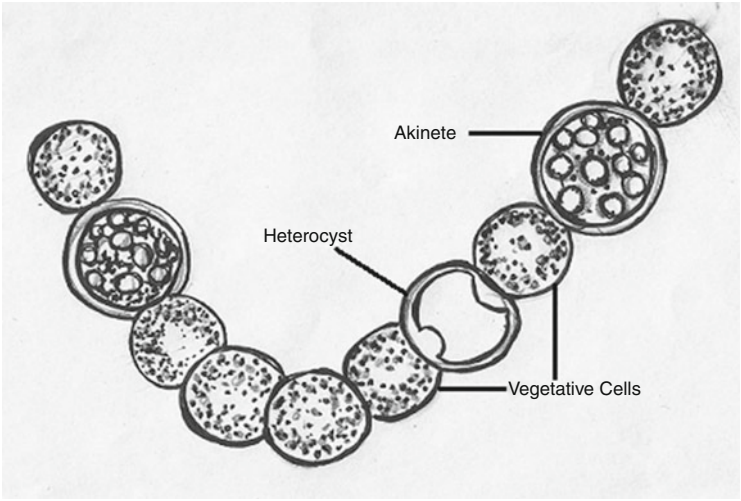

Fig. 10 Diagrammatic representation showing akinetes, vegetative and heterocyst cells in *Anabaena* (After Smith 1950)

Oscillatoria (Fig. 4(iv)d)

The species of *Oscillatoria* found in channels, ponds, swimming pool, drainage system. Few types are also formed on moist soil and moist rocks. They are set up in the form of layers on the surface of water in moving condition or basements of pools and lagoons. A small number of varieties are usually found in contaminated water. The structure is filamentous which are arranged in a single row and not forked. Each monofilament is bringing into being either singly or in masses and contains a trichome enclosed by a viscous covering which are some extent rectangular. Cells of the filament are positioned one after another like staircase. The final cell of filament might be slightly curved at the top. In *Oscillatoria proboscidea* the peak of the filament make a snout like appearance. The nucleus and the cytoplasm of each cell might simply be distinguished into binary areas. The marginal coloured area is named as chromoplast and not only the pigments but pseudovacuoles are also seen in the province. The key pale area of the cells is termed as centroplasm or central body. True nucleus is absent. The main area contains chromatin granules in discrete form. The central body signifies the actual primeval type of nucleus. Absence of nucleoli and its membrane. Cell wall is distinguished into two layers the external coating comprises of pectic materials while the coating following to protoplast made of cellulose. The filaments of Oscillatoria have fluctuation in its drive. The asexual replication is done by ways of hormogones. At numerous places of the filament monochrome biconcave parting discs are formed and disrupt at these sockets forming hormogones. Each hormogones contains additional cells and grows into a fresh organism.

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Green Algae

Mani Arora and Dinabandhu Sahoo

1 Introduction

Green algae are oxygenic photosynthetic eukaryotes characterized by the presence of chloroplast with a double- membrane envelope, chlorophyll *a* and *b*, stacked thylakoids and interplastidial starch. Plant body of green algae has a simple construction and shows no differentiation into true root, stem and leaves. For this reason plant body is called a thallus. The cells constituting the thallus are eukaryotic and thus contain all the cell organelles such as a definitely organized nucleus, membrane bound plastids, mitochondria, Golgi bodies, endoplasmic reticulum, and true vesicles. Green algae have been included in a single class Chlorophyceae by Fritsch (1935). This is considered as the most diverse group of algae with more than 7,500 species growing in a variety of habitats.

2 Origin of Green Algae: Evolutionary Significance of *Prochloron*

The green algae originated by an endosymbiotic event in which a heterotrophic eukaryotic host cell that already contained a mitochondrion, captured a photosynthetic prokaryote that ultimately turned into a photosynthetic plastid (Archibald 2009; Keeling 2010). This endosymbiotic event happened between 1 and 1.5 billion

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D. Sahoo, J. Seckbach (eds.), *The Algae World*, Cellular Origin, Life in Extreme Habitats and Astrobiology 26, DOI 10.1007/978-94-017-7321-8_4

years ago (Hedges et al. 2004; Yoon et al. 2004) and marked the origin of green algae, the most primitive oxygenic photosynthetic eukaryotes. This event ultimately resulted in a cell, containing a photosynthetic plastid surrounded by two membranes with a highly reduced genome derived from photosynthetic bacteria (Margulis 1993; Kutschera and Niklas 2005; Gould et al. 2008; Parker et al. 2008). It is believed that the outer membrane of the chloroplast envelope represents the vacuolar membrane of the host cell and the inner membrane represents the cell membrane of the photosynthetic bacteria. All green algae contain plastids with photosynthetic pigments – chl *a*, chl *b* and carotenoids. Hence the green algae must have acquired their plastids from the endosymbiosis of a unique photosynthetic bacterium having both chl *a*/*b*.

2.1 Discovery of Prochloron

Evolutionary pathways for the origin of plastids (chloroplasts) in support with the endosymbiotic hypothesis is one of the most interesting and complex problems in the field of evolution and development. It was a great finding to discover a photosynthetic bacterium *Prochloron* (Lewin 1975, 1977) which contains all the light harvesting pigments including Chl *b* which are present in chloroplasts of green algae. *Prochloron* is closely related to cyanobacteria but differ from other cyanobacteria because it contains both chl *a*/*b* and lacks phycobiliproteins. There are three known prochlorophytes (*Prochloron*, *Prochlorothrix*, and *Prochlorococcus*), that have both chl *a* and chl *b*. No other prokaryotic life forms contain both chl *a*/*b*, but all photosynthetic eukaryotes (land plants and green algae) contain both chl *a*/*b*. At present, there are studies being carried out on the photosynthetic machinery of *Prochloron*, because its morphology is distinctive among prokaryotes.

2.2 **Prochloron:** *Fine Structure*

Prochloron is a unicellular prokaryotic alga containing both chl *a* and chl *b*. The cell wall of *Prochloron* resembles that of cyanobacteria. The thylakoids and cytoplasm are in the form of a wide peripheral band. The thylakoids are not present as single lamellae, as in cyanobacteria, but in pairs or, occasionally, in thicker stacks. A large electron-transparent central zone is characterized by the absence of thylakoids and cytoplasm (Fig. 1b). This zone may contain fibrils and electron-dense granules. *Prochloron* cells are structurally comparable to the photosynthetic machinery of green plastids, having the thylakoids placed in the cytoplasm of the bacterial cells (Fig. 1). This feature forms the bases for the assumption that *Prochloron* is similar to the **symbiotic bacteria** which evolved into the first chloroplast within the autotrophic eukaryotes. Land plants like green algae also have chl *b* containing chloroplast surrounded by a double membrane. Hence the whole green lineage can be assumed to have developed originally by the endosymbiosis of *Prochloron*-like prokaryotic cells (Fig. 2). The light harvesting pigment composition of *Prochloron*

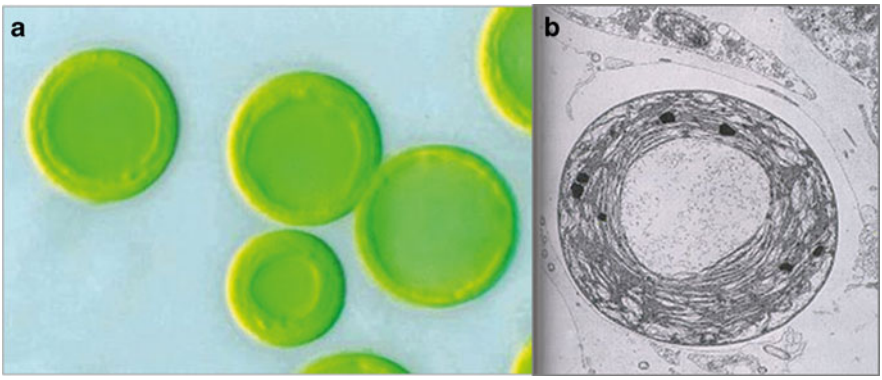


Fig. 1 (**a**) Light micrograph of *Prochloron* cells. (**b**) Transmission electron micrograph of *Prochloron* cells. These cells are structurally comparable to the photosynthetic machinery of green plastids, having the thylakoids placed at the periphery in the cytoplasm of the bacterial cells (Image courtesy: **a** College of Biological Sciences, University of Tsukuba. **b** College of Arts and Science, Miami University)

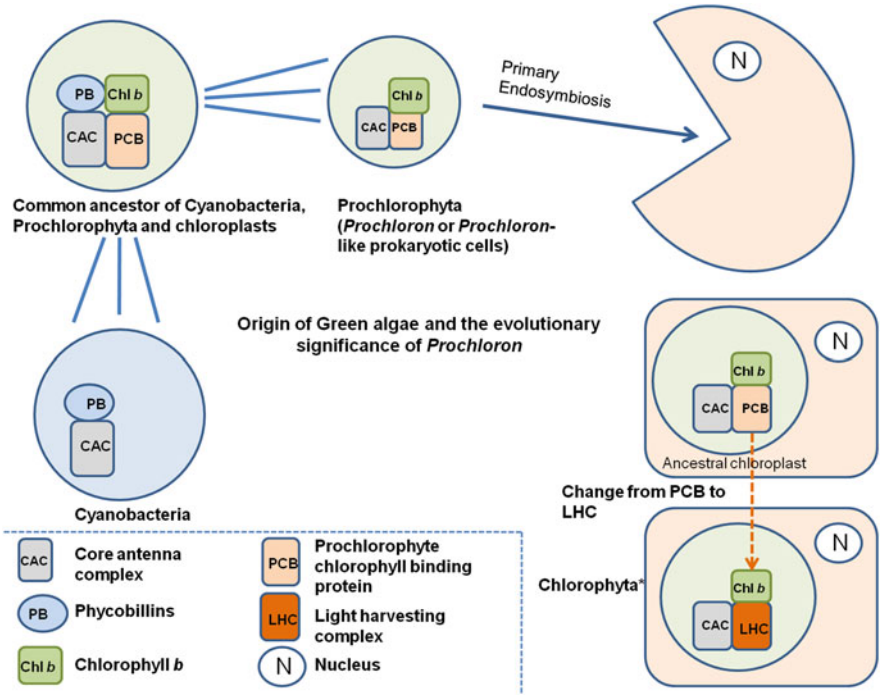


Fig. 2 Endosymbiotic origin of chloroplast: primary endosymbiosis involving a **non-photosynthetic** eukaryote and a ***Prochloron*- like cyanobacterium** containing core antenna complex and chl *b*, chloroplast in green algae is structurally comparable to the *Prochloron* like cyanobacterium. The outer membrane of the chloroplast envelope represents the vacuolar membrane of the host cell and the inner membrane represents the cell membrane of the cyanobacterium (Adapted from Tomitani et al. 1999)

has similarity with both cyanobacteria and chlorophytes. The unique photosynthetic machinery of *Prochloron* among cyanobacteria make them fascinating to scientists who investigate the evolution of the characters in photosynthetic organisms.

3 Systematics of Green Algae

The classification of algae into taxonomic groups relies upon their morphological, ultrastructural, and molecular characters. Classification of green algae has undergone considerable changes over the years. Earlier workers entirely relied upon colour and form. Later, cytological information and types of life histories were added. The development of further techniques helped in a wider understanding of the cell wall, flagellar structures and the physiology and biochemistry of functions, all of which proved useful in classifying different taxa. The introduction of molecular studies which are especially comparative provided a new framework for the phylogenetic relationships among the green lineage. For many algal groups, form alone can still be used for differentiation. For some algae form combined with function will prove useful. A detailed knowledge of the life-history is essential to understand the concept of taxa. Molecular, physiological and ecological characters likewise are useful in the systematics of green algae.

From early times algae have been variously classified. As far back as 1836, Harvey recognized three algal groups- Chlorospermae, Melanospermae, Rhodospermae and placed all green algae into Chlorospermae. Subsequently many flagellates were discovered and described and even today changes are being made to accommodate these forms properly. Some of the important systems of classification of algae are those of Engler and Gilg (1924), Fritsch (1935), Smith (1938), Round (1963, 1965, 1971, 1984), Prescott (1969), Chapman and Chapman (1973), Stewart and Mattox (1975), Pickett-Heaps (1975), Bold and Wynne (1978, 1985), and Lee (2008).

Fritsch (1935) classified algae into 11 classes and placed all green algae in the class **Chlorophyceae** (Fig. 3). Fritsch sub-divided Chlorophyceae into nine orders, Volvocales, Chlorococcales, Ulotrichales, Cladophorales, Chaetophorales, Oedogoniales, Conjugales, Siphonales, and Charales. However majority of modern phycologists favour the independent status for Charophytes. Fritsch considered the following characters while classifying algae: (i) Structure of plant body (ii) Habitat or occurrence (iii) Nature of photosynthesizing pigment (iv) Type of reserve food material (v) Method of reproduction etc.

In newer classifications (Fig. 4), Green lineage refers to the two clades making up the Viridiplantae (Leliaert et al. 2012). Viridiplantae has two clades, Chlorophyta and Streptophyta (Streptophyta includes land plants and Charophyte green algae). Hence green algae refer to the Chlorophytes and Charophytes. Chlorophyta includes the prasinophytes and core chlorophytes. Prasinophytes represent the early diverging chlorophytes. Ulvophyceae, Trebouxiophyceae and Chlorophyceae are together known as 'UTC clade'. The three major classes of the UTC clade along with Chlorodendrophyceae are considered as 'The Core Chlorophyta'. Chlorodendrophyceae represents the early diverging core chlorophytes. Chlorodendrophyceae is a small class of green algae, comprising the genera *Tetraselmis* and *Scherffelia* (Massjuk and Lilitska 2006; Leliaert et al. 2012).

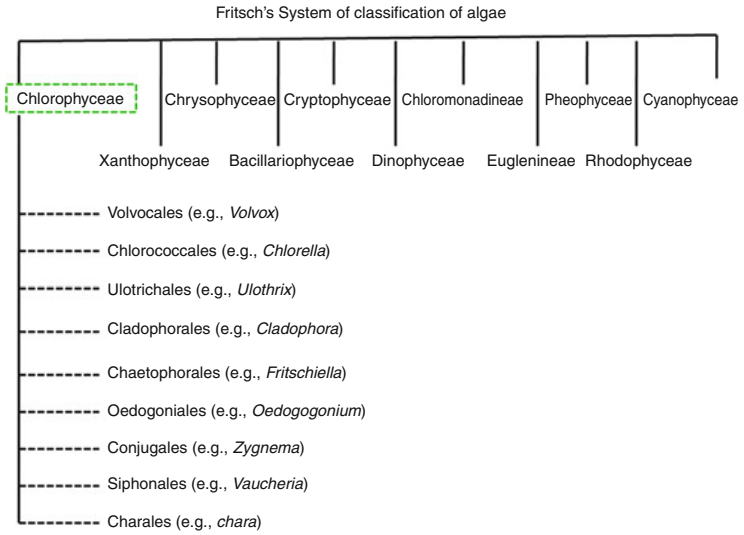


Fig. 3 Fritsch system of classification detailed for Chlorophyceae

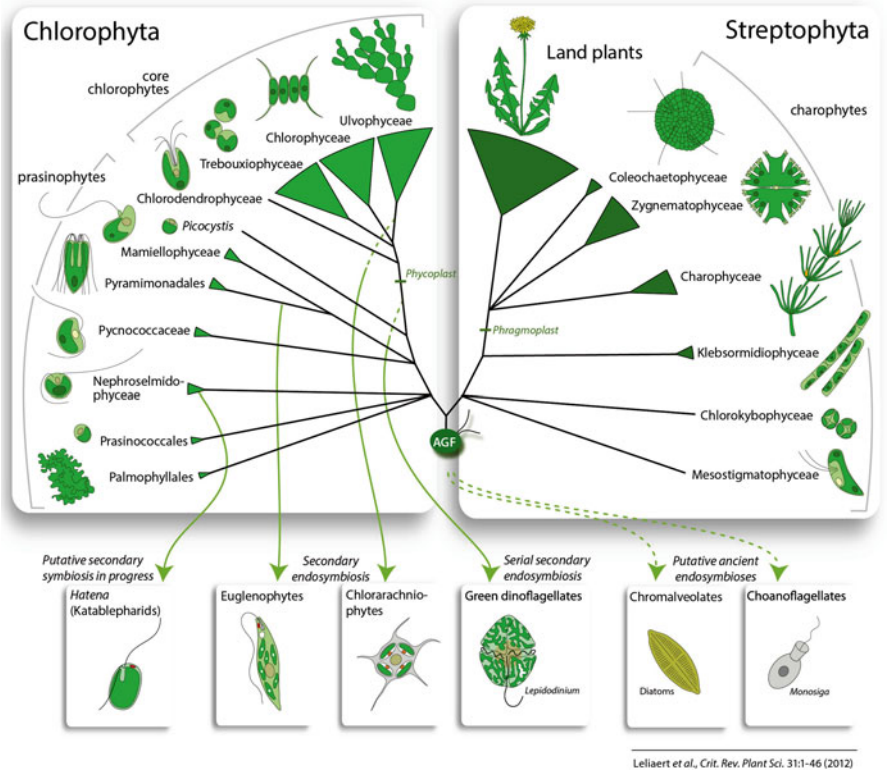


Fig. 4 Chlorophyta and Streptophyta, the Chlorophyta comprises most of the described species of green algae. The Streptophyta includes charophytes, a few freshwater algae, and the land plants (Picture courtesy: Dr. Frederik leliaert) Figure from Leliaert et al. (2012)

4 Occurrence

Most of the green algae are aquatic and are predominantly freshwater; only about 10 % of the aquatic forms are marine. Some are terrestrial as well.

4.1 Freshwater Aquatic Forms

These algae grow in river streams, lakes, ponds, puddles, ditches and other kinds of freshwater bodies (Fig. 5a). Most of the Ulotrichales have freshwater forms. Oedogoniales have exclusively freshwater forms. Among the freshwater aquatic forms, *Spirogyra* and *Hydrodictyon* frequently form noticeable but harmless blooms that may cover the surface of freshwater ponds. Many species of *Chlamydomonas, Volvox* and *Chlorella* are frequently found in freshwaters. *Chara* is also found in fresh water, particularly in the water flowing over limestone areas, where it grows submerged and remains attached to the muddy bottom.

4.2 Terrestrial and Subaerial Forms

These forms grow on moist aerated soils, rocks and cliffs (Fig. 5b). *Fritschiella* grow on moist acidic soil whereas *Stichococcus, Hormidium* and *Chlorella* are found on loam cultivated soils. Sub aerial forms occur on moist tree trunks, moist walls, rocks and other moist situations. Species of *Ulothrix*, and *Zygogonium* are sub-aerial, and are usually found on damp soil in the form of sheets. *Trentepohlia* forms orange-red growths on moist rocks or cliff faces due to the accumulation of carotenoid pigments which obscure chlorophyll.

4.3 Marine Forms

These algae occur in marine waters (Fig. 5c, d). Most Chlorodendrophyceae (*Tetraselmis, Scherffelia*) are found as planktonic (adapted to floatation) organisms in marine environments, where they sometimes occur in dense populations causing blooms in tidal pools or bays. Members of Caulerpales are predominantly marine. A few members commonly occur in near shore marine environments forming nuisance macroscopic growths e.g. *Ulva* (sea lettuce), *Codium* (dead man's fingers), *Enteromorpha, Cladophora*.

Fig. 5 (**a**) Freshwater aquatic. (**b**) Terrestrial/subaerial. (**c**) Marine planktonic. (**d**) Marine benthic. (**e–g**) Specialized habitats: (**e**) *Chlorella* cells inside the cytoplasm of *Paramecium bursaria* (**f**) *Cephaleuros virescens*, a parasitic alga causing red rust on tea leaves. (**g**) *Chlamydomonas nivalis,* an alga that accumulates red carotenoid pigments and causes "Watermelon snow"- red colour of the snow is due to *Chlamydomonas nivalis* (Image courtesy: **b** Lee Nachtigal. **e** Prof. Yuuji Tsukii, Hosei University, Japan. **f** Dr. Joey Williamson, Horticulture Extension Agent, Home & Garden Information Center, Clemson University)

4.4 Specialized Habitats

Species of *Protococcus* and *Trentepohlia* are epiphytic on sea weeds or on the bark of trees, whereas *Chlorella* is endophytic i.e. they live inside a host organism (Fig. 5e). *Cephaleuros* and *Rhodochytrium* are parasitic, and they lack green colour. *Cephaleuros* is a parasitic form which causes red-rust disease of tea leaves (Fig. 5f). Some species occur in unusual habitats; for example, *Chlamydomonas yellowstonensis, Chlamydomonas nivalis* and *Haematococcus nivalis* occur on the snow covered mountain tops and can impart a characteristic colour (which varies from red to orange to yellow) to the snow due to the accumulation of carotenoid pigments. *Chlamydomonas nivalis* is cryophilic (cold loving) and flourishes in freezing waters. It contains a red carotenoid pigment known as astaxanthin in addition to chlorophyll and imparts a red colour to the snow referred to as watermelon snow (Fig. 5g). Some green algae in association with certain fungi form lichens. Some species have been described as endosymbionts of marine animals, including *Tetraselmis convolutae* which is a facultative symbiont of the acoel flatworm *Symsagittifera roscoffensis*, and an undescribed *Tetraselmis* species that has been isolated from the radiolarian *Spongodrymus*.

5 Range of Thallus Organization in Green Algae

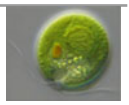
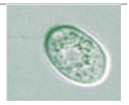
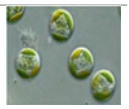
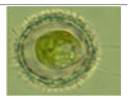
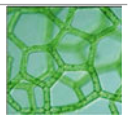
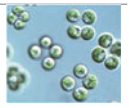
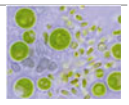
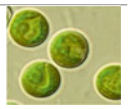
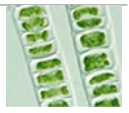
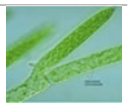
Green algae are a heterogenous group exhibiting a wide range in their thallus structure and morphology beginning from simple microscopic motile unicellular forms through multicellular flagellated or non flagellated colonies, palmelloid forms, dendroid forms, filamentous forms, heterotrichous forms, siphonous forms, well developed parenchymatous thalli to a thallus with well differentiated tissues which bear leaf and stem like structures and resemble land plants (Table 1 and Fig. 6). Unicellular thallus is the simplest type of construction within green algae.

5.1 Unicellular Forms

Unicellular forms may be motile or non-motile.

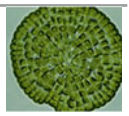
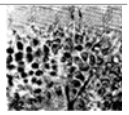
(i) *Unicellular motile thallus:* In terms of organisation of thallus, unicellular motile forms of algae are the simplest. Vegetative cells have two or four flagella and are motile (e.g., *Chlamydomonas, Tetraselmis, Sphaerella*)
(ii) *Unicellular non-motile thallus:* These cells do not possess flagella, eyespot etc., meant for locomotion. (e.g., *Chlorella*)

Table 1 Thallus organization in different genera

Thallus organisation	Genera	
Motile unicells	*Chlamydomonas*	
Motile colourless unicells	*Polytoma*	
Nonmotile unicells	*Chlorella*	
Encapsulated unicells	*Phacotus*	
Motile colony	*Pandorina*	
Dendroid colony	*Tetraselmis*	
Nonmotile net like colony	*Hydrodictyon*	
Palmelloid colony	*Tetraspora*	
Coccoid (Zoosporic)	*Chlorococcum*	
Coccoid (Azoosporic)	*Chlorella*	
Simple filament	*Ulothrix*	
Branched filament	*Cladophora*	
Heterotrichous filament	*Stigeoclonium*	

(continued)

Table 1 (continued)

Thallus organisation	Genera	
Discoid (prostrate type)	*Coleochaete*	
Crusts or cushions	*Pseudopringsheimia*	
Erect type	*Draparnaldia*	
Pseudoparenchymatous (uniaxial)	*Dasycladus*	
Pseudoparenchymatous (multiaxial)	*Codium*	
Foliose, parenchymatous	*Ulva*	
Tubular, parenchymatous	*Enteromorpha*	
Plant like thallus with dimorphic branches	*Chara*	

(Picture courtesy: *Chlamydomonas*- Mr. Wolfgang Bettighofer; *Cladophora*, *Stigeoclonium*, *Draparnaldia*- Prof. Yuuji Tsukii, Hosei University, Japan; *Coleochaete*- Dr. Linda Graham; *Dasycladus*- Dr. S. Berger; *Ulva*- Dr. Kjersti Sjøtun)

5.2 Colonial Forms

The colonial habit is achieved by aggregation of the products of cell division within a mucilaginous mass, by aggregation of motile cells or juxtaposition of cells subsequent to cell divisions.

(i) *Coenobium:* A colony with a definite shape, size and arrangement of cells. The number of cells in a coenobium is determined at the juvenile stage and subsequently there is only increase in size. Coenobia may be motile with flagellated

Fig. 6 Range of thallus structure in Chlorophyta. (**a**) *Pterosperma* (**b**) *Nephroselmis* (**c**) *Palmophyllum* (**d**) *Tetraselmis* (**e**) *Chlorella* (**f**) *Oocystis* (**g**) *Haematococcus* (**h**) *Pediastrum* (**i**) *Bulbochaete* (**j**) *Chaetophora* (**k**) *Ulothrix* (**l**) *Ulva* (**m**) *Cladophora* (**n**) *Boergesenia* (**o**) *Acetabularia* (**p**) *Caulerpa* (**q**) *Klebsormidium* (**r**) *Spirotaenia* (**t**) *Micrasterias* (**u**) *Coleochaete* (Photo courtesy: Dr. Frederik Leliaert; for photo credits see Leliaert et al. 2012) Figure from Leliaert et al. (2012)

cells (e.g., *Eudorina, Pandorina, Volvox*) or non-motile having cells without flagella (*Pediastrum, Hydrodictyon*).

(ii) *Palmelloid:* In contrast to coenobial forms, in a palmelloid colony the number of cells, their shape and size is not definite. The cells remain irregularly aggregated within a common mucilaginous matrix, but they are independent and function as individuals. In some palmelloid forms it is a temporary phase (e.g. *Chlamydomonas*), whereas in others it is a permanent feature (e.g., *Tetraspora*)

(iii) *Dendroid:* The colony appears like a microscopic tree. The number, shape and size of cells is indefinite and a mucilaginous thread is present at the base of each cell. Threads of different cells are united to form a branched structure (e.g., *Ecballocystis*).

5.3 Filamentous Forms

(i) *Filamentous unbranched:* Simple unbranched filaments may be free floating (e.g., *Spirogyra*) or attached to some substratum (e.g., *Ulothrix, Oedogonium*)
(ii) *Filamentous branched:* In *Cladophora* simple branched filaments remain attached to the substratum by a basal cell. In such filaments branches arise just below the septa between any two adjacent cells except the basal cell.
(iii) *Heterotrichous:* The thallus is very much evolved and differentiated into prostrate and erect systems (e.g., *Fritschiella, Coleochaete, Stigeoclonium, Draparnaldiopsis*). Both the prostrate and the erect systems may be well developed or there is progressive elimination of prostrate or erect systems.
(iv) *Siphonaceous:* The thallus is made up of branched, aseptate, coenocytic, tubular filaments as the nuclear divisions are not accompanied by wall formation (e.g. *Protosiphon, Codium*)
(v) *Parenchymatous:* Thallus is formed by the divisions of cells in two or more planes. The daughter cells do not separate from the parent and give rise to parenchymatous thalli of various shapes (e.g., *Ulva, Enteromorpha*). Leaf like or foliaceous thallus as seen in *Ulva* has evolved due to transverse as well as longitudinal septation in the filament.
(vi) *Complex forms:* The thallus is highly developed with well differentiated tissues. Plant looks like Angiosperm because of the presence of leaf and stem like structures (e.g., *Chara*).

This designates that there is a progressive evolution in the vegetative thallus of growth forms from simple (unicellular) to complex (multicellular) forms.

6 Cell Structure of Green Algae

Green algae are eukaryotic algae with an organized (membrane enclosed) cell nucleus containing DNA and organised plastids (chloroplasts) and all the cell organelles such as mitochondria, Golgi bodies, endoplasmic reticulum, and true vesicles.

The cells are often delimited by a cell wall of nonliving material i.e., polysaccharides that are partly produced and secreted by the Golgi body. The protoplast is bounded by a thin and semipermeable plasma membrane and this membrane is a living structure. The protoplast is characterized by the presence of a definite nucleus, a distinct cytoplasm, membrane bound chloroplast, mitochondria, dictyosomes, endoplasmic reticulum and vacuoles. The cytoplasm contains 80 S ribosomes and lipid bodies. The flagella have a precise number and orientation of microtubules. The streaming movements in the cytoplasm are of frequent occurrence.

6.1 Cell Wall

Except naked flagellates, zoospores and gametes, in most members of Chlorophyceae the cytoplasm is bounded by a definite cell wall (Fig. 7). Cell walls usually have cellulose as the main structural polysaccharide, although xylans and mannans often replace cellulose in some members. The submicroscopic morphology of the cell wall shows a fibrillar structure composed of 30–200 Å wide cellulose microfibrills embedded in a smooth or slightly granular matrix. The microfibrils composed of cellulose are either laid down in two layers at right angles to each other (as shown in the ultramicrograph of a green algal cell) or in three layers, the third layer running in an obtuse angle to the other two. Algae in Chlamydomonadales have walls composed of glycoproteins (Goodenough and Heuser 1985).

6.2 Mucilages

Algal mucilages are mainly constituents of the continuous amorphous phase of cell walls. Mucilages have been examined from a number of genera in Chlorophyceae. Polysaccharides containing rhamnose, galactan sulphate and uronic acid have been isolated. Some mucilages have highly branched polysaccharides found to yield galactose, mannose and arabinose on hydrolysis.

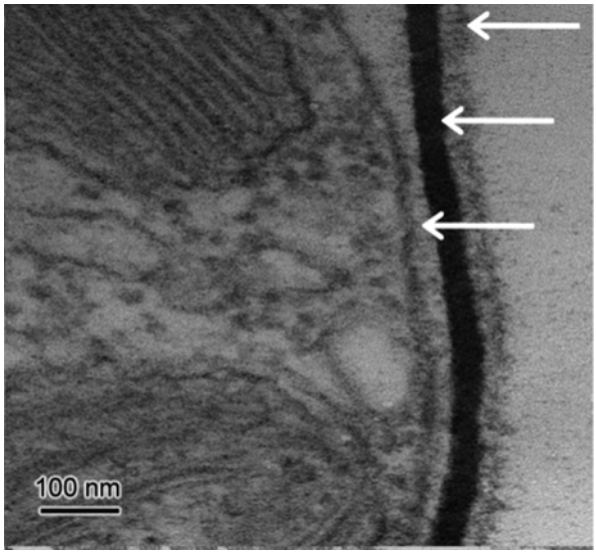


Fig. 7 Two layers of the cell wall in *Tetraselmis indica* (Chlorophyta) (Represented by *arrowheads*, the third *arrow* points towards the plasmalemma). Inner layer appears more electron dense than the outermost layer (Arora 2011)

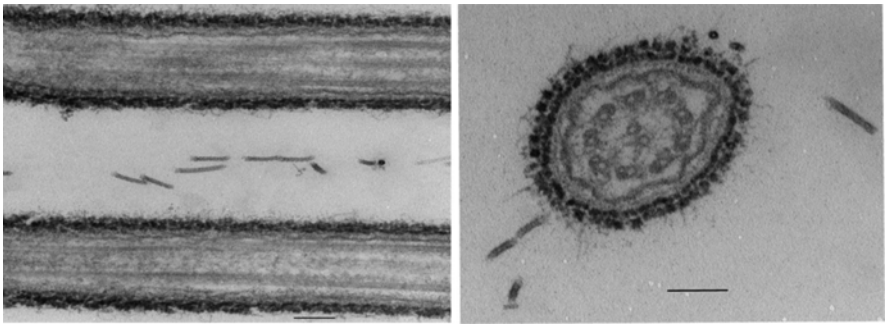


Fig. 8 The longitudinal and a transverse section of flagella from a green alga, 9+2 arrangement of microtubules can be seen in the transverse section (Arora 2011)

6.3 Flagella

In Chlorophyceae, the motility of cells is due to small protoplasmic whiplike threads called flagella. They function as locomotory structure of the cell. The number of flagella varies from one to four to many. Flagella are of equal length and are inserted at the anterior or apical end of the motile cells. The flagella usually have a smooth surface and hence are of whiplash type. There is a single granule at the base of each flagellum. It is known as basal body or blepharoplast. Each flagellum consists of a thin axial filament of axoneme surrounded by a cytoplasmic membrane or sheath. In a transverse or cross section the axoneme consists of 11 (9 peripheral + 2 central) microtubules. Two of these are situated in the centre and are called central tubules. They are single, consisting of 13 protofilaments each and lie side by side. These central microtubules are surrounded by nine, peripheral, doublet microtubules arranged in a circle. Each peripheral doublet microtubule consists of A and B tubules: the A tubule is a complete microtubule with 13 protofilaments, whereas the B tubule have 11 protofilaments. All the peripheral doublet microtubules are surrounded by a common cytoplasmic sheath (Fig. 8).

6.4 Nucleus

Cells in Chlorophyceae have a well organized nucleus (Fig. 9). The number of nuclei per cell varies. Both uninucleate and multinucleate cells have been reported in Chlorophyceae. In uninucleate algae nucleus usually lies alongside the cell wall but sometimes it is suspended in the centre of the cell by fine cytoplasmic threads (e.g., *Spirogyra*). Nuclear membrane is a double layered structure made up of proteins and lipids. The outer membrane is continuous with the membranes of endoplasmic reticulum. The two nuclear membranes are separated by a perinuclear space. Nuclear pores provide aqueous channels through the nuclear envelope and

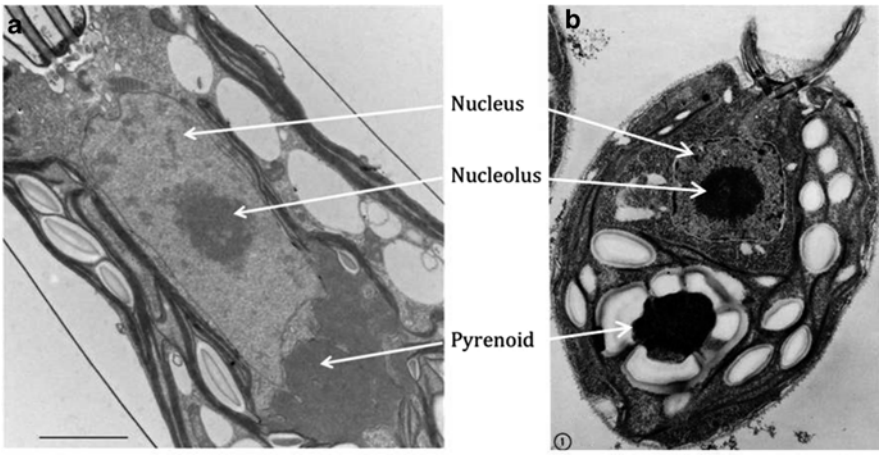


Fig. 9 Ultramicrographs of green algae (**a**) *Tetraselmis indica* (**b**) *Chlamydomonas* sp., showing nucleus, nucleolus and pyrenoid

are composed of nucleoporin proteins. Each nucleus contains one or more pronounced nucleoli. Each nucleolus is made up of RNA and proteins.

6.5 Mitochondria, Golgi Bodies, Endoplasmic Reticulum and Contractile Vacuoles

Mitochondria, Golgi bodies and endoplasmic reticulum have been observed in members of Chlorophyceae (Fig. 10). Mitochondria are bounded by a double membrane, inner one projecting into the lumen in the form of many folds. They function as sites of enzyme action. In some members, the mitochondria have plate-like cristae which are long extensions of inner lamella. Golgi bodies or dictyosomes are encountered in a number of green algae. In *Chlamydomonas*, they are found in the region of the nucleus, whereas in some algae they are associated with the flagellar base. Golgi bodies are composed of stacks of flat vesicles. About 10–20 Golgi bodies can be seen in the cytoplasmic matrix without any apparent association with any particular organelle. The endoplasmic reticulum (ER) of green algae traverses the ground substance of the cells. The ER in *Chlamydomonas* has various membranous elements and is concentrated mostly in the anterior half of the cell. The system does not penetrate either the chloroplast or the pyrenoid. The membrane of the ER is often studded with small particles (ribosomes). Mature cells of most of the Chlorophyceae possess one or more vacuoles. Each vacuole is bounded by a distinct membrane, called tonoplast. Some of the vacuoles may be contractile and are considered to be excretory in function. These vacuoles show periodic contractions and throw out the waste products of the cell.

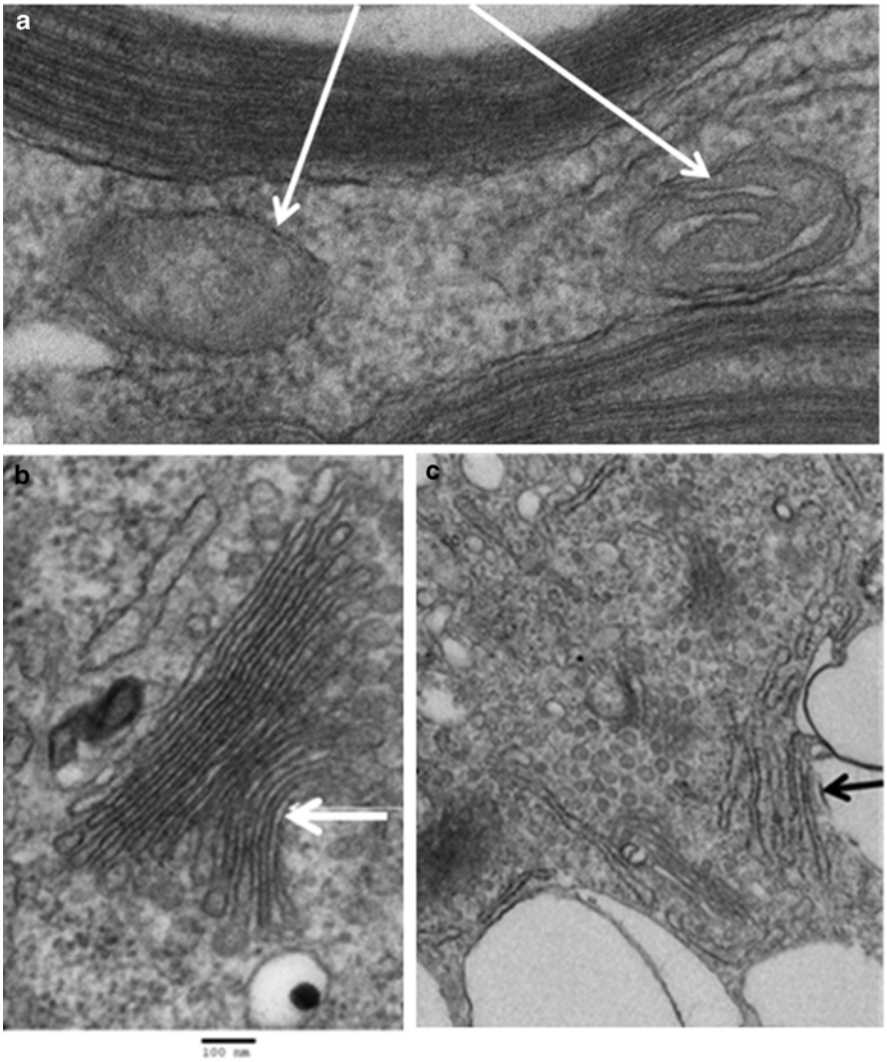


Fig. 10 (**a**) Electron micrograph of mitochondria bounded by double membrane and the inner membrane is projecting into the lumen in a Chlorophycean alga, (**b**) Electron micrograph of Golgi bodies composed of stacked vesicles in a Chlorophycean alga, (**c**) Electron micrograph of endoplasmic reticulum in a Chlorophycean alga (Arora 2011)

6.6 Chloroplast

Chloroplasts are autonomous cytoplasmic bodies containing the complete cellular apparatus needed for photosynthesis. Each chloroplast is surrounded by a double membrane system. In all chloroplasts internal membrane system is embedded in a matrix called stroma. The internal membranes are actually closed, flattened sacs termed thylakoids. The thylakoids of green algae are much larger than those of

higher plants. In green algae they are stacked in groups of 2, 3 or 4. Chloroplast consists of a matrix or stroma containing a number of small discs or thylakoids. The thylakoids contain all the chlorophyll pigment of the chloroplast and are made up of proteins and lipid substances. The stroma contains small ribosomes and osmiophillic globules. The chloroplasts in green algae exhibit remarkable variation in shape and size (Fig. 11). They are either axile (situated along the central axis of the cell) or parietal and can be grouped as follows:

(i) *Cup shaped:Chlamydomonas, Chlorella*
(ii) *Stellate (star shaped):Cylindrocapsa involuta, Zygnema*
(iii) *Laminate (in the form of a thin plate or sheet extending full cell length):Mougeotia*
(iv) *Parietal, Girdle-shaped (Partially or fully encircles the cell circumference): Ulothrix*
(v) *Reticulate (Mesh like network):Oedogonium, Hydrodictyon*
(vi) *Spiral (Chloroplast is ribbon shaped and spirally arranged):Spirogyra*

6.7 Pyrenoid and Eyespot

In green algae starch is formed within the chloroplasts, in association with one or more distinct, rounded, proteinaceous body called pyrenoid. Pyrenoid consists of a central granular core surrounded by tightly packed starch plates (Fig. 12). Presence of starch grains within the chloroplast is a distinguishing feature of green algae. In some genera amyloplasts containing starch grains are present in the chloroplasts. Pyrenoids become reduced or diminish if the alga experience conditions of starvation and reappear once the conditions become favourable.

Pyrenoids are considered to be important components of the carbon concentrating mechanism (CCM) in algae. Pyrenoid is not a membrane-bound structure, though it has a definite physical outline and is usually surrounded by starch cells or starch plates. A large amount of enzyme RuBisCO (Ribulose-1, 5-bisphosphate carboxylase oxygenase) is located in the pyrenoid. In the pyrenoid CO_2 is concentrated around this RuBisCO, permitting the enzyme to work at a higher efficiency. Inflated tubule-like structures of the thylakoids that penetrate into the pyrenoid appear to be the path of CO_2 into the pyrenoid.

The motile vegetative or reproductive cells of algae have a small pigmented bright reddish or brownish red eye-spot or stigma (Fig. 13). It is usually associated with the chloroplast. It is considered as a photoreceptive organ. It consists of a curved pigment plate carrying the pigment and a biconvex hyaline lens in front. The motile vegetative and reproductive cells of *Chlamydomonas* have a small pigmented bright reddish or brownish red eye-spot or stigma which senses light. Eyespot is considered as a photoreceptive organ or "eye" of the green algal cell and is involved in phototaxis (light dependent movement responses). It can be easily seen in the light microscope because of the huge accumulation of carotenoids. Using electron microscopic studies it has been revealed that in a vertical section the eye-spot consists of one to four rows of globules. Each row comprises closely packed globules containing carotenoid pigments.

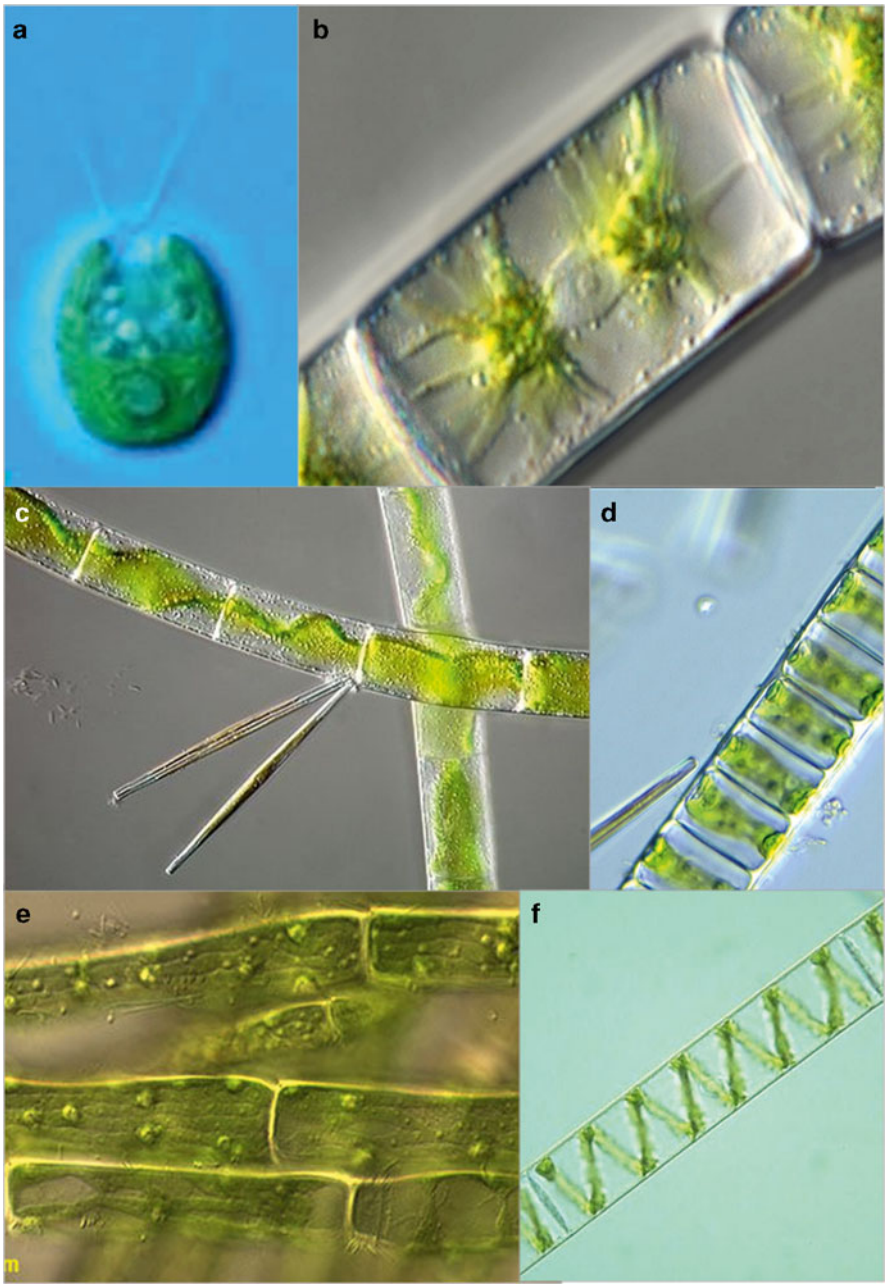


Fig. 11 Variation in the shape of chloroplasts in green algae. (**a**) Cup shaped, (**b**) Stellate, (**c**) Laminate, (**d**) Parietal, (**e**) Reticulate, (**f**) Spiral (Image courtesy: **a**, **e** Prof. David J. Patterson. **b**, **c** Mr. Wolfgang Bettighofer. **d** Jason Oyadomari)

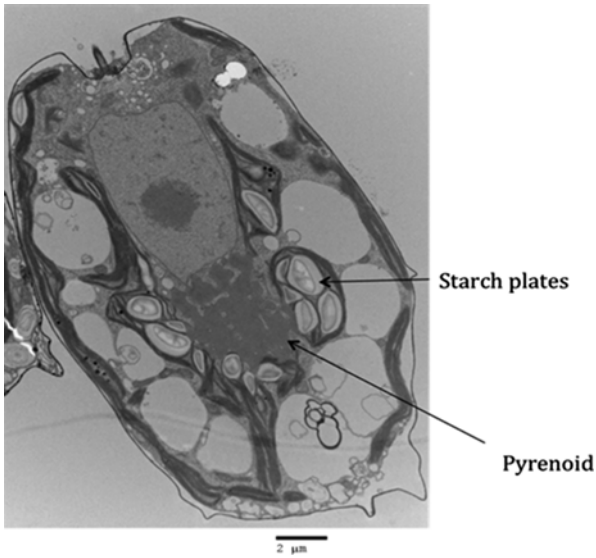


Fig. 12 The pyrenoid in longitudinal section of a green alga appears as a large, finely granular mass of polygonal profile surrounded by discontinuous shell of starch plates and penetrated by a system of tubules. Contractile vacuoles are also visible (Arora 2011)

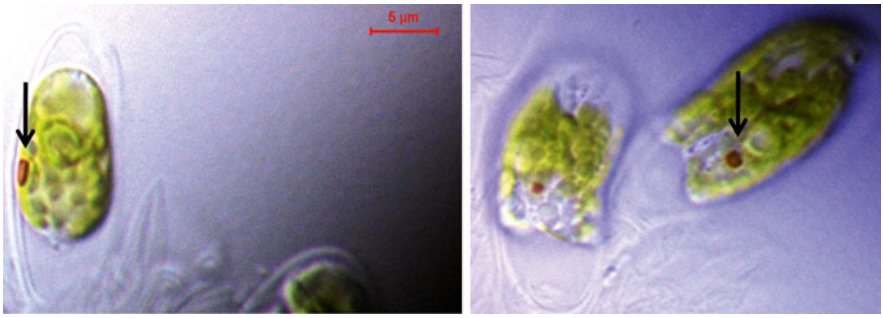


Fig. 13 Light micrograph of a green alga showing the presence of eyespot- marked by arrowheads (Arora 2011)

7 Pigments of Green Algae

Chlorophyll *a*, chlorophyll *b* and carotenoids have been described from green algae. Carotenoids are grouped into carotenes (Oxygen-free hydrocarbons) and xanthophylls (oxygen derivatives of carotenes). Both carotenes and xanthophylls are present in green algae.

Chloroplast pigments in green algae are similar to those of the higher plants; chlorophyll *a* and *b* are present. In all photosynthetic algae, chlorophyll *a* is the major pigment and there is no member known to lack this pigment. Chlorophyll *b*, lutein and β-carotene are important general accessory pigments of Chlorophyceae (Larkum and Howe 1997). Lutein is the main carotenoid. Some genera are found to

have siphonoxanthin and its esters siphonein (Yoshi et al. 2003). Carotenoid pigments also commonly occur outside the chloroplasts, especially in resting cells and in the terrestrial *Trentepohlia*, as well as in the eye-spots of the motile stages. These yellow or red coloured substances, known as haematochrome are represented by two or more carotenoid pigments. In many cases they occur dissolved in fat globules.

Accumulation of carotenoids occurs under conditions of nitrogen deficiency, high irradiance or salinity. This is particularly true in *Dunaliella* where β-carotene accumulates between thylakoids in the chloroplast, and *Haematococcus*, where astaxanthin accumulates in lipid globules outside the chloroplast (Hagen et al. 2000; Wang et al. 2003). Haematochrome is a general term for these carotenoids. Accumulation of haematochromes colour the cells orange or red, with haematochrome accumulating up to 8–12 % of the cellular contents in *Dunaliella* (Orset and Young 1999). Animals cannot synthesize carotenoids and they acquire the pigments through the food chain from primary producers. Haematochromes are responsible for the coloring in fish, crustaceans and birds (such as the pink flamingos).

The plastids of green algae and land plants lack the phycobilin accessory pigments and thylakoid bound phycobilisomes (a characteristic pigment of cyanobacteria, red algae and glaucophytes). No genetic traces of phycobilisomes have been found in green algae or land plant genomes.

8 Reproduction

There are three common ways of reproduction in green algae: vegetative, asexual and sexual. In addition several perennating bodies are also formed to face adverse environmental conditions. In the vegetative mode of reproduction, the algal body cuts off or break and gives rise to new individuals. This process is known as fragmentation. Vegetative reproduction also occurs by the formation of akinetes which are specially thickened vegetative cells. In the colonial *Dictyosphaerium* and in some filamentous forms, fragmentation is common. Multiplication by ordinary cell division is a characteristic feature of some Chlorophyceans. It is also called fission and is a common method of reproduction in unicellular forms (Fig. 14). Forms like *Pleurococcus* and desmids are characterized by multiplication by ordinary cell division.

Asexual reproduction by zoospores is widespread in *Ulothrix, Chlorococcum*. In some cases, the zoospores are non-motile and are known as arrested zoospores or aplanospores. These spores in some forms have thickened wall and are capable of enduring prolonged desiccation and these are called hypnospores. In a number of algae, production of zoospores never takes place, although reproductive cells are formed. The cell contents divide and new cell walls are formed around the divided protoplasts. These cells acquire all the distinctive features of the parent, while still enclosed in the parent cell. These bodies are known as autospores. The formation of autospores is common in Chlorococcales.

Sexual reproduction in green algae takes place by fusion of gametes (Fig. 15) or by exchange of genetic information through conjugation. Available evidences indi-

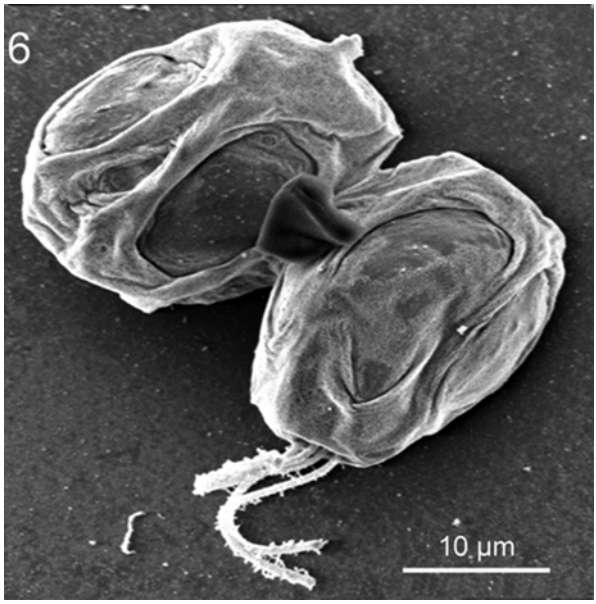


Fig. 14 Cell division in a green alga by fission (Arora et al. 2013)

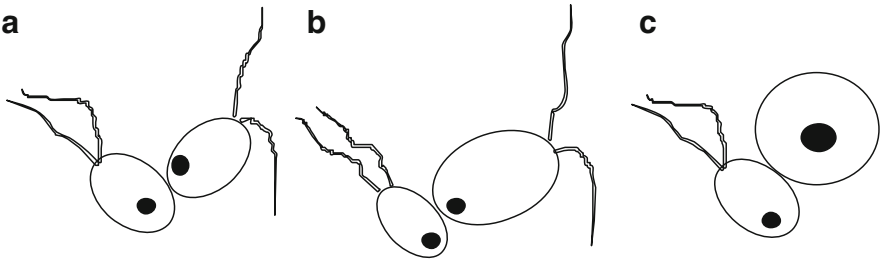


Fig. 15 Various types of sexual reproduction in Chlorophyceae: (**a**) Isogamy, (**b**) Anisogamy, (**c**) Oogamy

cate that sexuality in green algae is largely controlled by environmental factors such as light, temperature and nutrition. In *Chlamydomonas and Pandorina*, sexually active cells appear at the end of exponential phase. In certain cases depletion of nitrogen results in the formation of sexual gametes, whereas a rapid loss of sexuality occurs as a result of high concentrations of nitrogen. Temperature has a remarkable influence on mating. Under certain conditions, a rise in temperature increases the proportion of gametes in the population of *Chlamydomonas*. In addition, light also plays an important role in inducing sexuality. In *Pandorina morum* subjected to 16:8 h light-dark period, mating occurs only during a certain time span of light period. It has been suggested that light acts through photosynthetic assimilation leading to the depletion of the available nitrogen supply in the medium, which triggers sexuality. The production of gametes in light also requires oxygen. A rapid reduction in the

proportion of active gametes has been observed in *Chlamydomonas eugametos*. In oogamous chlorophytes like *Oedogonium,* active egg substances attractive to the spermatozoids have been observed. Mating involves a certain degree of clumping of flagellum to flagellum, as in *Chlamydomonas.* The clump inducing substance in *Chlamydomonas eugametos* has been shown to be glycoproteins. Temperature also has a critical effect in clumping.

9 Importance of Green Algae

Green algae have played a crucial role in the global ecosystem for hundreds of millions of years (Falkowski et al. 2004; O'Kelly 2007; Leliaert et al. 2011). The structure and function of marine ecosystems are significantly dependent on green algae and these organisms are accountable for the majority of the flux of organic matter to higher trophic levels and the ocean interior (Falkowski et al. 2004). They generate oxygen through photosynthesis and sequester large amounts of atmospheric CO_2 in the ocean interior and provide food for other organisms. Due to their role as primary producers in aquatic food webs, green algae have a high ecological relevance. The liberation of extracellular products by green algae is of quite ecological importance in many ways. In most simple cases, the nutrient cycle is short circuited, i.e., photosynthetic products of algae liberated by healthy cells are used directly by bacteria and some animals as food material. Extracellular products such as organic acids and polysaccharides are utilized quickly (Fig. 16).

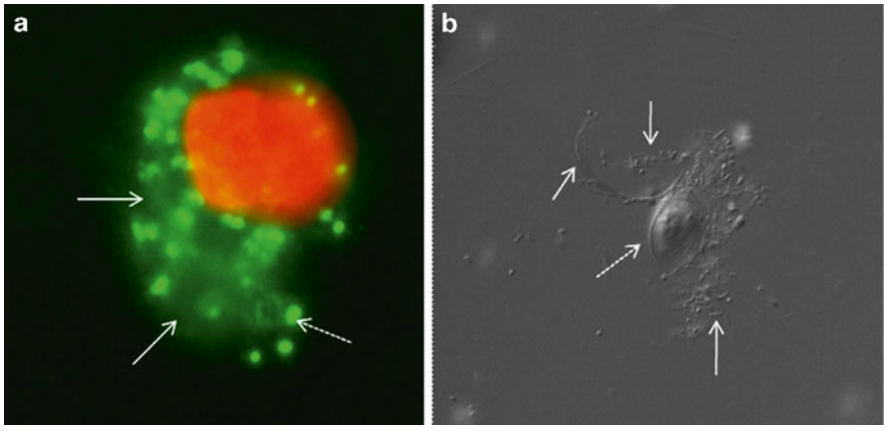


Fig. 16 (**a**) Epifluorescent micrograph showing the attachment of bacteria to the discarded cell wall of *T. indica*; *solid arrows* point towards the cell wall and the dotted arrow shows bacterial cells. (**b**) Phase contrast micrograph of the discarded and disintegrating cell wall showing the presence of bacteria; *solid arrows* point towards the disintegrating cell wall and bacteria and the *dotted arrow* shows a live algal cell (Arora et al. 2012)

Land plants have evolved from a green algal ancestor and this key event has led to spectacular transformations in the earth's environment, beginning the development of the whole terrestrial ecosystem (Kenrick and Crane 1997).

Some of the green algae such as *Chlamydomonas, Chlorella, Volvox, Acetabularia, Halicyctis* etc. are also being used as model organisms in laboratories. A few well-known Green algae are: *Ostreococcus taurii* (the smallest known free living eukaryote), *Chlorella* (used by Melvin Calvin for the elucidation of light-dependent reactions of photosynthesis -Calvin cycle), *Acetabularia* (was used by Joachim Hammerling for the transplantation experiments to demonstrate the role of nucleus in carrying genetic information and cellular development), *Chlamydomonas reinhardtii* (used as a model system for studying chloroplast biogenesis, photosynthesis, flagellar assembly and function, cell cycle control and circadian rhythms), the colonial green alga *Volvox* (has served as a model for the evolution of multicellularity, cell differentiation, and colony motility) (Kirk 1998, 2003; Herron and Michod 2008; Herron et al. 2009), *Mougeotia* (has been used in elucidating the role of phytochrome in plant development). *Halicystis* has been found to be suitable for experiments on membrane permeability.

Practical utilization of green algae includes a diverse array of fields including algal culture and seaweed farming, in making commercial products such as carotenoids, hydrocolloids, and pharmaceuticals, as soil conditioners to improve the physical qualities of the soil, in biofertilizers, in the treatment of wastewater, as environmental indicators, in reducing CO_2 emissions, in renewable energy production and environmental bioremediation. The commercial production of green algae including green seaweeds and the products derived from them is a huge and well established industry. Novel algal species along with new and improved yield of products and with effective bioremediation property are being continuously developed. Selection of algal genera and species and their genetic manipulation leads to a high production of desired chemicals (e.g. biofuel) or activity (e.g. bioremediation, sewage reclamation), hence creating a new biotechnological field. On the other hand the uncontrolled growth of algae can cause pond epidemics, water fouling and fouling of marine vessels.

9.1 Green Algae as Food and Dietary Supplements

Ulva and *Chlorella* are generally used as food in different forms. *Ulva lactuca* is used as salad and soups in Scotland and Ireland. Many green algae produce a variety of bioactive compounds with nutraceutical value. *Chlorella* is very useful in terms of its nutritive value. Its nutritional value is comparable to that of a mixture of soybean and spinach. Many species of *Chlorella* are rich in lipids and proteins. It is very easy to culture *Chlorella* in the presence of light, carbon dioxide and minerals. The rate of growth is very rapid for this alga. *Chlorella* contains about 30 % carbohydrates, 30 % proteins and 15 % lipids. Freshwater *Chlorella* is a carotenoid rich alga used for food supplements or health foods. *Chlorella* is also important as

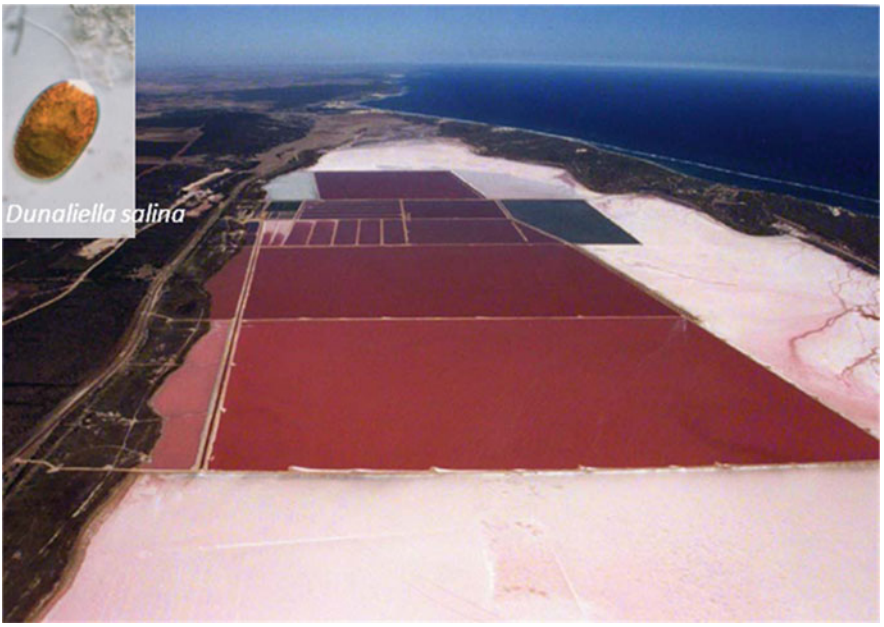


Fig. 17 *Dunaliella salina* production plant in Australia (operated by Cognis Nutrition & Health at Hutt Lagoon), it is the largest production plant of algae in the world

aquaculture feeds. *Chlorella* is of great use in space flights and nuclear submarines as food and also in recycling oxygen regenerating system. In Japan *Chlorella ellipsoidea* is used while mixing with tea. The carotenoids produced by algae are β-carotene by *Dunaliella salina* along with astaxanthin by *Haematococcus pluvialis*. Carotenoids are used in pharmaceuticals, food colourings and cosmetics. Accumulation of carotenoids occurs under conditions of nitrogen deficiency, high irradiance or salinity. This is particularly true in *Dunaliella* where β-carotene accumulates between thylakoids in the chloroplast, and *Haematococcus*, where astaxanthin accumulates in lipid globules outside the chloroplast. Haematochrome is a general term for these carotenoids. Accumulation of haematochromes colour the cells orange or red, with haematochrome accumulating up to 8–12 % of the cellular contents in *Dunaliella. Dunaliella salina* is the star amongst the commercially important algae and is being grown commercially in Israel, India, Australia and China (Fig. 17). It has a very high content β-carotene which is the precursor of Vitamin A.

Haematococcus pluvialis, in its life history has a red colored resting stage followed by a green swimming stage followed over again by a red resting stage. The alga is harvested after the reddening cycle and the red pigment astaxanthin is col-

lected. Astaxanthin also has numerous health benefits which include enhancing eye health, increasing muscle strength and endurance, skin protection from premature ageing UVA damage and inflammation, immune function and regeneration. Astaxanthin also protects corpse tissues from oxidative damage and has a capacity to fight free radicals many times than that of vitamin E. Tocopherols have been reported from several green algal genera i.e. *Chlorella protothecoides* and *Chlamydomonas reinhardtii*. In addition, green algae such as *Tetraselmis*, *Chlorella* and *Dunaliella* also synthesize omega-3 (ω-3) long chain polyunsaturated fatty acids (LC-PUFAs) to a certain extent. Green algae such as *Ulva* and *Enteromorpha* are rich sources of Vitamin B and C. The proteins of some green algae contain all essential amino acids required for human and animal nutrition. Green algae are also used as fish food. The fish larvae are reared in special tanks and small flagellate algae are served as food. *Tetraselmis* and *Chlorella* are widely used in aquaculture facilities as feed for juvenile molluscs, shrimp larvae and rotifers. After a few months fishes are transferred into a pond in which *Spirogyra, Ulothrix, Oedogonium, Enteromorpha* grow as fish food. These algae are very important in sustaining fish life in water because they liberate oxygen and remove carbon dioxide by the photosynthetic process.

9.2 Antibiotics

A variety of algae have been screened for antibiotics and some pharmacologically active compounds with antibacterial and antiviral activity have been isolated from *Chlorella* spp., *Chlamydomonas* spp., *Scenedesmus* spp., *Dunaliella primolecta, Cladophora.* The first such antibiotic was prepared form *Chlorella* and is called Chlorellin.

9.3 In Cosmetics

Some green algae are well recognized in the skin care products such as anti-ageing creams, emollients, in peelers or peel off masks, sun protection products, refreshing and regenerant care products (Fig. 18). For example, an extract from *Chlorella vulgaris* (Dermochlorella, France) stimulates collagen synthesis in skin, thus supporting tissue regeneration and wrinkle reduction. An ingredient from *Dunaliella salina*, shows the ability to stimulate cell proliferation and positively influences the energy metabolism of skin (PephaCtive, Switzerland).

9.4 In Sewage Disposal

Sewage reclamation is mainly an aerobic process and oxidation of organic compounds of sewage is brought about by certain bacteria. Lack of oxygen prevents their complete oxidation. Algae photosynthesize and release oxygen if light is

Fig. 18 Green algae in cosmetics

available to sewage oxidation tanks. The algae which thrive under such conditions are *Chlamydomonas, Pandorina, Chlorella, Scenedesmus* etc. Oxygen of sewage is enhanced to a great extent by these algae. This oxygen helps in complete oxidation of the sewage into simple and soluble inorganic compounds, which is a biological process.

When water bodies are polluted by domestic sewage or organic wastes, certain very specific algae such as *Chlorella pyrenoidosa*, *Chlamydomonas* etc. become noticeable. The high content of nitrogen generally encourages the algal forms like *Scenedesmus*. Therefore green algae are also an important part of biological monitoring programs meant for evaluating water quality.

9.5 As a Source of Renewable Energy

Green algae are of enormous interest in biofuel production because of their fast growth along with relatively high lipid and carbohydrate content. All these properties make them a brilliant source for biofuel production. Algae biofuel is one of the substitutes to fossil fuel. Hydrogen is used as a source of gaseous biofuel as it causes no pollution. Several government agencies are giving emphasis to make gaseous biofuel production from algae commercially viable. When water is subjected to photolysis it splits into oxygen, electrons and hydrogen ions (H^+). Hydrogen ions

are converted into hydrogen gas. Algae like *Chlorella vulgaris, Chlamydomonas reinhardtii* and *Scenedesmus* have the enzyme hydrogenase. This hydrogenase can be used effectively to produce hydrogen from water. *Botryococcus braunii* is the oil producing green alga. It produces large quantity of long-chain hydrocarbons which can provide as liquid or gaseous fuel. High lipid-containing strains of *Tetraselmis* have potential to be used in biofuel production.

9.6 To Reduce CO_2 Emissions

Green algae have significant potential for biological carbon dioxide fixation to reduce greenhouse gas emissions. A few green algal species have been characterized which can grow at low pH, high CO_2 concentrations. Algae bioreactors are used by some power plants to reduce CO_2 emissions (Fig. 19). Released CO_2 can be pumped into a tank or pond, on which the algae feed. The bioreactor can also be installed directly on a smokestack. Countries like US, China, South Korea, Canada have invested heavily on technologies to capture carbon with the help of algae to produce bio-fuels. The first CO_2 capture for algae biofuel has been built beside a large coal-fired power station in Australia. In the same way green algae has also become a solution for capturing carbon from vehicular emissions, for providing oxygen to soldiers in high peaks, and for reducing oceanic acidification. *Chlorella* is mainly being used for these purposes because of its rapid rate of photosynthesis and its ability to efficiently utilize the released CO_2 .

In addition *Chlorella, Spirogyra, Cladophora and Scenedesmus* have been found to absorb certain radioactive wastes. The mucilaginous species of *Chlorella* are utilized as soil conditioners. Rapid growth of *Zygogonium* reduces soil erosion on disturbed soils. In Japan *Spirogyra* is used in the manufacture of lens cleaning paper utilized for cleaning optical articles.

Fig. 19 Carbon reduction technology-Flue gas from the CO_2 emission plant provides carbon dioxide to feed algae in fuel tanks

Acknowledgement The authors are grateful to Dr. Frederik Leliaert for his valuable suggestions towards the preparation of this manuscript.

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Growth Forms and Life Histories in Green Algae

Mani Arora and Dinabandhu Sahoo

1 Chlamydomonas

1.1 Systematic Position

Fritsch (1935)	Bold and Wynne (1978)	Lee (2008)
Division– Algae	Division – Chlorophycophyta	Phylum –Chlorophyta
Class– Chlorophyceae	Class– Chlorophyceae	Class –Chlorophyceae
Order –Volvocales	Order –Volvocales	Order –Volvocales
Family– Chlamydomonadaceae	Family – Chlamydomonadaceae	Family – Chlamydomonadaceae
Genus–*Chlamydomonas*	Genus – *Chlamydomonas*	Genus–*Chlamydomonas*

1.2 Introduction

The name of the genus is derived from the Greek words "**chlamys**" (mantle) + "**monas**" (unit). *Chlamydomonas* is a unicellular motile freshwater green alga (Fig. 1). There are about 500 described species of *Chlamydomonas. Chlamydomonas*

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D. Sahoo, J. Seckbach (eds.), *The Algae World*, Cellular Origin, Life in Extreme Habitats and Astrobiology 26, DOI 10.1007/978-94-017-7321-8_5

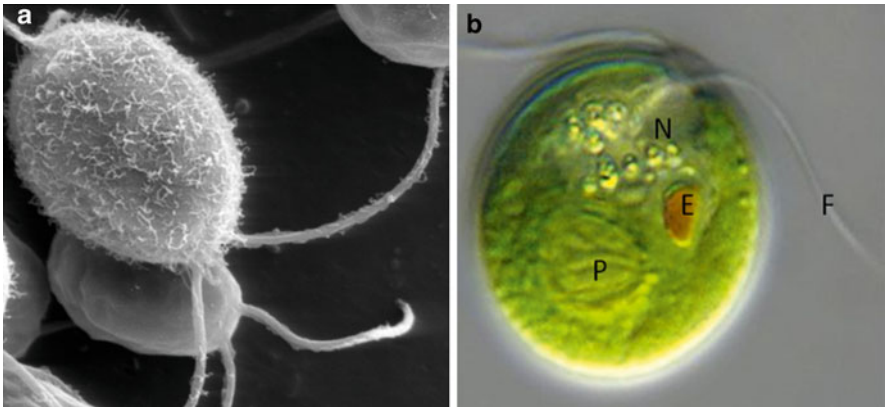

Fig. 1 (**a**) Scanning electron micrograph (SEM) of *Chlamydomonas* cells, (**b**) Light micrograph (LM) of *Chlamydomonas* cell showing the position of nucleus (*N*), pyrenoid (*P*) and eyespot (*E*) (Image courtesy: (**a**) Louisa Howard, Dartmouth College, Hanover, NH (**b**) Mr. Wolfgang Bettighofer)

unicells are biflagellate and have a single cup-shaped chloroplast. *Chlamydomonas* belongs to the order Volvocales. The Volvocales constitute the largest group of the Class Chlorophyceae. It mainly include freshwater and terrestrial green algal forms, including several salt tolerant or halotolerant species and/or psychrophilic species (*Dunaliella* and *Chlamydomonas nivalis*). Since Volvocales include unicellular forms (e.g., *Chlamydomonas*) as well as colonial forms (e.g., *Volvox*), its members have been extensively studied as model organisms to shed light on the evolution of primitive multicellular condition.

All *Chlamydomonas* species are capable of autotrophic growth. *Chlamydomonas reinhardtii* can grow in the dark utilizing acetate as a source of organic carbon. *Chlamydomonas reinhardtii* has been mainly well studied, and has become a model system for the study of eukaryotic cell structure and function. *Chlamydomonas* is commonly used in the laboratory work, mostly in molecular genetics for the reason that it is haploid, has a simple life cycle, easy to cultivate, grows rapidly, offers easy isolation of mutants and sexual reproduction can easily be induced. *Chlamydomonas* is an excellent system for studying chloroplast genetics, chloroplast biogenesis, photosynthesis, light perception, cell cycle, cell-cell recognition, maternal inheritance, role of dynein in flagellar function and centrin in the cytoskeleton.

1.3 Occurrence

Chlamydomonas is frequently found free swimming in freshwater of all types such as in ponds, pools and lakes. A few species occur in brackish and marine waters. *C. braunii* is a fresh-water species, whereas *C. perpusilla* is found in brackish waters.

C. ehrenbergii occurs in marine waters. *C. reinhardtii* is common in both fresh water and brackish water. Some species of *Chlamydomonas* form a green scum above the surface of water. *Chlamydomonas* generally prefers habitat rich in ammonium compounds. Some species are also found in terrestrial habitats such as moist soil, in snow and ice. *C. nivalis* occurs on snow and can colour the snow red due to the accumulation of pigment known as haematochrome or astaxanthin (red carotenoid) in its cells. A few species of *Chlamydomonas* are epizoic, living symbiotically on the external surface of animals (e.g., *C. hydrae* on the surface of fresh-water polyp).

1.4 Morphology and Ultrastructure

Chlamydomonas thallus is a unicellular, green, motile, biflagellate structure. Shape of the *Chlamydomonas* cell could be oval, spherical, ellipsoidal, oblong or pear shaped in different species. Dimensions of the Chlamydomonas vegetative cell are: about 30 μm in length and about 20 μm in diameter. Flagella emerge through pores in the cell wall. Inner to the cell wall is present a protoplast which is differentiated into a plasma membrane, a single distinct nucleus, cytoplasm, two contractile

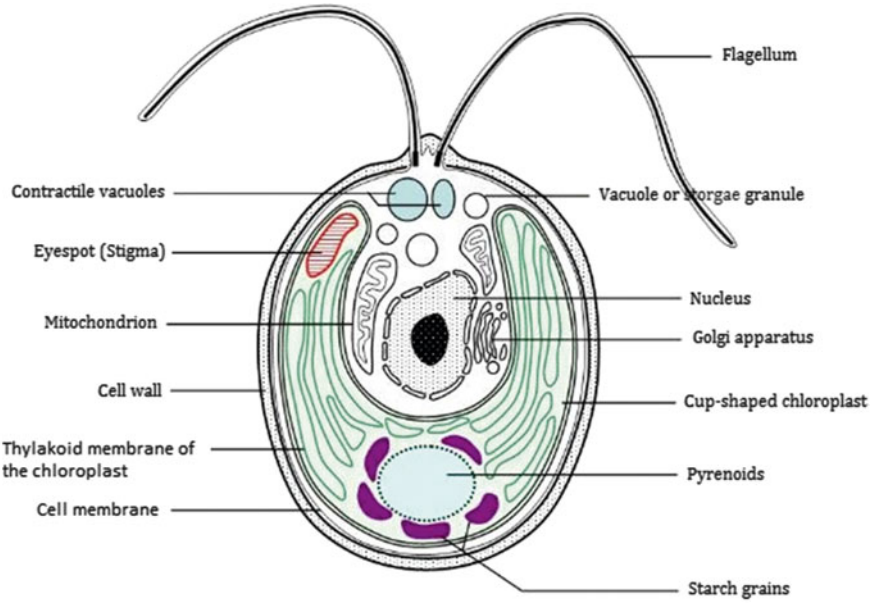

Fig. 2 *Chlamydomonas reinhardtii*, diagrammatic representation of a cell showing ultrastructural details(Image courtesy: Dr. Christopher Skilbeck)

vacuoles, a massive cup shaped chloroplast, pyrenoid, an eyespot and other cell organelles such as mitochondria, endoplasmic reticulum, Golgi bodies, and true vesicles (Fig. 2). Vacuoles and numerous dense granules called volutin granules (polyphosphate bodies) are present in the cytoplasm.

1.4.1 Cell Wall

The cell is delimited by a definite cell wall. It is pinched out anteriorly into a papilla in several species. Early reports referred to *Chlamydomonas* cell wall as cellulosic. The multilayered cell wall is not composed of cellulose, but rather the major constituents are hydroxyproline rich glycoproteins. In *C. reinhardtii*, the wall is a complex structure containing seven layers (Roberts et al. 1972).

When growing in conditions in which liquid water is limiting, cells may occur as palmelloid stages that are groups of nonflagellate cells held together by common mucilage rich in hydroxyproline and sugars. When palmelloid aggregates are exposed to water, the mucilage gets dissolved and the cells typically transform to flagellates.

1.4.2 Flagella and Neuromotor Apparatus

The anterior side of the cell has two whiplash type (the flagella with a smooth surface without hairs) of flagella which are of equal length (Fig. 3). Each flagellum originates from a basal granule/body or blepharoplast and shows a typical 9+2 arrangement of microtubules. The flagella of motile cells have a tendency to be shed under several circumstances. This abscission of flagella is termed as 'Autotomy of flagella' which is a common phenomenon among the Volvocales (Lewin and Lee

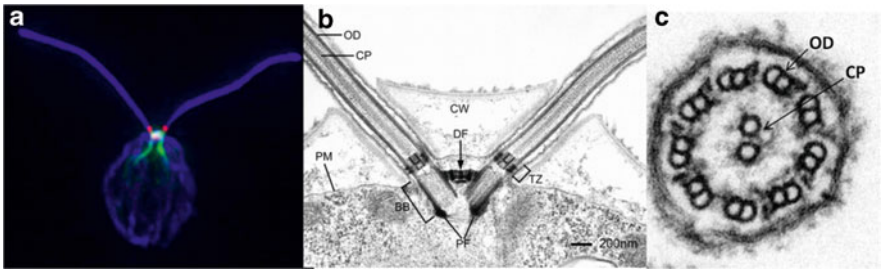

Fig. 3 *Chlamydomonas reinhardtii* flagella. (**a**) Fluorescence micrograph, (**b**). Transmission electron micrograph showing the longitudinal section, Cell wall (*CW*); plasma membrane (*PM*); outer doublet microtubule (*OD*); central pair of microtubules (*CP*); basal body (*BB*); distal striated fiber (*DF*); transition zone (*TZ*); proximal fiber (*PF*) which connects the two basal bodies. (**c**) TEM showing the cross section (Image courtesy: (**a**) Prof. Lynne Quarmby, Simon Fraser University. (**b**) Prof. William Dentler, (**c**) Prof. G.J. Pazour)

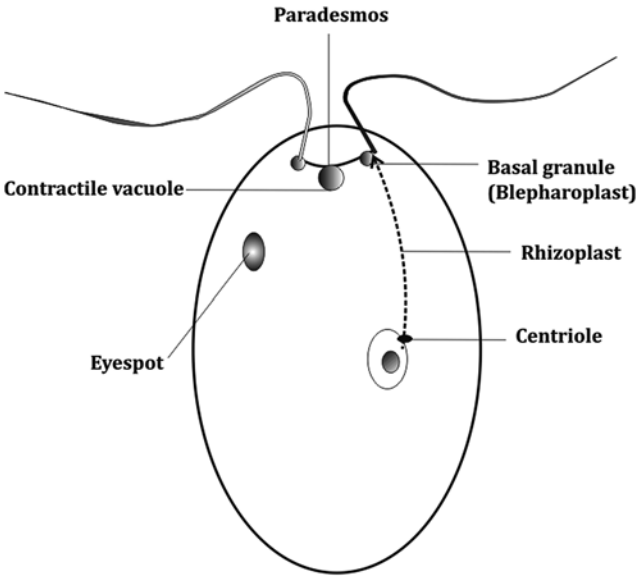

Fig. 4 Neuromotor apparatus in *Chlamydomonas* cell

1985). Abscission occurs at a precise site at the base of shaft immediately before the transition zone. Two contractile vacuoles are present at the anterior end of the cell, one near the base of each flagellum.

Flagella are associated with neuromotor apparatus which provides a connection between the flagella and the nucleus. Flagella are connected with the nucleus through the neuromotor apparatus of the cell (Fig. 4). The neuromotor apparatus consists of three main parts: (1) Basal body or blepharoplast (2) Paradesmos, a transverse fibre connecting the two blepharoplasts (3) Rhizoplast, a delicate, descending thread connecting one of the blepharoplasts with the centrosome. The centrosome stays connected with the nucleolus by many thin fibrils. Centrosome could be intranuclear or may be situated outside the nucleus.

1.4.3 Chloroplast, Pyrenoid and Eyespot

Chloroplast in *Chlamydomonas* is usually single, massive and cup-shaped and is needed for photosynthesis. Chloroplast is parietal in position (located in the peripheral part of the cytoplasm). In each chloroplast there is a pyrenoid with starch sheath. In *Chlamydomonas*, the pyrenoid is located in the lower part of the chloroplast. Usually a single pyrenoid is present, however, it may be two to several in some species. *Chlamydomonas* cells are phototactic. The motile vegetative and reproductive cells of *Chlamydomonas* have a small pigmented bright reddish or brownish red eye-spot or stigma which senses light. Eyespot is an oval photoreceptive organ and is present on the anterior side of the chloroplast. In *Chlamydomonas* the eyespot remains embedded in the chloroplast. The eyespot of *Chlamydomonas*

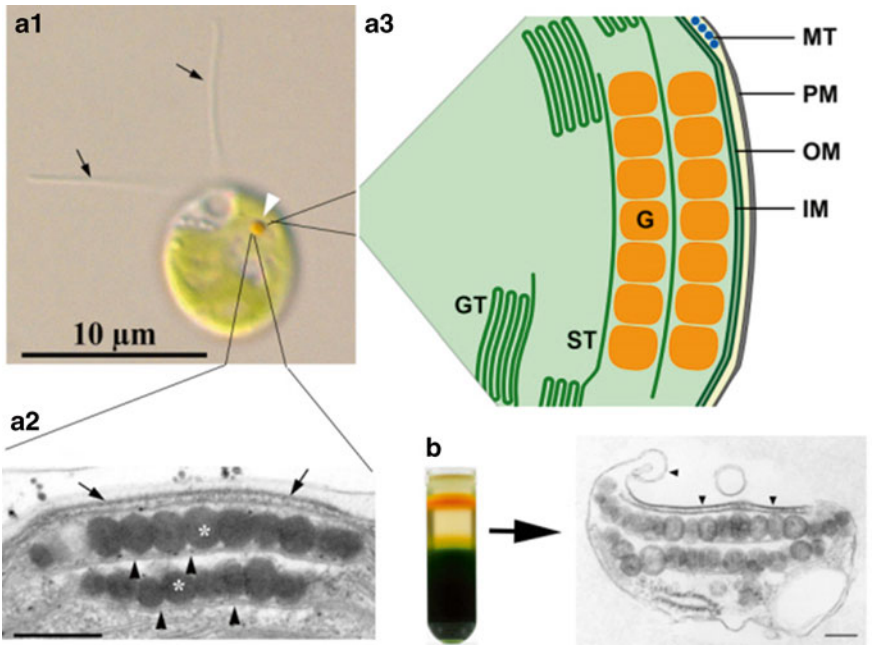

Fig. 5 *Chlamydomonas reinhardtii* (**a1**) eyespot apparatus as seen in the light microscope (*white arrow-head*), (**a2**) as seen in the transmission electron microscope (**a3**) a schematic drawing (**b**) eyespot apparatus can be isolated as a structural unit by sucrose density gradient centrifugation (Image courtesy: Prof. Georg Kreimer)

reinhardtii is composed of two ordered layers of lipid (carotenoid) globules surrounded by the chloroplast (Fig. 5). The outermost carotenoid rich globule layer is in contact with the specialized regions of the two surrounding chloroplast membranes and the bordering plasma membrane. Isolation of the eyespot apparatus as a structural unit can be done using sucrose density gradient centrifugation (Fig. 5). The plasma membrane and the surrounding outer chloroplast membrane above the eyespot are characterized by the presence of numerous intramembrane particles. The photoreceptors are believed to be situated in that plasma membrane patch. *Chlamydomonas* accomplishes phototaxis by the modulation of intensity of light reaching to its photoreceptors present in the eyespot apparatus. Many ribosomes are present in the cytoplasm. In addition *Chlamydomonas* cell also contains other usual membrane bound cell organelles such as mitochondria, Golgi bodies, endoplasmic reticulum and nucleus.

1.5 Reproduction

Chlamydomonas reproduces by asexual and sexual methods.

1.5.1 Asexual Reproduction

Asexual reproduction in *Chlamydomonas* takes place mainly by zoospores but can also occur through aplanospores, hypnospores and palmella stage. **Zoospore formation**: Generally zoospores are formed during the night. At the time of zoospore formation parent cell comes to rest, withdraws its flagella, contractile vacuoles disappear and the protoplast withdraws from the cell wall. In this immobile state the cytoplasm, chloroplast and the nucleus divide longitudinally into two daughter protoplasts. The second division is at right angles to the first division. In this way 4, 8 or 16 uninucleate protoplasts are formed by successive mitotic divisions within the parent cell wall. Mitosis is closed and the cytokinesis involves phycoplast microtubules. Cytokinesis occurs by furrowing. Each daughter protoplast secretes a new cell wall, develops flagella, contractile vacuoles and a neuromotor apparatus. The flagellate daughter cells are similar to the parent cell in shape and structure but are smaller in size. The parent cell wall ruptures or gelatinizes and the daughter cells are released. The daughter cells are released from the parental cell wall by production of specific wall autolysins that digest the cell wall. These daughter cells are called zoospores or mitozoospores. The liberated zoospores increase in size and are capable of producing new zoospores after 24 h, hence the process is repeated. Under certain conditions zoospores fail to escape from the gelatinous parent cell wall and remain clustered together. This stage is known as palmella stage.

Aplanospore Formation Under unfavorable conditions such as drought, the *Chlamydomonas* cells come to rest and withdraw their flagella. The protoplast withdraws from the parent wall and rounds up. Protoplast divides into daughter protoplasts and each daughter protoplast secretes thin wall around itself but does not develop flagella. Each aplanospore germinates into a new individual resembling the parent. To survive severe drought conditions aplanospore secretes a thick wall around it. This thick walled resting spore is called a hypnospore.

Palmella Stage Under certain unsuitable conditions the protoplast of the parent cell divides to form 4–8 daughter cells. The daughter cells do not develop flagella and thus fail to escape out. These non-motile cells stay clustered together within the mucilaginous matrix formed by gelatinization and swelling of the parent cell wall (Fig. 6). This assemblage of cells in a common gelatinous matrix is called ‘palmella stage’. This stage is temporary perennating stage and is of brief duration. Under favorable conditions (e.g., presence of water) the individual cells in these palmelloid phases readily develop flagella and return to the motile condition. These cells then escape out from the mucilaginous matrix and mature into large vegetative cells. Sometimes in severe drought conditions the individual cells of the palmella stage secrete a thick wall around them and develop into red non-motile resting spores called the hypnospores. Contents of hypnospores include a red pigment called haematochrome which imparts red colour to the hypnospore.

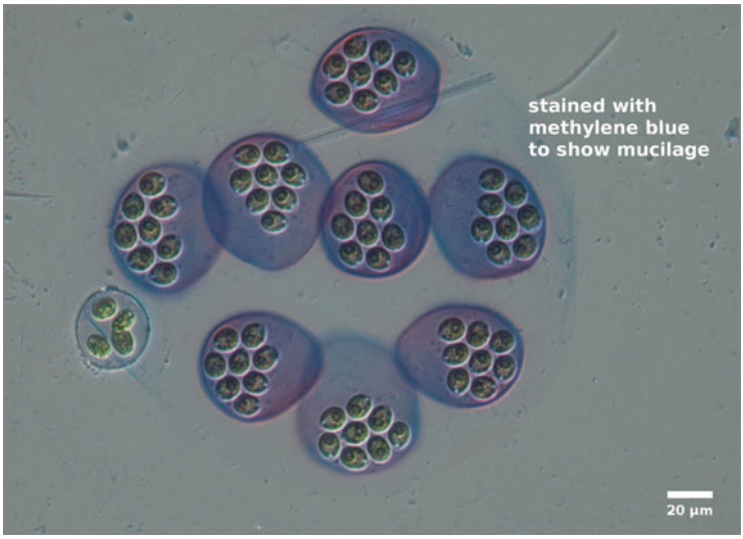

Fig. 6 Light micrograph (LM) of *Chlamydomonas*- Palmella stage (Image Copyright: Dr. Robin Matthews, Western Washington University, used with permission)

1.5.2 Sexual Reproduction

Sexual reproduction in *Chlamydomonas* is regulated by certain environmental factors such as:

1. Depletion of Nitrogen/ammonium
2. Absence or deficiency of nutritional substances
3. Light and temperature
4. High CO_2 concentration and presence of Calcium

Some species are isogamous, some are anisogamous and a few are primitive oogamous.

Isogamy It is a primitive type of sexual reproduction and most species of *Chlamydomonas* are isogamous. Isogamous reproduction is characterized by a similarity in size, form and structure between the fusing gametes. The sexual reproduction starts with the division of the cell protoplast into 8, 16, 32 or 64 daughter protoplasts. Each daughter protoplast acquires two flagella and is called a gamete or isogamete. The biflagellate gamete is usually naked and has no cell wall. These gametes are smaller than zoospores. The gametes are released from the parental cell wall by production of specific wall autolysins that digest the cell wall. The liberated gametes swim in the surrounding water for a while. The flagellar surface of gametes is covered by linear glycoprotein molecules called agglutinins. These chemical substances are involved in the recognition of gametes of the opposite strains and promote the adhesion of flagella of cells of opposite mating types. These molecules are absent in the flagella of the vegetative cells. In isogamous forms mating types are usually designated as mt^+ and mt^-. Tips of the flagella adhere initially; afterwards

the flagellar pairs become attached through their whole length. The gametes of both the strains fuse at their anterior ends. Isogamous sexually reproducing species can be classified into homothallic (*C. debaryanum*, *C. longistigma*, *C. media*) and heterothallic (*C. reinhardtii*, *C. moewusii*) (Goodenough and Thorner 1983; Wiese 1984; Snell 1985; Van Den Ende 1985). In homothallic species fusion takes place between the gametes produced in the same parent cell. Once the gametes are released from the parent cell wall they swim for a while in the surrounding water and then fuse in pairs to form a zygote. In heterothallic species fusion takes place between the gametes produced in two different parent cells of opposite mating strains. One of these is designated as a plus strain (+) and the other as minus (−) strain. Once the gametes of plus strain get into contact with a minus strain they fuse to form a zygote. Hence the isogametes are morphologically similar but functionally different as one belongs to the plus strain and the other to the minus strain.

Anisogamy In anisogamy the fusion takes place between dissimilar gametes, the anisogametes. Anisogamy in *Chlamydomonas* can be categorized into physiological anisogamy and morphological anisogamy. In physiological anisogamy (e.g., *C. monoica*) the fusing gametes are morphologically identical but different in their behavior i.e., one gamete may be more active than the other.

In morphological anisogamy the fusing gametes (male and female) differ noticeably in size e.g., *C. braunii*. The anisogametes differing in their size can be designated as male and female. Physiological anisogamy is a primitive type of anisogamy, whereas morphological anisogamy is an advanced type of anisogamy. Gametes are produced in specialized cells called gametangia. The male gamete is small and active, whereas the female gamete is large and passive. These anisogametes come in contact with each other through their anterior end and the wall at the point of fusion dissolves.

Fusion of the gametes takes place externally in the surrounding water. The protoplast of one of the gamete (microgamete) escapes from its cell wall and flows entirely into the envelope of the other gamete (the macrogamete) to fuse with it. A zygote is formed in the cell envelope of the macrogamete. The anisogamous species are all heterothallic or dioecious.

Oogamy Oogamous sexual reproduction is the most advanced type of sexual reproduction and has been observed in *C. coccifera and C. ooganum*. Distinct male and female sex organs are formed. The female mother cell withdraws its flagella and its protoplast rounds off to form a single globose macrogamete or female gamete which is considered equivalent to egg cell. The large globose macrogamete does not have any flagella and is immobile. Male parent cell divides by 4 divisions to form 16 spherical biflagellate microgametes or male gametes. Both microgamete and macrogamete have a cell wall. The male gametes or microgametes on maturity are released in the surrounding water. The active male gamete swim in the direction of immobile female gamete and the two unite at their anterior ends. The intervening walls at the point of union between the two gametes dissolve and the flagella of the male gamete are resorbed. The protoplast of the male gamete detaches from its cell wall and moves into the envelope of female gamete. Plasmogamy and karyogamy

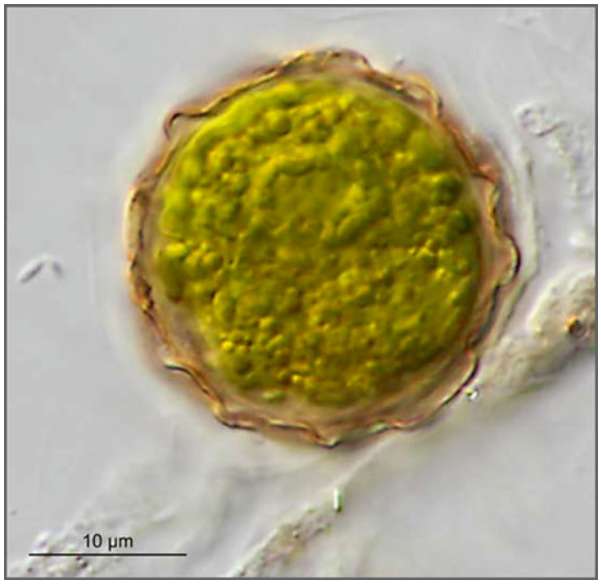

Fig. 7 A thick walled resting zygote (zygospore) (Picture courtesy: Dr. Bernd)

occurs and the two protoplasts fuse to form a non-motile zygote. It is a primitive type of oogamy as the motile male gametes or microgametes are not typical spermatozoids.

Zygospore The newly formed zygote in isogamy remains motile for few hours to several days depending upon the species. The zygote has four flagella, two chloroplasts, two nuclei and two eyespots. Shortly the flagella are withdrawn and the zygote comes to rest by settling down on some substratum. Soon the cytoplasm, nuclei and chloroplast of the gametes fuse. The non-motile zygote secretes a primary and a secondary wall around it and is converted into a resting zygospore (Cavalier-Smith 1976). The zygote or zygospore is the only diploid structure in the life cycle. Zygote always passes through a resting stage (Fig. 7). The zygote accumulates fats and reserve food materials and turns orange red in colour. Zygote can endure drought and waits for the return of favorable conditions for germination.

Germination of Zygospore When the zygospore encounters favorable conditions (water) it germinates. The red colour of the zygospore changes to green before germination. The diploid nucleus of the zygospore undergoes meiosis resulting into the formation of four to eight haploid nuclei. In the heterothallic species + and − strains become distinct at this point by segregation of the nuclei of opposite mating types (+ and −). Each haploid nucleus is incorporated into some protoplast and forms a biflagellate meiozoospore which is motile. Each meiozoospore secretes a cell wall around it. At this stage the inner wall of the zygospore gets dissolved and the outer wall splits open, liberating the meiozoospores. Each released meiozoospore grows into a fully developed *Chlamydomonas* cell.

1.6 *Origin, Evolution and Differentiation of Sex in* Chlamydomonas

Under favorable environmental conditions *Chlamydomonas* reproduces by the formation of biflagellate, motile zoospores. Each zoospore is capable of growing into a new *Chlamydomonas* individual resembling the parent. In this process there is no fusion of gametic cells and hence this process is called as asexual reproduction.

The origin of gametic cells is the origin of sex. At the end of a growing season when environmental conditions become unfavorable (e.g., nitrogen depletion), *Chlamydomonas* resorts to sexual reproduction. Isogamy is the primitive form of sexual reproduction in which fusion occurs between similar gametic cells. Isogamy occurs in *C. debaryanum*, *C. longistigma*, *C. media* and *C. reinhardtii*. Anisogamy is a further advanced form of sexual reproduction in which fusion occurs between dissimilar gametes. Anisogamy in *Chlamydomonas* can be categorized into physiological anisogamy and morphological anisogamy. Physiological anisogamy occurs in *C. monoica* and the fusing gametes are morphologically identical but different in their behavior. In morphological anisogamy occurs in *C. braunii* and the fusing gametes differ noticeably in size. Physiological anisogamy is a primitive type of anisogamy, whereas morphological anisogamy is an advanced type of anisogamy.

Oogamy is the most advanced level of sexual reproduction and can be traced in *Chlamydomonas. C. coccifera* exhibits oogamous reproduction in which the non-motile female gamete or egg cell is fertilized by biflagellate microgametes. In *C. coccifera* it is a primitive type of oogamy as the motile male gametes or microgametes are not typical spermatozoids.

1.7 *Life Cycle*

The life cycle of *Chlamydomonas* consists of haploid and diploid phases. The motile vegetative *Chlamydomonas* cell is haploid. It produces haploid gametes. The haploid gametes fuse and the diploid phase is attained in the zygote. The diploid nucleus of the zygote undergoes meiotic division followed by formation of haploid meiozoospores. This way *Chlamydomonas* terminates its diploid phase. Each haploid meiozoospore grows into a haploid vegetative *Chlamydomonas* cell. The life cycle of *Chlamydomonas* having haploid phases represented by unicellular vegetative thallus, zoospores, gametes and meiozoospores and a diploid phase which is only restricted to the zygote (zygotic meiosis) is described as haplontic.

Vegetative cells of *C. reinhardtii* are haploid with 17 chromosomes (the size of nuclear genome is 121 Mb or 121,000,000 base pairs and it is divided into 17 linkage groups or chromosomes). Under unfavorable conditions haploid gametes are produced. Haploid gametes are identical in appearance and are designated as mt(+) and mt(−). These haploid gametes fuse and form a diploid zygote. The diploid zygote perennates in soil and germinates on the return of favorable conditions. The

diploid zygote undergoes meiotic division and liberates four biflagellate haploid cells. These cells grow into fully developed haploid vegetative cells and resume the life cycle of *C. reihardtii*. Under favorable growth conditions, haploid vegetative cells of *C. reinhardtii* undergo two or three mitotic divisions resulting into the formation of four or eight haploid daughter cells or zoospores per mother cell. These zoospores give rise to haploid vegetative cells of *C. reinhardtii* and resume the life cycle.

2 Volvox

2.1 Systematic Position

Fritsch (1935)	Bold and Wynne (1978)	Lee (2008)
Division – Algae	Division – Chlorophycophyta	Phylum – Chlorophyta
Class– Chlorophyceae	Class – Chlorophyceae	Class – Chlorophyceae
Order–Volvocales	Order – Volvocales	Order – Volvocales
Family– Sphaerellaceae	Family – Volvocaceae	Family – Volvocaceae
Genus – *Volvox*	Genus–*Volvox*	Genus–*Volvox*

The name of the genus is derived from the latin word "**Volvere**" (to roll). *Volvox* is a very large, green, motile colonial alga. An integrated colony having a definite number of cells arranged in a precise manner is called a coenobium (Fig. 8).

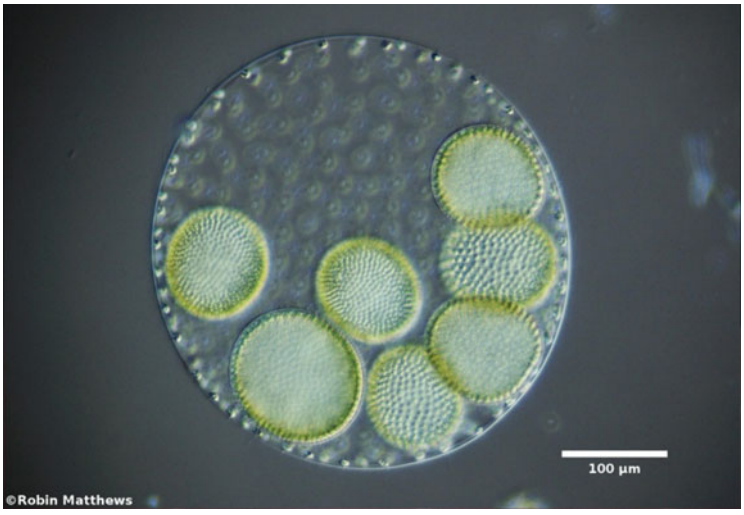

Fig. 8 Light micrograph (LM) of *Volvox* coenobia (Image Copyright: Dr. Robin Matthews, Western Washington University, used with permission)

2.2 Occurrence

It commonly occurs in the fresh waters of ponds, lakes and ditches, in warm weather in the form of pin-head sized balls. Motile coenobia grow vigorously in the rainy season forming blooms. It perennates in winter with the help of resting stages such as zygospore or oospore. The genus has around 20 described species worldwide.

2.3 Morphology

Volvox coenobium is a hollow sphere of mucilage containing 500 to several thousand cells arranged at its periphery (Fig. 9). Individual cells of the coenobium are Chlamydomonad type (similar to *Chlamydomonas* cells) and are embedded in a gelatinous matrix. These cells are interconnected by protoplasmic bridges. The protoplasmic bridges are the extended remnants of protoplasm of incompletely separated protoplasts. Hence the cytoplasm of all the cells are continuous with each other through protoplasmic bridges. This allows the cells to coordinate with each other and trigger the flagellar motion in an organized manner. As the coenobia

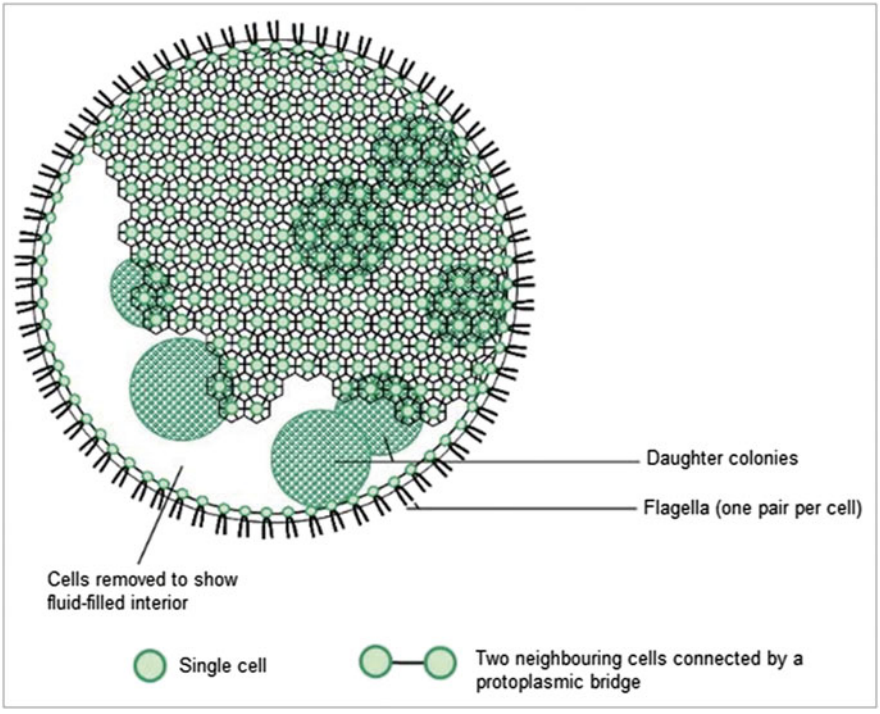

Fig. 9 A coenobium of *Volvox* (Image courtesy: Dr. Christopher Skilbeck)

approach the reproductive phase these bridges are withdrawn. Extracellular matrix (ECM) in *Volvox* is divided into four main zones: the flagellar zone (FZ), boundary zone (BZ), cellular zone (CZ) and deep zone (DZ) (Kirk et al. 1986). Each zone is characterized by the presence of characteristic glycoproteins. Outside the mucilage there is a boundary layer which shows similarity with the hydroxyproline rich glycoprotein cell wall of *Chlamydomonas reinhardtii*, however, the innermost areas of the extracellular matrix are chemically different in *Volvox* (Schmitt et al. 1992). *Volvox* ECM is modified in response to developmental control or external stimuli such as the stress factors or sex inducing pheromone (Sumper and Hallmann 1998). *Volvox* coenobia are so large that they can be easily seen with the naked eye. It is suitable to use hanging drop preparations while performing microscopic visualization of the coenobia because cells are not crushed in this process. *Volvox* colonies have polar organization, and the cells in the anterior region have larger eyespots, suggestive of their enhanced phototactic ability.

Each cell of the coenobium is biflagellate. Flagella project out from the surface of the coenobium and keep it motile. Each cell of the coenobium has its individual gelatinous sheath. Cells in a young coenobium are green and somatic or vegetative in function. In most species, adjacent flagellate vegetative cells or somatic cells are interconnected by protoplasmic strands. Somatic cells cannot divide and are differentiated at the anterior region of the coenobium. The flagellar activity of these somatic cells is crucial to keep the large coenobium motile in order to provide all cells with light and mineral resources. These cells undergo ageing and die consequently. In the posterior part of coenobium certain larger, nonflagellate cells called gonidia arise by asymmetric divisions and this event takes place early in the colony development. Gonidia are able to divide and generate new colonies either by asexual reproduction or produce gametes for sexual reproduction.

2.4 Reproduction

2.4.1 Asexual Reproduction

An asexual colony consists of two types of cells: somatic or vegetative cells which are non-reproducing and gonidia, which are able to divide and produce new colonies by repeated longitudinal divisions. Gonidia are present in the posterior part of the colony and are immotile and specialized for reproduction; Once a gonidium is mature, it behaves like a stem cell (Hoops et al. 2006; Kirk et al. 1999) and divides to produce a young spheroid containing a new cohort of gonidia and somatic cells (Starr 1969, 1970). Somatic cells, on the contrary, are crucial for motility, however they never divide and have no reproductive potential; they eventually undergo programmed cell death (Pommerville and Kochert 1981, 1982).

Asexually reproducing gonidial cells first divide longitudinally. The second division is also longitudinal but in a plane perpendicular to the first one. Division of gonidia ultimately produces hollow balls of cells i.e., young colonies that develop

within the parental colony. As the young colonies are formed, flagella are produced from the anterior ends of the constituent cells which are facing towards the centre of young colony. For these young colonies to be able to swim, a process called 'inversion' occurs (Powers 1908). An opening termed as phialopore, becomes visible at the eight-celled stage. As soon as the number of cells in the young colony reaches a characteristic number for that particular species, the colony inverts through the pore. Daughter colonies are finally released into the surrounding water with the rupture of the parental colony.

2.4.2 Sexual Reproduction

Sexual reproduction in *Volvox* is strictly oogamous (Coleman 1979; Starr 1980). Most of the species are dioecious (*V. aureus*) although some are monoecious (*V. globator*). Monoecous species are generally protandrous. Sexual reproduction is often induced by environmental stress such as nitrogen starvation and high temperature. However, in *V. carterii*, sexual reproduction occurs under optimal or most favorable growth conditions (Jaenicke and Gilles 1985). Sexual reproduction is generally induced when water temperature has attained a significant high level. One or a few colonies are induced to develop male gametes. These male colonies produce a sex hormone called pheromone, a glycoprotein which serves as a chemical sexual attractant (Starr and Jaenicke 1974; Gilles et al. 1981). It diffuses through water, inducing other *Volvox* colonies to turn them sexually competent. Gonidia exposed to pheromone produce either a single egg cell or a packet of 16–64 small, pale, biflagellate sperms or antherozoids. Sperm packets get liberated from parental colonies and swim towards female colonies. They produce a hole in the female colony mucilage by enzymatic lysis, and gets dissociated into individual sperms or antherozoids that fertilize the eggs. The unfertilized eggs are capable of developing into new colonies. Zygote secretes a thick spiny wall around it that serves in perennation. Zygote may also develop haematochrome or red coloration by production of carotenoid pigments. In *Volvox* species, just one meiotic product- a single biflagellate cell survives; the other three cells apparently die. Consecutive divisions of this biflagellate cell regenerate the coenobium or colonial form of *Volvox*.

2.5 Life Cycle

The life cycle of *Volvox* consists of haploid and diploid phases. In *Volvox* both asexual and sexual reproduction occurs. In a coenobium some kind of differentiation takes place between vegetative and reproductive cells, though all the cells are haploid. Asexual reproduction occurs by repeated cellular division and the consequent detachment of the daughter colony from the mother wall. The daughter colony floats inside the parental colony. Eventually, the old parental colony disintegrates and the daughter colony with haploid cells is released. In sexual reproduction a few

vegetative cells enlarge to form egg cells, while some other cells divide to form antherozoids. These antherozoids or sperm cells reach the egg cell and fertilization can occur. The haploid gametes fuse and the diploid phase is attained in the zygote. The diploid zygote develops a thick wall and remains in the hollow space of the colony. After being liberated from the colony the naked zygote undergoes meiosis. Just one product of meiosis survives and generates a new colony. The life cycle of *Volvox* having haploid phases represented by unicellular vegetative cells arranged in the colony, zoospores, gametes (egg cell and antherozoids) and a diploid phase which is only restricted to the zygote (zygotic meiosis) is described as haplontic.

3 Chlorella

3.1 Systevmatic Position

Fritsch (1935)	Bold and Wynne (1978)	Lee (2008)
Division – Algae	Division – Chlorophycophyta	Phylum – Chlorophyta
Class – Chlorophyceae	Class – Chlorophyceae	Class – Chlorophyceae
Order – Chlorococcales	Order – Chlorellales	Order – Chlorellales
Family– Chlorellaceae	Family – Chlorellaceae	Family – Chlorellaceae
Genus – *Chlorella*	Genus – *Chlorella*	Genus – *Chlorella*

3.2 Introduction

The name of the genus is derived from the Greek words "**chloros**" (green) + "**ella**" (minute). *Chlorella* is an extremely simple, easy to cultivate, rapidly growing, small spherical (coccoid) green unicellular alga. *Chlorella* is commonly used in physiological research especially in photosynthesis and respiration. The cells are rich in amino acids, mineral and vitamins hence possess a very high food value. *Chlorella* species are capable of autotrophic growth and can also grow in the dark utilizing glucose as a source of organic carbon.

3.3 Occurrence

Chlorella occurs on damp soils, walls, bark of trees, in freshwater pools and sewage. Some species of *Chlorella* live as symbionts in invertebrates such as *Hydra*, *Paramecium* and sponges and are described as Zoochlorella. These *Chlorella* cells have an ability to escape digestion. Zoochlorellae utilize the CO_2 released during

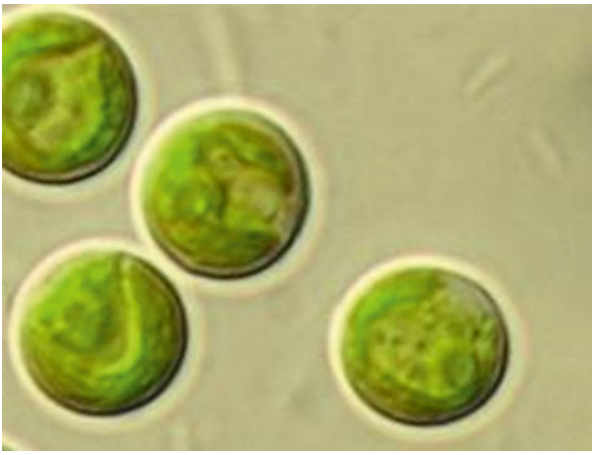

Fig. 10 *Chlorella* cells under light microscope (Image courtesy: Prof. Marvin W. Fawley)

the respiration of animal tissue, photosynthesize and oxygen is released. Some common species of *Chlorella* include *C. vulgaris*, *C. variegata*, *C. lobophora*, *C. parasitica*, *C. conglomerata*, and *C. conductrix*.

3.4 Morphology and Ultrastructure

Chlorella is an extremely simple alga. The cells are non-motile, small (2–10 μm in diameter), spherical to ellipsoid, solitary or occasionally in small colonies of irregular shape (Fig. 10). Each cell is surrounded by a cellulose cell wall. The protoplast of the cell is enclosed in a cell membrane. A single parietal cup shaped chloroplast is present with or without a pyrenoid. The thylakoids are present in the stacks of 2–6. Starch and some small electron dense granules accumulate between the stacks of thylakoid. A single nucleus is present in the colourless central cytoplasm in the cavity of the chloroplast. Stigma and contractile vacuoles are usually absent. Tubular and branched mitochondria, dictyosomes, a few vacuoles, endoplasmic reticulum and lipid bodies are present in the cytoplasm.

3.5 Reproduction

Chlorella reproduces exclusively by asexual means i.e., autospore formation. Sexual reproduction is not known. *Chlorella* is non-motile and it does not produce zoospores or gametes i.e., motile cells. Each cell produces by successive divisions, 2, 4, 8 or 16 daughter protoplasts (Fig. 11). Each daughter protoplast rounds off to form an autospore (a non-motile spore). These autospores escape out by the rupture of the parent cell wall. On release each autospore grows into a new individual.

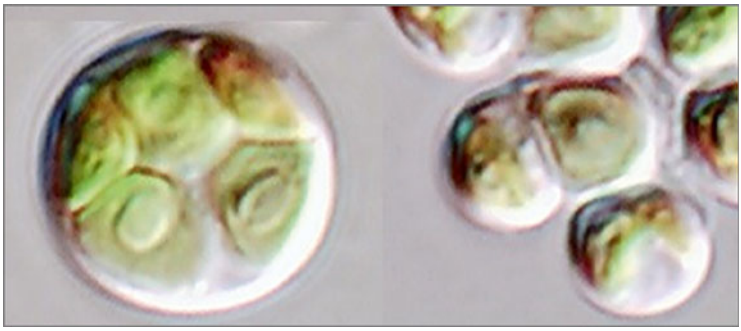

Fig. 11 Autospore formation in *Chlorella* (Image courtesy: Prof. Marvin W. Fawley)

3.6 Life Cycle

The life cycle of *Chlorella* indicates that there is no distinct alternation of generations. The cells are haploid and reproduce asexually by bearing haploid autospores which ultimately develop into non-motile mature cells. Four phases have been recorded in the life cycle of *Chlorella*. (1) Growth phase in which the autospores grow larger in size (2) Mature stage when the cell prepares for cell division (3) The cell undergoes cell division (4) The parent cell wall ruptures and releases the autospores.

4 Ulothrix

4.1 Systematic Position

Fritsch (1935)	Bold and Wynne (1978)	Lee (2008)
Division – Algae	Division – Chlorophycophyta	Phylum – Chlorophyta
Class – Chlorophyceae	Class – Chlorophyceae	Class – Ulvophyceae
Order – Ulotrichales	Order – Ulotrichales	Order – Ulotrichales
Family– Ulotrichaceae	Family – Ulotrichaceae	Family – Ulotrichaceae
Genus – *Ulothrix*	Genus – *Ulothrix*	Genus – *Ulothrix*

4.2 Occurrence

Ulothrix is a thread like alga found in a variety of habitats. Usually it occurs in cold, slow running freshwater streams. Some species of *Ulothrix* such as *U. flacca* are marine. It is usually found attached to the substratum such as rocks and stones.

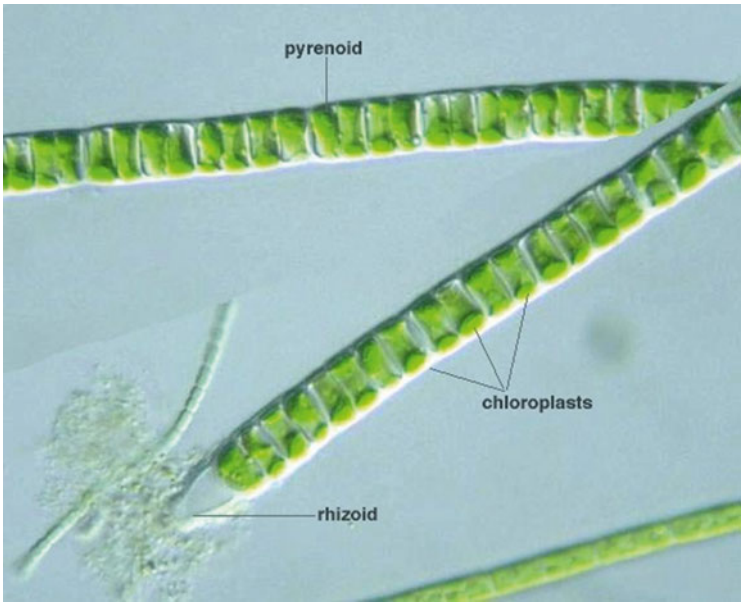

Fig. 12 *Ulothrix* filaments showing rhizoid along with cells having parietal chloroplasts and pyrenoids (Image courtesy: Prof. Yuuji Tsukii, Hosei University, Japan)

4.3 Thallus Structure

Plant body of *Ulothrix* is filamentous, and the filaments are multicellular and unbranched having a single row of cylindrical uninucleate cells (Fig. 12). Young filaments are bright green and remain attached to the substratum by means of its lowermost cell called rhizoidal cell. These filaments may become free floating later. Three types of cells can be distinguished in each filament: (1) Apical cell, which is present at the tip of the filament. It is green, rounded and dome-shaped. (2) Basal cell or holdfast, which is present at the base and remains attached to the substratum. It is elongated, narrow and colourless. (3) Middle cells, which are preset between the apical cell and basal cell and hence are also called intercalary cells. The intercalary cells are often shorter than broad, however, they can be as long as broad as well. These cells are green and cylindrical. Apical cell and intercalary cells have the capacity to divide.

4.4 Cell Structure

The cells are surrounded by a cell wall, which generally consists of two layers. The outer layer is mostly consists of protopectin and the inner layer of cellulose. In the centre of the cell is present a single nucleus. A single parietal chloroplast is present in the cytoplasm which is girdle- shaped or ring shaped, collar shaped or C- shaped. Chloroplast may contain one or more pyrenoids.

4.5 Reproduction

Ulothrix reproduces by all three means, i.e. vegetative, asexual and sexual.

4.5.1 Vegetative Reproduction

It takes place by fragmentation or akinete formation. The filaments of *Ulothrix* often get fragmented due to mechanical injury. Fragmentation starts in the vicinity of the apex and progresses towards the base of the filament. Each fragment develops into a new filament of *Ulothrix* by mitotic divisions.

4.5.2 Asexual Reproduction

The alga reproduces asexually by the formation of zoospores, aplanospores, hypnospores, akinetes and palmella stage. During favourable conditions the alga multiplies by zoospore formation, whereas under unfavourable conditions asexual reproduction occurs by means of aplanospores, hypnospores, akinetes and palmella stage. Any cell of the filament except the holdfast is capable to act as zoosporangium. The protoplast of the zoosporangium divides to form 2, 4, 8, 16 or 32 daughter protoplasts. The first division is longitudinal. All these daughter protoplast contain nucleus and cytoplasm, and get transformed into zoospores (Fig. 13). The

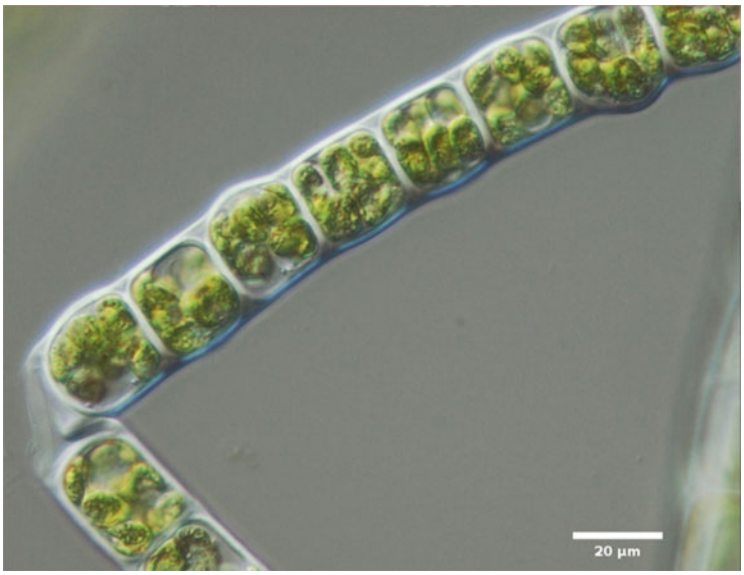

Fig. 13 *Ulothrix* filament bearing zoospores (Image Copyright: Dr. Robin Matthews, Western Washington University, used with permission)

zoospores are of two types: Macrozoospores which are bigger, oval and quadriflagellate and Microzoospores which are smaller, pear shaped, and bi- or quadriflagellate. Macrozoospores are generally 2, 4 or 8 in number in a cell, whereas microzoospores are generally 8–32 in number in a cell.

The zoospores are initially released into a mucilaginous vesicle through a pore in the wall. The wall of the mucilaginous vesicle dissolves soon and the zoospores are liberated in water. The zoospores swim for some time after which they settle down on the substratum. The zoospores elongate and divide to produce a basal cell or holdfast on the lower side and a narrow filament having a single row of cells on the upper side. In *Ulothrix* asexual reproduction also occurs through hypnospores. In *U. zonata* hypnospores are formed by protoplast divisions similar to that of Zoosporogenesis (Bullock 1978). The hypnospores lack flagella and eyespot but have thick cell wall.

4.5.3 Sexual Reproduction

Sexual reproduction occurs under unfavourable conditions. Sexual reproduction is of isogamous type and occurs by means of biflagellate gametes. Majority of the *Ulothrix* species are heterothallic and the fusion takes place between two gametes released from two different filaments. Gametes are produced inside a gametangium by the divisions of the protoplasts of cells. Generally 8, 16, 32 or 64 gametes are formed per gametangium depending on the species. These gametes are called isogametes and are smaller than microzoospores. Liberation of gametes occurs in the morning hours. The liberated gametes swim for some time. Two isogametes of different strains (+ and −) fuse and form a quadriflagellate zygote. The zygote keeps on swimming for some time, settles down on some substratum, becomes rounded off, withdraws its flagella, gets surrounded by a thick wall and represents the zygospore. Germination of zygospore occurs after a long resting period i.e. 5–9 months. The diploid nucleus of the zygospore divides meiotically to form four nuclei (two of + and two of − strains) which develop into meiozoospores or aplanospores. On liberation these haploid cells develop into (+) or (−) type of *Ulothrix* filaments.

4.6 Life Cycle

The life cycle of *Ulothrix* consists of haploid and diploid phases. The multicellular filament is haploid. It produces haploid gametes. The haploid gametes released from two different filaments (of + and −strains) fuse and the diploid phase is attained in the zygote. Hence diploid phase is represented by a one-celled zygote. The diploid zygospore divides meiotically to form four haploid zoospores (two of each strain). Each haploid meiozoospore grows into a haploid vegetative *Ulothrix* filament. The life cycle of *Ulothrix* having haploid *phases* represented by multicellular vegetative filament, zoospores, gametes and meiozoospores and a diploid phase which is only restricted to the zygote (zygotic meiosis) is described as haplontic.

5 *Ulva*

5.1 Systematic Position

Fritsch (1935)	Bold and Wynne (1978)	Lee (2008)
Division – Algae	Division – Chlorophycophyta	Phylum – Chlorophyta
Class – Chlorophyceae	Class – Chlorophyceae	Class – Ulvophyceae
Order – Ulotrichales	Order – Ulvales	Order – Ulvales
Family– Ulvaceae	Family – Ulvaceae	Family – Ulvaceae
Genus – *Ulva*	Genus – *Ulva*	Genus – *Ulva*

5.2 Occurrence

Ulva is an essentially marine alga generally found in rocky shores where it occurs attached to stones, rocks etc. Some species of *Ulva* are also found in brackish water and polluted estuaries. The genus *Ulva* has around 30 described species, of which *U. lactuca* is the most common species.

5.3 Thallus Structure

Ulva is commonly known as 'sea lettuce' as the blades constituting the thallus are expanded leaf like structures which resemble garden lettuce in appearance. Thallus is conspicuous, macroscopic and consists of broad and flat blades. Blades are composed of two cell layers (distromatic) (Fig. 14). Each cell is isodiametric in shape, uninucleate with one parietal laminate to cup shaped chloroplast and a single pyrenoid. Blades of *Ulva* can be as long as 1 m. *Ulva* is attached to substrates in marine coastal waters by means of rhizoidal branches, and it also occurs in free-floating masses. The distromatic blades arise from zoospores. The blade is narrowed into a short basal stalk. Stalk is attached to the substratum with the help of an attaching disc. The attaching disc is formed by the rhizoidal outgrowths of the cells present on the lower side of the thallus.

5.4 Reproduction

Reproduction in *Ulva* occurs by all three means: vegetative, asexual as well as sexual.

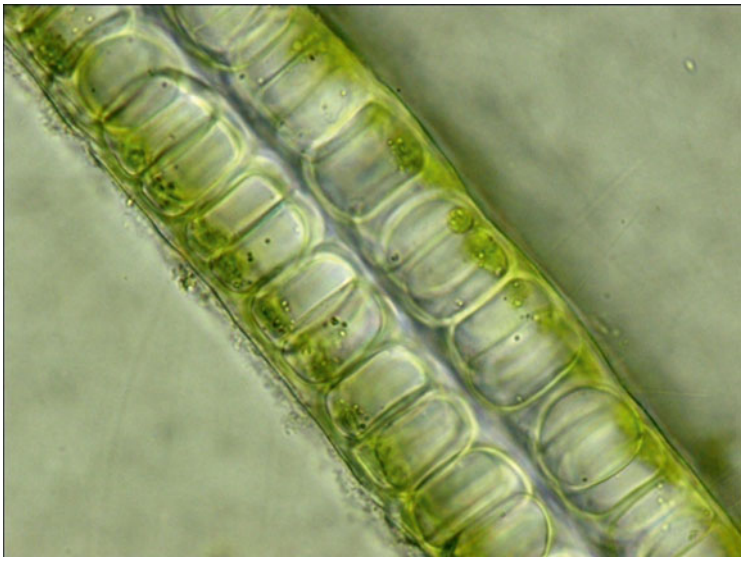

Fig. 14 *Ulva*, vertical section (V.S.) through a distromatic thallus (Image courtesy: Dr. Cindy Fernández)

5.4.1 Vegetative Reproduction

Vegetative reproduction is entirely by fragmentation.

5.4.2 Asexual Reproduction

Asexual reproduction occurs by quadriflagellate zoospores (Fig. 15). These zoospores are produced only in the sporophytic (2n) plants. Zoospores are formed by the division of protoplasts into 4–8 daughter protoplasts. The first division is a reduction division hence the daughter protoplasts are haploid. These daughter protoplasts develop into uninucleate and quadriflagellate zoospores. After liberation these zoospores settle down on some substratum and germinate to produce the haploid gametophytic thalli.

5.4.3 Sexual Reproduction

Ulva is heterothallic or dioecious and gametes from plants of different mating types fuse with each other. Gametes are produced only in the haploid gametophytic thalli. Gametes are biflagellate, pyriform and are produced in the marginal cells of the thallus. Hence reproduction can occur without complete breakdown of the parental thallus. Gametes are formed by the successive mitotic divisions of the cell protoplast. Each daughter protoplast develops into a biflagellate uninucleate gamete. The male gametes are narrower and smaller and possess a yellowish green chloroplast with an

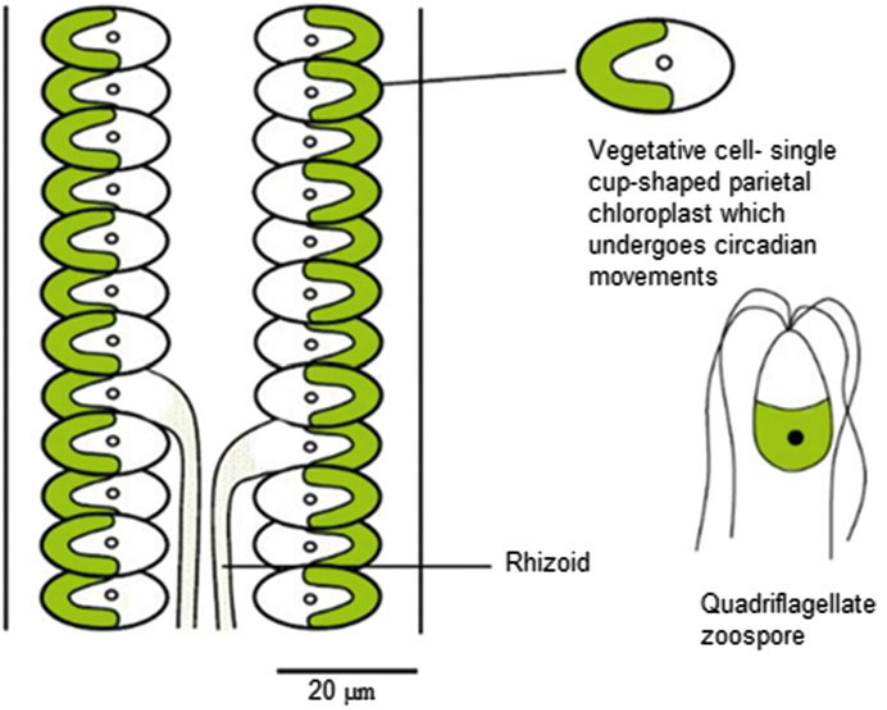

Fig. 15 A section through an *Ulva* distromatic thallus showing cells having a single cup-shaped, parietal chloroplast. Some of the cells, give out appendages called rhizoids (Picture courtesy: Dr. Christopher Skilbeck)

indistinguishable pyrenoid. The female gametes are larger and possess a green chloroplast with a distinct pyrenoid. The gametes escape through an apical aperture formed at the tip of the gametangium. The fusion takes place between the gametes coming from plants of different mating types in the surrounding water. The freshly formed zygote is motile at first and has four flagella, one diploid nucleus and two chloroplasts. One of the chloroplasts disintegrates during maturity. After a short period of activity, zygote comes to rest, retracts its flagella and secretes a wall around it.

The diploid zygote germinates within a few days. It does not undergo reduction division on germination. By repeated transverse divisions which are all mitotic, in a single direction, a simple filament is formed. It is attached to the substratum by a basal holdfast and is diploid. During further growth the cells of the filament divide both by transverse and vertical divisions to form the leaf like thallus of *Ulva*.

5.5 Life Cycle

There are two types of *Ulva* thalli: (i) haploid gametophytic or sexual plants and (ii) diploid sporophytic or asexual plants. The gametophytic plants produce haploid gametes by repeated mitotic divisions. Fusion between two gametes takes place and

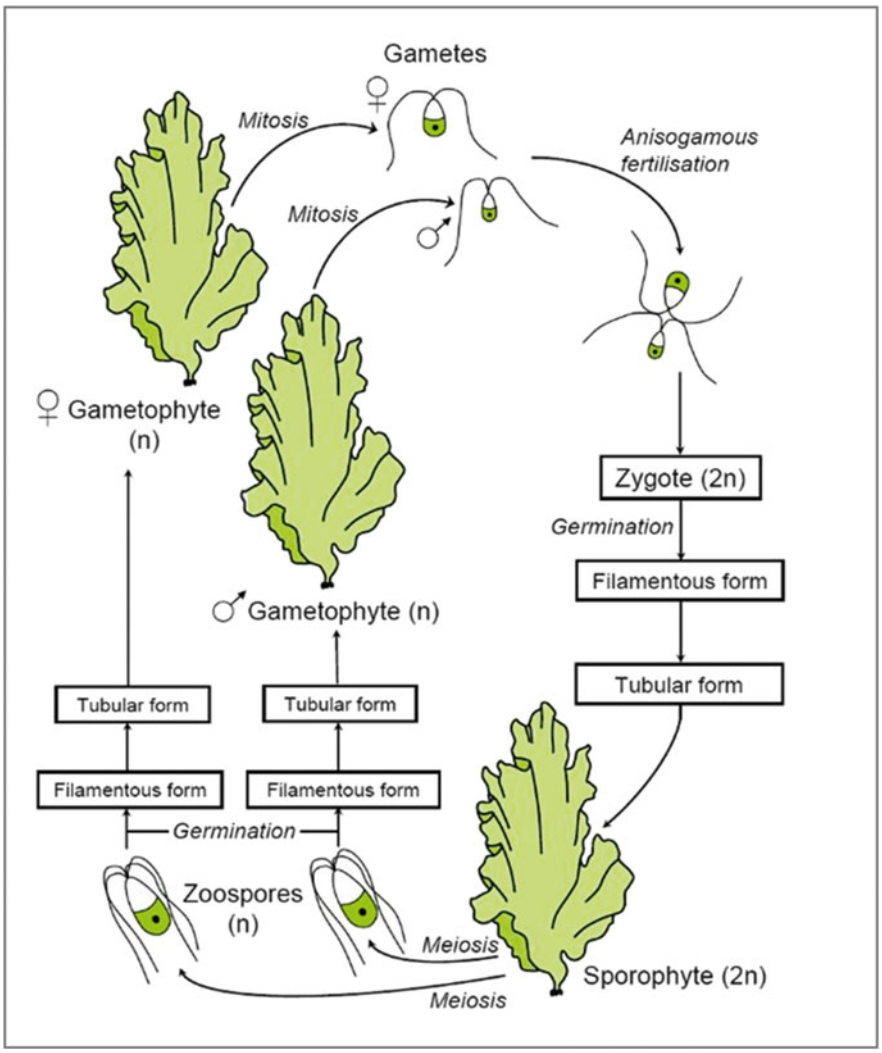

Fig. 16 Life cycle of *Ulva* (Picture courtesy: Dr. Christopher Skilbeck)

a diploid quadriflagellate zygote is formed which settles on a substratum and develops into diploid sporophytic thallus of *Ulva*. The morphology of these diploid plants resembles exactly with the haploid gametophytic plants.

Diploid sporophytic plants produce many spores and the first division of the diploid nucleus during spore formation is a reduction division resulting into the formation of haploid quadriflagellate zoomeiospores. These spores after liberation develop into haploid gametophytic thalli of *Ulva*. The life cycle is thus diplohaplontic showing isomorphic alternation of generations and the diploid sporophytic and haploid gametophytic generations come alternately (Fig. 16).

6 Acetabularia

6.1 Systematic Position

Fritsch (1935)	Bold and Wynne (1978)	Lee (2008)
Division – Algae	Division – Chlorophycophyta	Phylum – Chlorophyta
Class – Chlorophyceae	Class – Chlorophyceae	Class – Ulvophyceae
Order – Siphonales	Order –Dasycladales	Order – Dasycladales
Family–Dasycladaceae	Family –Dasychladaceae	Family – Dasychladaceae
Genus – *Acetabularia*	Genus – *Acetabularia*	Genus – *Acetabularia*

6.2 Introduction

The name of the genus is derived from the latin word "**acetabulum**" (a broad, shallow cup used for dipping bread); the inverted cap of *Acetabularia* bear a resemblance to such a cup and is popularly known as '**mermaid's wineglass**'. *Acetabularia* plant is shaped like an umbrella because of the unique shape of their disks and hence it is also called 'umbrella algae'. *Acetabularia* is one of the largest single-celled organisms (ranges from a few mm to 10 cm), and also has an extraordinarily large nucleus. Plant body is large in size and has a complex structure. These features make it an excellent model system for studying morphogenesis, cell biology, circadian rhythms and gene expression. Joachim Hammerling (1930s–1950s) studied the morphogenetic and biochemical aspects of *Acetabularia*, and demonstrated that nucleus of a cell contains the genetic information and directs the development, differentiation and general metabolism of the eukaryotic cell.

6.3 Occurrence

Acetabularia is a warm water alga found attached to stones, in shallow protected lagoons, on shell fragments, on the borders of seagrass beds, mangrove swamps and in shallow protected areas of coral reefs. The plant body of *Acetabularia* may grow singly or in groups.

6.4 Morphology and Ultrastructure

The unicellular plant body of *Acetabularia* is composed of three parts: a lobed rhizoidal holdfast which anchors the plant to the substrate; a middle upright tubular stalk; and a cap at the top which is composed of slightly concave disc of fused rays

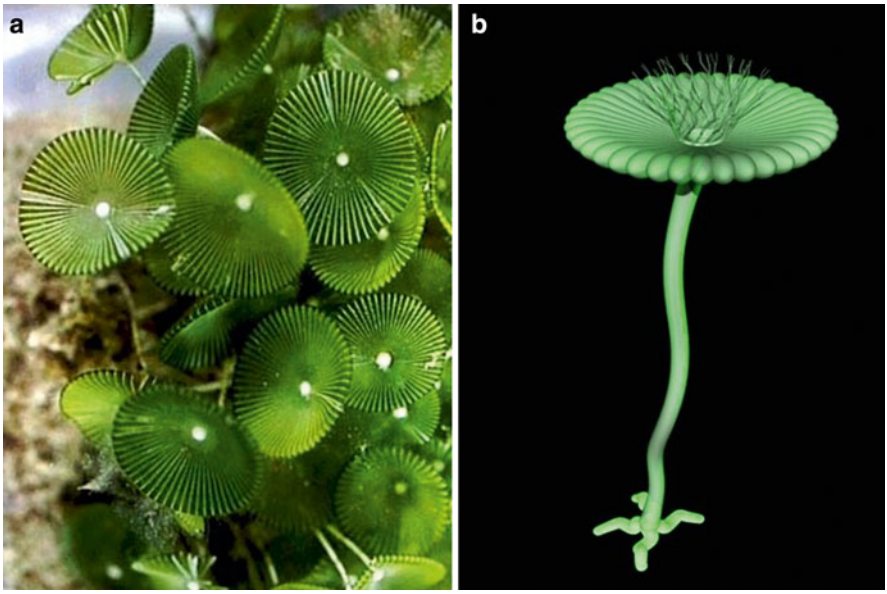

Fig. 17 *Acetabularia* thalli showing fertile disc, sterile hairs, stalk and rhizoidal system (Picture courtesy: (**a**) Prof. Isao Inouye, University of Tsukuba, Tsukuba, Japan, (**b**) Dr. Christopher Skilbeck)

having crenulate edge. The single nucleus of *Acetabularia* is located in the rhizoidal holdfast during the vegetative growth of the thallus and allows the cell to regenerate completely if its cap is removed. The middle long stalk grows out from the holdfast and produces a whorl of rays at its apex. The rays radiate from the center and a whorl of vertically arranged sterile hairs occur on the middle of the disc (Fig. 17). The stalks and rays are lime encrusted or calcified. Stalks grow upto 7.5 cm tall, and the discs can grow upto 2 cm in diameter.

Acetabularia cell contains two types of chloroplasts: long and globular. The long type of chloroplast is simple and has an external double membrane with peripheral lamellae and polysaccharide grains in the interior. The thylakoids of the globular (round type) of chloroplasts are poorly developed.

6.5 Reproduction

6.5.1 Vegetative Reproduction

Vegetative reproduction occurs by regeneration, fragmentation or development from basal rhizoid. *Acetabularia* can regenerate from sections of the parent plant.

6.5.2 Asexual Reproduction

Species of the *Acetabularia* reproduce asexually through cysts, which release flagellated, motile zoospores.

6.5.3 Sexual Reproduction

Sexual reproduction is the principal method of propagation in *Acetabularia*. A mature plant body forms many gametangial rays attached together at the apex of the thallus. Once the gametangial rays have reached full size, the single nucleus located in the rhizoidal lobe enlarges in size, undergoes meiosis and divides repeatedly resulting into the formation of many small secondary nuclei. These nuclei reach the gametangial rays by cytoplasmic streaming. In the gametangial rays cytoplasm contracts around each nucleus and a wall is synthesized resulting into the formation of a cyst. Mature plants release gamete-filled bright green cysts from the rays of the umbrella-shaped reproductive cap into the water column. The cysts have a small operculum that opens only when the gametes are ready to be liberated. Each cyst has biflagellate gametes of only one strain (+ or −). The cyst nuclei possess the same amount of DNA as the gametes, but half the amount as the zygote, showing that meiosis occurs prior to gamete formation (Koop 1979). Gametes of + or − strains (from the same thallus) fuse to form the zygote. The resulting zygote is able to germinate soon after meiosis to form a new *Acetabularia* plant, its nucleus gradually swell and develop into the giant primary nucleus.

6.6 Life Cycle

The life cycle of *Acetabularia* consists of haploid and diploid phases. *Acetabularia* has a dominant diploid generation. The unicellular plant body is diploid. The single diploid nucleus enlarges in size and the gametangial rays develop at the top of the cell. The nucleus undergoes meiosis and then divides repetitively resulting in the formation of many haploid nuclei (secondary nuclei) that reach the gametangial rays by cytoplasmic streaming. There, they secrete some cytoplasm and wall to form haploid cysts (the gametophyte), which undergo further mitotic divisions to form around 20 haploid nuclei. Mature rays, each with many bright-green cysts, release the cysts into the water column. The cysts have biflagellate gametes, which liberate through an operculum. The zygote is formed by the fusion of the biflagellate isogametes of different strains (+ and −) which are produced in the same thallus. Hence *Acetabularia* has a sexual life history that is isogamous. The zygote germinates to make a diploid uninucleate sporophyte. The life cycle of *Acetabularia* having a dominant diploid phase and a haploid phase which is only restricted to the gametes (gametic meiosis) is described as diplontic.

7 Oedogonium

7.1 Systematic Position

Fritsch (1935)	Bold and Wynne (1978)	Lee (2008)
Division – Algae	Division – Chlorophycophyta	Phylum – Chlorophyta
Class – Chlorophyceae	Class – Chlorophyceae	Class – Chlorophyceae
Order – Oedogoniales	Order – Oedogoniales	Order – Oedogoniales
Family – Oedogoniaceae	Family – Oedogoniaceae	Family – Oedogoniaceae
Genus – *Oedogonium*	Genus – *Oedogonium*	Genus – *Oedogonium*

7.2 Introduction

The name of the genus is derived from the Greek words "**oidos**" (swelling) + "**gonos**" (reproductive structures). *Oedogonium* is a filamentous alga (Fig. 18). *Oedogonium* thallus is an unbranched filament, which can be easily identified by characteristic rings at the apical ends of certain cells. Different species of *Oedogonium* vary in dimensions of the vegetative cells, structure of oogonia, zygote wall ornamentation, and position of antheridia (Prescott 1951). Some species of *Oedogonium* are edible and are consumed in a few subtropical areas. Occasionally, the alga can form conspicuous blooms.

Fig. 18 Filaments of *Oedogonium* (Image courtesy: Prof. Yuuji Tsukii, Hosei University, Japan)

7.3 Occurrence

Oedogonium is a submerged epiphytic freshwater alga. *Oedogonium* commonly occurs attached to the submerged plants and other substrates such as stone, wood etc. in freshwater ponds, pools, lakes, rivers and slow running streams. The filaments remain attached to the substratum with the help of a basal cell called holdfast, which is specially differentiated for this purpose. Young filaments remain attached to substratum, whereas older filaments of *Oedogonium* get detached and freely float on the water surface. *Oedogonium* is more common in standing or stagnant water than that of running water and forms green silk or scum of standing water. Some *Oedogonium* species are terrestrial also e.g., *O. terrestris* and *O. randhawae* grow on moist soil. *O. oblengellum*, *O. cardiacum*, *O. tenuis* and *O. elegans* are some common species of *Oedogonium*.

7.4 Organization of Thallus

7.4.1 Morphology

The plant body is a thallus showing apical basal polarity. Thallus is a long unbranched filament made up of a single row of elongated cells placed end to end and are connected by plasmodesmata. The filament has three types of cells:

1. A club shaped basal cell or holdfast
2. Dome shaped apical cell
3. Intercalary cells

The filament is generally attached at the lower end by means of a basal cell or holdfast which is achlorophyllous and is expanded into a flattened disc with finger like projections. In terrestrial species the holdfast shows rhizoid like outgrowths. The free end of the terminal cell at the upper part of the filament is broadly rounded forming a dome shaped apical cell which is chlorophyllous. The apical cell does not show division. Cells present between the basal cell and apical cell are termed as intercalary cells. A distinctive feature of *Oedogonium* is the presence of characteristic fragile rings at the apical ends of some intercalary cells (Fig. 19). These rings are formed at the time of cell division and are called apical rings or apical caps. Only those cells which have the apical rings divide again and the cells that have undergone many such divisions display several rings.

7.4.2 Cell Structure

Cells of the vegetative filament are elongated and cylindrical with a rigid cell wall enclosing the protoplast. The cell wall (except that of the holdfast) is differentiated into three layers. The outer layer is formed of chitin, middle layer of pectin material

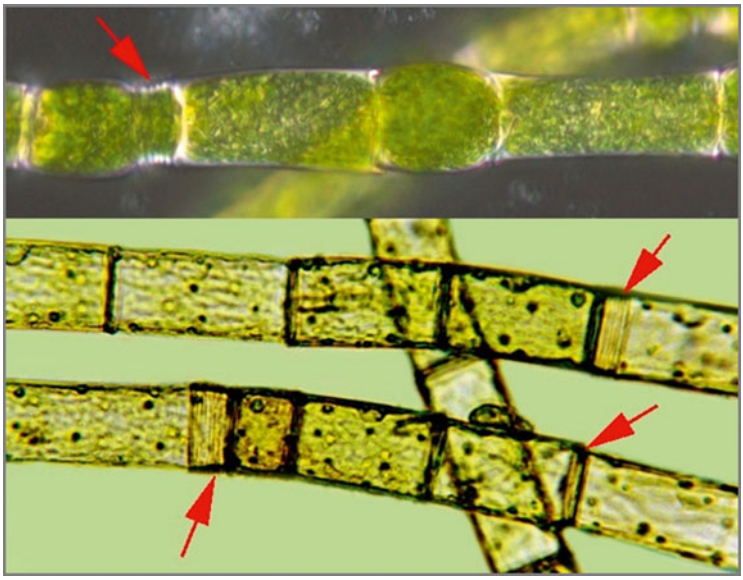

Fig. 19 Filaments of *Oedogonium* showing cap cells (*arrows*) (Image courtesy: Phil Novis, Allan Herbarium, Landcare Research New Zealand Ltd)

and inner cellulose layer. The outer chitinous layer prevents the dissolving away of the pectic layer therefore the filaments remains wet. Inner to the cell wall is present a plasma membrane. The plasma membrane encloses the cytoplasm, a single large parietal chloroplast with a number of pyrenoids, a nucleus and other membrane bound organelles such as mitochondria, Golgi bodies and endoplasmic reticulum. There is a large central vacuole filled with cell sap. In *Oedogonium*, the chloroplast is a large, reticulate structure made up of hollow cylindrical network with meshes (meshes may be broad or narrow). The chloroplast is parietal in position and extends from one cell to the other. The chloroplast of *Oedogonium* contains microtubules which provide support to the large chloroplast. Pyrenoids are present at the anastamoses or intersecting points of the meshes of the reticulate chloroplast. Pyrenoids accumulate starch and are surrounded by starch sheath. A series of transverse parallel fragile rings run across the apical ends of intercalary cells. These rings are formed as a result of cell division and the cells with these rings are known as cap cells.

7.4.3 Growth and Cell Division in *Oedogonium*

Oedogonium exhibits a unique mode of cell division (Fig. 20) which results into the formation of ring like scars near the anterior end of a cell.

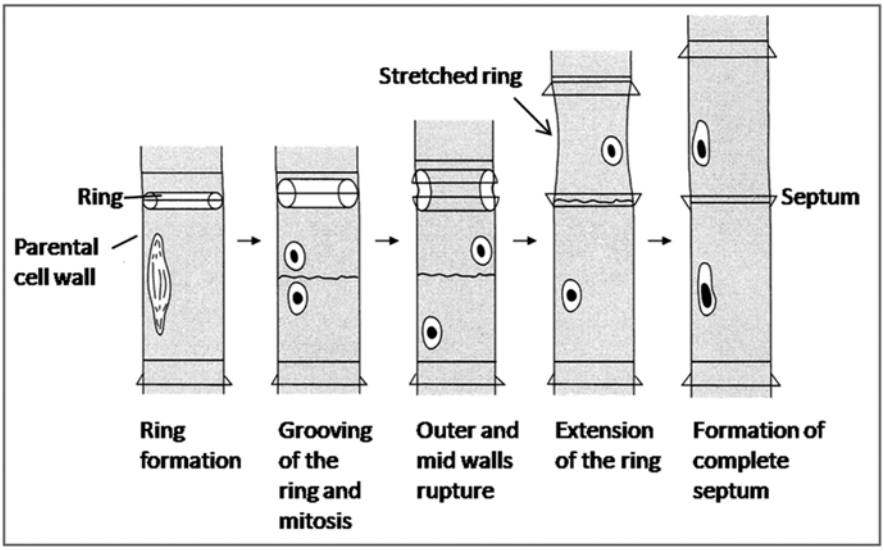

Fig. 20 Various stages of cell division in *Oedogonium* (Source: Pickett-Heaps 1975)

1. Once a cell is ready to divide, two main events occur at the initiation of a division. (i) Movement of the peripherally located nucleus into the centre of the cell. (ii) A transverse thickened ring like ingrowth of the wall material appears from the inner face of the lateral wall in the anterior portion of the cell. The ring is formed by intussusception (the increase in the surface area of a cell due to the accumulation of new wall material between the existing materials of the cell wall).
2. The ring gradually increases in thickness and turns into a grooved structure due to the continuous buildup of wall material transported from the Golgi-derived vesicles (Hill and Machilis 1968; Pickett-Heaps and Fowke 1970). About the same time, the nucleus migrates towards the upper part of the cell. Here the nucleus begins to divide mitotically.
3. Nuclear division is followed by formation of an incomplete septation of floating cytoplasmic strands between the two daughter nuclei. This results into the formation of a floating septum which is not connected with the lateral walls.
4. After some time the outer and middle wall layers of the mother cell external to the grooved inner wall ring rupture due to the gradual increase in the size of the ring. The grooved ring expands completely and forms a cylindrical structure.
5. By the expansion of the inner wall, the parent cell elongates to approximately double its length. Progressively the floating septum between the two daughter nuclei migrates upwards to the base of the daughter cell and develops into a complete septum of mature cross wall. Consequently the process of cell division is completed resulting into the formation of two daughter cells. The wall of the upper cell is completely made up of the extended inner wall material.

6. The remnants of the ruptured parent cell wall form an apical cap at the upper end of the newly formed daughter cell and a bottom sheath formed by the ruptured portion of the parent wall on the lower part of the same daughter cell.

Cell division takes place only in those cells which have cap-like apical rings or apical caps. These cells are called cap cells. Therefore, after each cell division one of the daughter cells (the upper one) divides actively, whereas the other daughter cell remains quiescent. A new cap develops after each cell division. Thus the number of caps on a cell denotes the number of times a cell has divided.

7.5 Reproduction

Oedogonium reproduces by vegetative, asexual and sexual methods.

7.5.1 Vegetative Reproduction

It occurs by fragmentation and akinete formation. Fragmentation takes place by dying out of some intercalary cells, due to accidental breaking of the filament, through the conversion of intercalary cells into zoosporangia or gametangia, or in adverse environmental conditions. In some species the vegetative akinetes are developed during unfavorable conditions for vegetative growth. Under favorable conditions akinetes germinate into new filaments.

7.5.2 Asexual Reproduction

Asexual reproduction in *Oedogonium* takes place by the formation of stephanokontean type (numerous short flagella arranged in the subapical portion) of zoospores (Fig. 21). Around 120 flagella have been observed in a zoospore of *O. cardiacum*

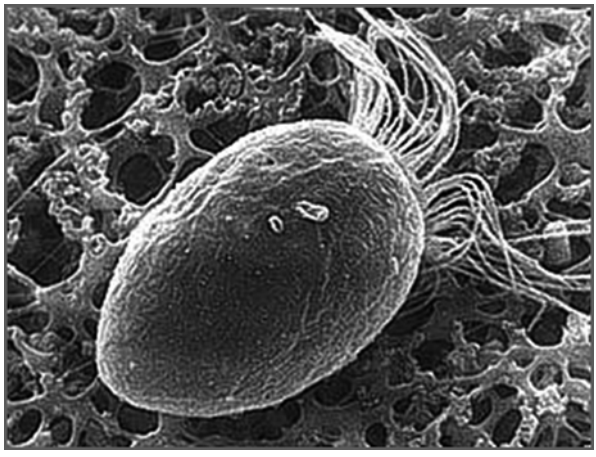

Fig. 21 *Oedogonium* zoospore (SEM) with stephanokont flagella (numerous small flagella placed in the subapical portion) (Source: Pickett-Heaps 1975)

(Hoffman and Manton 1962). All cap cells of the filament are capable of producing zoospores. The cap cell becomes a zoosporangium and zoospore is produced singly inside the zoosporangium. The zoosporangial cell is usually rich in food materials. At the time of zoospore formation the entire protoplast of the zoosporangium withdraws from the cell wall as a single unit. Its nucleus retracts somewhat and moves towards one side. The entire protoplast rounds up. On one side, a hyaline region appears adjacent to the nucleus (in between the cell wall and the nucleus). Along the margin of this hyaline region a circular ring of blepharoplast granules is developed. One single flagellum develops from each of these granules thus forming a ring of flagella and the entire structure becomes a multiflagellate zoospore. Once the zoospore is fully developed, it breaks the cell wall of the zoosporangium transversely in the cap region and starts to come out in a thin mucilaginous vesicle. The vesicle dissolves soon and the zoospore escapes in water within a few minutes from the vesicle. The released zoospore is a green ovoid or pear shaped structure and have an eyespot, a chloroplast and many contractile vacuoles. The zoospore swims for about an hour and then settles down on some solid substratum with its colourless anterior side (flagellar side with the hyaline spot) downwards. At this stage flagella are withdrawn and the structure elongates considerably. This elongated structure divides transversely by an apical ring and the apical (upper) cell forms a new filament by repeated cell divisions.

7.5.3 Sexual Reproduction

Sexual reproduction in *Oedogonium* is always advanced oogamous. There are distinct male and female gametes showing a drastic difference in their structure and function. The sexual gametes are formed in specific reproductive organs called gametangia. These gametangia are formed by the modification and differentiation of certain vegetative cells of the filament (Fig. 22). Male gametangium is termed as antheridium and the female gametangium is called the oogonium. The species can be homothallic or heterothallic. Sexual reproduction may be of macrandrous or nannandrous type depending upon the pattern of distribution of sex organs (Fritsch 1935). Sexual reproduction is of common occurrence in still water and rarely occurs in flowing waters.

1. **Oogonia**: Oogonia are borne on normal filaments and develop by terminal or intercalary oogonial mother cells. The oogonial mother cell divides transversely into two daughter cells. The upper daughter cell always develops into an oogonium, whereas the lower daughter cell forms the supporting cell or 'suffultory cell'. Oogonium contains a large non-motile spherical egg cell in it. The egg cell is uninucleate with a centrally placed nucleus and is green in colour because of the presence of chlorophyll. On maturity the nucleus migrates to the periphery of the egg cell and a hyaline spot called receptive spot is visible near the upper end. Oogonium has one or more distinct caps on its upper end. Mature oogonium develops a small slit or pore near its upper end above the receptive hyaline spot.

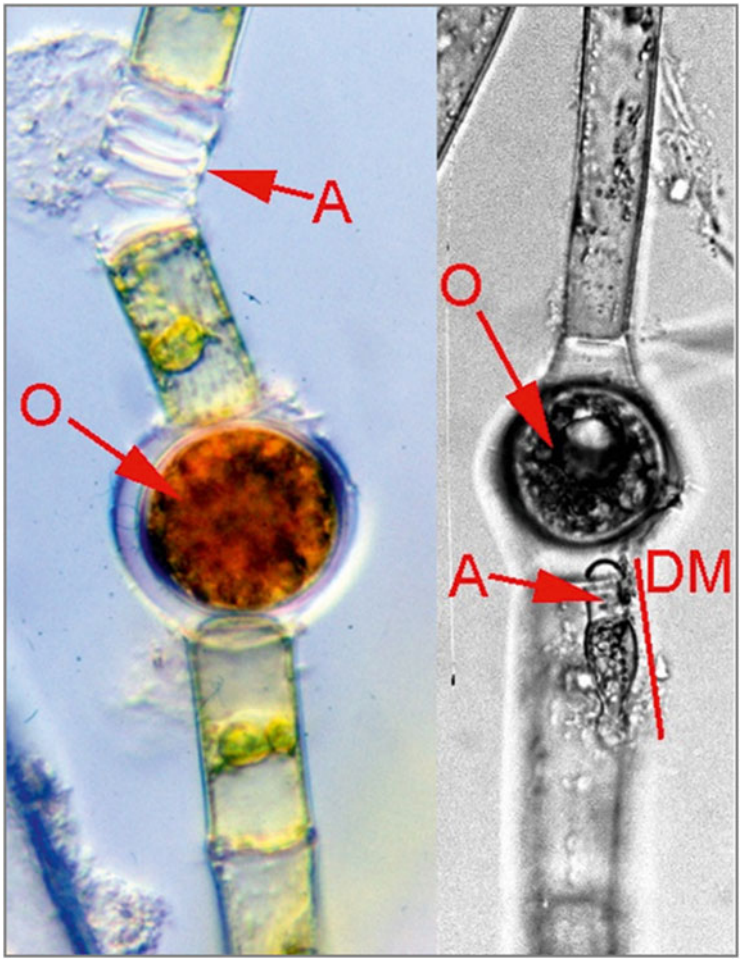

Fig. 22 A dioecious filament of *Oedogonium* showing oogonia (*O*) and antheridia (*A*) and Dwarf male (*DM*) (Image courtesy: Phil Novis, Allan Herbarium, Landcare Research New Zealand Ltd)

2. **Antheridia:** The filament is classified into two types on the basis of structure and position of antheridia: Macrandrous and nannandrous type.

   Macrandrous Type: The antheridia are borne on the filaments of normal size. In macrandrous monoecious species oogonia or antheridia develop on the same filament (monoecious or homothallic or bisexual) e.g., *O. nodulosum*. In macrandrous dioecious antheridia and oogonia are borne on two different or

separate filaments (dioecious or heterothallic or unisexual) e.g., *O. aquaticum*. The antheridia are produced by the rapid and repeated transverse division of antheridial mother cell. Any cap cell of the filament can function as antheridial mother cell. This cell divides transversely into two unequal cells, the upper smaller antheridial cell and a lower larger sister cell. The lower sister cell divides repeatedly resulting into the formation of a row of flat cells and a chain of 2–40 uninucleate antheridia is produced. The nucleus of each antheridium divides mitotically into two nuclei. These nuclei are enclosed by some cytoplasm and metamorphose to form two antherozoids or sperms. The antherozooids are unicellular, uninucleate and multiflagellate structure which resemble zoospores in structure and shape only with the difference that they are somewhat smaller in size and with few numbers of flagella. Unlike zoospores, antherozoids are yellowish because of their reduced plastids. The antherozooids liberate in the same way as zoospores coming out by transverse splitting of the wall of the antheridium and are enclosed in thin hyaline mucilaginous vesicles.

Nannandrous Type: The antheridia are produced on special very small 2–4 celled filaments called dwarf males or nannandria. These dwarf males grow epiphytically attached to the female filaments. Hence nannandrous species are always heterothallic or dioecious, i.e. antheridia and oogonia develop on separate filaments. A dwarf male is produced by germination of a particular type of spore called androspore. The development of androspores takes place within androsporangia. In nannandrous species if the androsporangia and oogonia are borne on same filament, the species is called 'gynandrosporous'. If the androsporangia and oogonia are borne on different filaments, the species is called 'idioandrosporous' 'Androsporangia' are produced by repeated transverse divisions of the vegetative cells of the *Oedogonium* filament. In each androsporangium develops an androspore.

The androspore is a unicellular, uninucleate and multiflagellate structure and is similar to zoospores in shape and structure. Androspores are rather smaller than zoospores and bigger than antherozooids. On being liberated from the androsporangium, the androspores are enclosed in a thin vesicle which soon disappears and the androspore swims briefly. Following their brief swarming period, androspores attach either to the oogonium or to one of the adjacent cells by its anterior end (Tiffany 1957). Then the androspore germinates by undergoing elongation and cuts off one or more flat cells and finally develops into a dwarf male or nannandria. The tip cells or flat cells of nannandria are called antheridia. Hence the nannandrium consists of one basal cell attached to the oogonium or adjacent cell, and one or more flat cells, the antheridia. The nucleus of each antheridium undergoes mitotic division to form two nuclei which along with some cytoplasm metamorphose into two **antherozoids**. These antherozoids are similar in structure to the antherozoids produced by macrandrous species. Antherozoid and egg fuse to form a diploid zygote.

3. **Fertilization** occurs both in macrandrous and nannandrous species by the swimming antherozoid or sperm which approaches the oogonium at the receptive hyaline spot. The antherozoid swims through the pore or slit in the oogonial wall. The antherozoids are attracted chemotactically towards the oogonium due to the extrusion of some chemical substance by the mature egg (Hoffman 1960). After entering into the oogonium through the pore, only one antherozoid (the one which arrives first) penetrates the egg at the hyaline receptive spot and the subsequent entry of any other antherozoid is prevented by the egg membrane.

   The flagella of the antherozoid are retracted and plasmogamy takes place, which is soon followed by fusion of male and female nucleus resulting into the formation of a diploid zygote.

   Shortly after fertilization the zygote retracts from the oogonial wall and secretes thick three layered wall around it. When the zygote is young it has a green colour due to the presence of chloroplast but as it passes into the resting stage, its colour change from green to red or brown. The zygote liberates by the decay of oogonial wall and undergoes a period of rest which varies from 12 to 14 months.
4. **Germination of Zygote**
   The zygote germinates under favorable conditions. Prior to germination, during the resting period diploid nucleus of the zygote undergoes meiotic division resulting into the formation of four haploid nuclei. Hence zygotic meiosis re-establishes the haploid nucleus of the vegetative stage of the *Oedogonium*. The haploid protoplast turns green and divides into four daughter cells. Each of the daughter protoplasts acquires a crown of flagella and metamorphoses into a meiozoospore. These meiozoospores are liberated by rupturing the zygote wall and remain enclosed in a vesicle which soon disappears. These meiozoospores resemble with the vegetatively produced zoospores. The liberated multiflagellate meiozoospores swim freely in water for sometime and then settle on some substratum by their anterior ends, withdraw their flagella and germinate into new haploid vegetative filaments of *Oedogonium*. In macrandrous diecious species out of the four meiozoospores, two meiozoospores germinate into male filaments and the remaining two germinate into female filaments.

7.6 Life Cycle

Oedogonium has a haplontic life cycle. The filamentous plant body in all species is always haploid. Haploid male and female gametes fuse to form a diploid zygote. Zygote is the only diploid stage in the life cycle. The zygote soon undergoes meiosis to form haploid meiozoospores. The meiozoospores germinate into new haploid filaments.

7.6.1 Life Cycle of Macrandrous Monoecious Species

The antheridia and oogonia develop on the same plant of normal size and produce haploid male gamete (antherozoids) and haploid female gamete (egg). Both these gametes fuse to form a diploid zygote. The zygote divides by meiotic division to form four haploid meiozoospores. These meiozoospores germinate into new haploid filaments of *Oedogonium*.

7.6.2 Life Cycle of Macrandrous Dioecious Species

The antheridia and oogonia develop on two separate haploid filaments of normal size and produce haploid male gamete (antherozoids) and haploid female gamete (egg) in separate filaments. Both these gametes fuse to form a diploid zygote. The zygote divides by meiotic division to form four haploid meiozoospores. Out of these four meiozoospores, two germinate into male filaments and the other two into female filaments.

7.6.3 Life Cycle of Nannandrous Gynandrosporous Species

A few cells of the haploid filament of *Oedogonium* divide repeatedly to form a row of androsporangia. The same filament also bears the oogonium. In each androsporangium develops an androspore. The androspore is released and settles either on oogonium or on the suffultory cell of the filament. After getting settled the androspore develops into a short filament which is called dwarf male or nannandrium. Tip cell of the dwarf male develop into antheridia, in each of which develop two antherozoids. The antherozoids are liberated, fuse with eggs and develop into zygotes. The zygote divides by meiotic division to form four haploid meiozoospores. These meiozoospores germinate into new haploid filaments of *Oedogonium*.

7.6.4 Life Cycle of Nannandrous Idioandrosporous Species

Oogonium and androsporangia develop on two separate haploid filaments. In each androsporangium develops an androspore. The androspore gets liberated and settles either on oogonium or on the suffultory or supporting cell of the female filament, and develops into dwarf male filament. Tip cells of nannandria develop into antheridia. Each antheridia produce two antherozoids. Antherozoid and egg fuse to form a diploid zygote. The zygote divides by meiotic division to form four haploid meiozoospores. Out of these four meiozoospores, two germinate into androsporangiate male filaments and the other two into oogonia producing female filaments.

8 Spirogyra

8.1 Systematic Position

Fritsch (1935)	Bold and Wynne (1978)	Lee (2008)
Division – Algae	Division – Chlorophycophyta	Phylum – Chlorophyta
Class – Chlorophyceae	Class – Chlorophyceae	Class – Charophyceae
Order – Conjugales	Order – Zygnematales	Order – Zygnematales
Family– Zygnemaceae	Family – Zygnemataceae	Family – Zygnemataceae
Genus – *Spirogyra*	Genus – *Spirogyra*	Genus – *Spirogyra*

The name of the genus is derived from the Greek words "**speira**" (coil) + "**gyros**" (twisted). *Spirogyra* is a freshwater filamentous alga and the filaments are usually free floating. *Spirogyra* is commonly known as "water silk" or "pond scum".

8.2 Morphology and Ultrastructure

Spirogyra thallus is a green, unbranched filament composed of a row of cells having 1–16 spiral type or ribbon-shaped chloroplasts per cell (Fig. 23). The cells are usually longer than broad. The cell wall in *Spirogyra* is made up of two layers; an outer pectic layer and an inner cellulose layer. Pectin from the outermost layer dissolves and provides a slippery texture to the filaments. The chloroplasts are in the form of left-handed spirals whose edges are beautifully sculptured. The nucleus is suspended in the centre of the cytoplasm with the help of cytoplasmic strands. Numerous pyrenoids are present in the chloroplasts. *Spirogyra* cell also contains several mitochondria, endoplasmic reticulum and dictyosomes. In a few species rhizoidal processes or haptera are present at the basal end of the filament and help in the attachment to the substrates. Cytoplasmic streaming can often be observed in the peripheral cytoplasm.

8.3 Reproduction

Spirogyra reproduces mostly by vegetative method of fragmentation, or sexually by the process of conjugation. Asexual reproduction takes place only in some species of *Spirogyra*.

8.3.1 Vegetative Reproduction

Vegetative reproduction takes place by fragmentation and akinete formation.

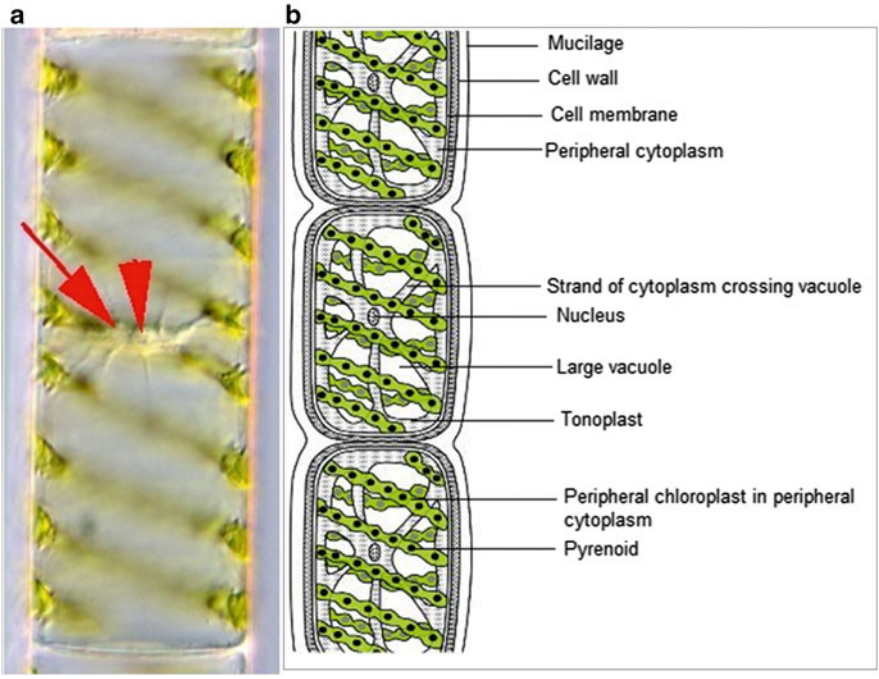

Fig. 23 *Spirogyra* filament (**a**) Light micrograph showing chloroplast and nucleus (*arrow*), (**b**) Diagrammatic (Image courtesy: (**a**) Phil Novis, Allan Herbarium, Landcare Research New Zealand Ltd and (**b**) Dr. Christopher Skilbeck)

8.3.2 Asexual Reproduction

Asexual reproduction occurs in some species by producing a few kinds of spores i.e., aplanospores, parthenospores and akinetes. Zoospore formation has not been observed in *Spirogyra*.

8.3.3 Sexual Reproduction

Sexual reproduction takes place by means of conjugation. Nitrogen depletion and light intensity have been suggested as major factors for the induction of conjugation (Yamashita and Sasaki 1979; Simons et al. 1984). The increased carbohydrate concentration in the conjugating cells suggests that light may have a role in increase of intracellular carbohydrates, which are necessary for the formation of zygospore (Yamashita and Sasaki 1979).

Both scalariform and lateral conjugations have been observed (Fig. 24). During conjugation, previously bright green filamentous masses turn noticeably brownish in colour, reflecting the loss of chlorophyll pigments from zygotes and development of brown zygote walls.

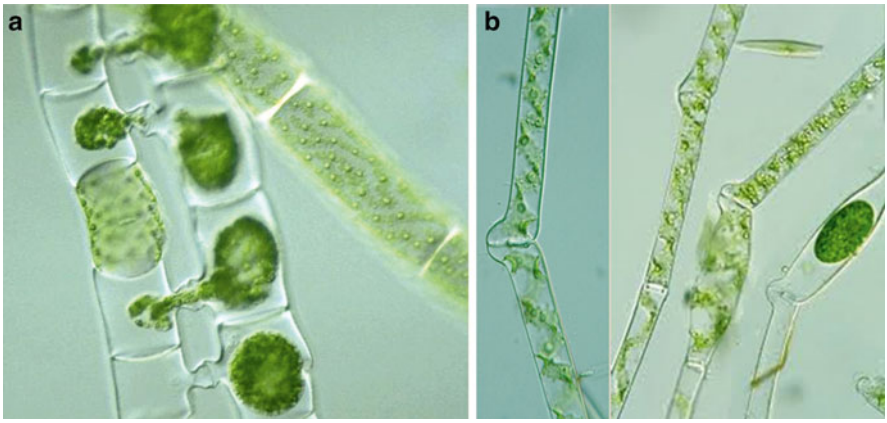

Fig. 24 *Spirogyra* filaments (**a**) Scalariform conjugation (**b**) Lateral conjugation (Image courtesy: Prof. Yuuji Tsukii, Hosei University, Japan)

Scalariform Conjugation The conjugation takes place between two cells of opposite filaments. It is very common in most of the species of *Spirogyra*. During scalariform conjugation, the filaments lie parallel to each other in common mucilage. At many points of proximity, opposite cells produce peg like outgrowths. They produce tube like structures, which soon come in intimate physical contact, dissolve, forming a passage between the opposite cells. Thus a conjugation tube is formed. Meanwhile the entire protoplast of each cell shrinks and is rounded up into a single non-flagellate gamete. One of the gametes moves from its cell into the opposite one by an amoeboid movement. The migrating gamete is the − type or the male one and the other stationary gamete is the + type or the female one, the cells forming them being called as male or female respectively. The male gamete after moving into the female cell, fuses with its gamete forming the zygote.

Lateral Conjugation It takes place between two adjacent cells of the same filament. It is not so common, but in *S. affinis* and *S. tenuissima* it is almost a rule. In this type a lateral protrusion is produced by cell of a filament towards another cell present immediately next in the same filament. Once the lateral peg comes in physical contact with the neighbouring cell, the septum dissolves and a passage is established between adjacent cells. Male gamete moves into the female cell through the conjugation tube and fuses with its gamete, forming the zygospore. After lateral conjugation female cell having a zygospore is always found to be adjacent to an empty male cell.

The zygospores are thick walled structures which may undergo a prolonged resting period. The zygospore liberates by decay of the wall of the female cells. The diploid nucleus of the zygospore divides reductionally before the germination of zygospore. Four haploid nuclei are formed as a result of the meiotic division, of which only one survives. The zygospore with a single haploid nucleus germinates and gives rise to a new filament of *Spirogyra*.

8.4 Life Cycle

The life cycle of *Spirogyra* consists of haploid and diploid phases. The unbranched multicellular filament is haploid. It produces haploid gametes. The gametes are non-flagellate and nonciliate (aplanogametes). Sexual reproduction in *Spirogyra* occurs by conjugation. Lateral conjugation takes place between two adjacent cells of the same filament. Haploid male gamete moves into the female cell through the conjugation tube and fuses with its haploid gamete, forming a diploid zygospore. The scalariform conjugation takes place between cells of two opposite filaments; hence, the species are heterothallic. In this case, two physiologically different filaments lie parallel to each other. One of the gametes (male, −) moves from its cell into the opposite one through conjugation tube by an amoeboid movement. The male gamete after moving into the female cell, fuses with its gamete (female, +) forming the diploid zygote. Hence diploid phase is represented by a one-celled zygote. The diploid zygospore divides meiotically to form four haploid nuclei, of which only one survives. The zygospore with a single haploid nucleus germinates to form a new filament of *Spirogyra*. The life cycle of *Spirogyra* having haploid *phases* represented by multicellular vegetative filament and gametes and a diploid phase which is only restricted to the zygote (zygotic meiosis) is described as haplontic.

9 Coleochaete

9.1 Systematic Position

Fritsch (1935)	Bold and Wynne (1978)	Lee (2008)
Division – Algae	Division – Chlorophycophyta	Phylum – Chlorophyta
Class – Chlorophyceae	Class – Chlorophyceae	Class – Charophyceae
Order – Chaetophorales	Order – Chaetophorales	Order – Coleochaetales
Family– Coleochaetaceae	Family – Coleochaetaceae	Family – Coleochaetaceae
Genus – *Coleochaete*	Genus – *Coleochaete*	Genus – *Coleochaete*

The name of the genus is derived from the Greek words “koleon” (sheath) + “chaetos” (hair). One of the most prominent feature of *Coleochaete* is the presence of sheathed hairs or ‘seta cells’ which are supposed to serve as protection against herbivores. Broken setae exude a substance which deters potential predators.

9.2 Occurrence

Coleochaete is a freshwater alga growing epiphytically on the submerged portions of aquatic angiosperms such as *Potamogeton*, *Nelumbo*, *Lemna*, *Trapa*, *Ipomoea*, *Polygonum*, *Typha*, *Sagittaria*, *Nymphaea*, *Hydrilla* etc. *Coleochate* grows

epiphytically upon other algae such as *Chara*, *Vaucheria*, *Oedogonium* etc. and can also grow endophytically within Charales such as *C. nitellarum* in the cells of *Nitella* and *Chara*. In addition they grow on non-living substrates in the vicinity of the boundaries of freshwater lakes and ponds. There are about ten species reported in this genus out of which three species namely *C. orbicularis*, *C. scutata* and *C. nitellarum* are common in India. Almost all of the species are fresh water in habitat.

9.3 Organisation of Thallus

Coleochaete is a remarkable genus exhibiting an unusual degree of thallus variability. Species in this genus are thalloid, multicellular, branched and show a variety of plant body types.

(i) *C. pulvinata* is a heterotrichous form in which the plant body is divided into erect and prostrate systems. A few algal filaments are placed prostrate on the substratum, whereas others stand upright forming a projecting system which is cushion like. Thalli of *C. pulvinata* are covered with a mucilaginous material.
(ii) In *C. divergens* and *C. nitellarum* only the prostrate system is present. It is represented by loosely set branching threads.
(iii) In discoid forms such as *C. scutata* and *C. orbicularis* only the prostrate system is present and is represented by laterally fused filaments which form a pseudoparenchymatous circular disc which resembles that of plant tissues (Fig. 25).

In all these types of thalli certain cells have single long unbrached cytoplasmic seta.

9.4 Cell Shape and Structure

The outline of the cell differs in prostrate and erect systems. In prostrate systems it is generally hexagonal or polygonal, whereas in erect systems cells are longer than broad. The cells are uninucleate having a single curved parietal chloroplast with one or more pyrenoid. The vegetative cells bear long cytoplasmic setae projecting right angles to the thallus. These cells are termed as seta cells and are specialized in their structure, i.e. their walls are composed of several layers and their chloroplasts have a C-shaped appearance. Base of the Seta is partly or wholly ensheathed by a gelatinous material. All or only certain cells of the thallus may have these setae and the development of a seta is due to a blepharoplast which lies just underneath a minute pore or opening in the outer cell wall. The cytoplasmic contents of the cells extend through the opening in the shape of a thread which eventually forms the bristle or seta.

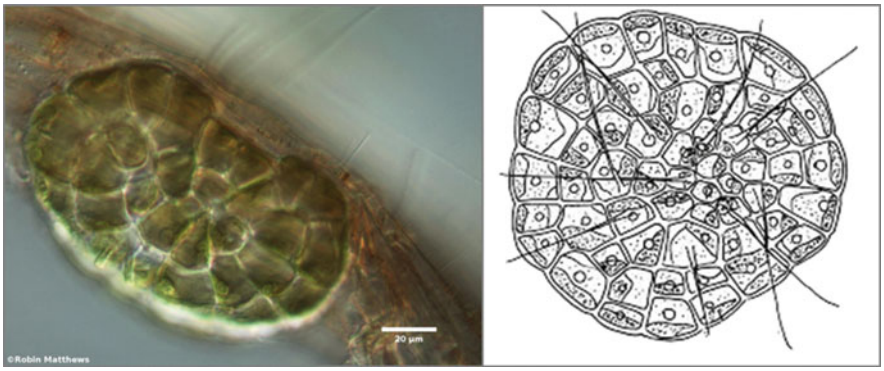

Fig. 25 External features of *Coleochate scutata* (Image courtesy: Phil Novis, Allan Herbarium, Landcare Research New Zealand Ltd)

9.5 Reproduction

Coleochaete reproduces both asexually or sexually.

9.5.1 Asexual Reproduction

Coleochaete produce biflagellate zoospores which lack eyespots and are formed singly within a cell. Zoospores generate new thalli of the same type. Zoospore formation can occur at any time of the year though it occurs frequently in spring or in early summer. Zoospores get discharged by moving in an amoeboid manner through a specialized pore in the parental cell wall which arises by the localized action of hydrolytic enzymes on the cell wall. They swim briefly and later come to rest. They retract their flagella and secrete a wall around it. This one-celled germling begins to divide and soon develops into a multicellular thallus characteristic of that particular species. Thick walled aplanospores may also be formed under unfavourable conditions and are produced singly within a cell.

9.5.2 Sexual Reproduction

Coleochaete also reproduce sexually by oogamous sexual reproduction and according to the species the plants may be heterothallic (dioecious) or homothallic (monoecious). The male sex organs are called antheridia and the female oogonia. In heterotrichous species i.e., *C. pulvinata* the antheridia are borne terminally in groups at the tips of the branches of the projecting system. The antheridia in *C. scutata* are developed from the intercalary cells situated midway between centre and margin of the discoid thallus. In this species parent intercalary cell divides into two daughter cells one of which functions as the antheridial mother cell and redivides to form antheridia. Each antheridium produces a single biflagellate green or colorless antherozooid or spermatozoid. Spermatozoid contains an anterior multilayered

structure (MLS) (Graham and McBride 1979) which is similar to that present in the land plants (Graham and Wedemayer 1984).

In *C. pulvinata* the oogonia are also borne terminally on the branches of the erect projecting system. Egg cells or oogonia of *Coleochaete* are not liberated from the thallus. They develop a cell wall protuberance in the form of a long neck called trichogyne. The oogonium of *C. pulvinata* is a flask shaped structure with a basal enlarged portion and a long colorless trichogyne. Trichogyne is filled with cytoplasmic contents. Oogonia are formed from marginal cells of the discoid thallus. Oogonia of *C. orbicularis* have a relatively short trichogyne, whereas it is inconspicuous in *C. scutata*. The tip of the trichogyne disintegrates when the egg is ready for fertilization and exudes cytoplasmic contents that come out to attract flagellated sperm.

Fertilization occurs by an antherozooid swimming towards the opened neck of an oogonium. Flagellated sperm appears to be attracted chemotactically by the cytoplasmic contents oozed out from the opened tip of the trichogyne. One antherozoid enters through the neck. The male and female gametes fusing to form a zygote have nuclei of fairly different size. However, as the male gamete move towards the female nucleus it increases significantly in size and at the time of fusion, they are roughly equal sized.

Post-fertilization Changes After fertilization the basal portion of the oogonium increases in size following the formation of a septum which cuts it off from the trichogyne. The fertilized egg or zygote stays within the oogonium, secretes a thick wall, and increases greatly in size. Meanwhile there is an upgrowth of filaments from the cell underlying the oogonium and from adjacent vegetative cells to form a parenchymatous layer that completely encloses the oogonium. In *C. orbicularis* the parenchyma cells that are connected with zygote bear a resemblance to placental transfer cells which are widely present in embryophytes (Graham and Wilcox 1983). The mature oogonium covered with its continuous sheath of cells rapidly becomes reddish brown in colour and is called a spermocarp. The spermocarp perennates over the winter season.

Germination of Zygote Under favourable conditions, the protoplast of the zygospore turns green and divides by meiotic division to re-establish the haploid state of the vegetative thallus. Meiosis is followed by mitosis until 8–32 daughter protoplasts are formed and then each of the daughter protoplast is metamorphosed into a biflagellate meiozoospore. The zoospores escape out by rupturing of the spermocarpic and zygosporic walls into two halves. The liberated meiozoospores swim for a short time and then withdraw their flagella. Zoospores come to rest and secrete a wall around them. They germinate directly and give rise to new thalli.

9.6 Life Cycle

The life cycle of *Coleochaete* indicates that it consists of both haploid and diploid phases. The filaments comprising the adult plant are haploid, bear haploid male and female sex organs (antheridia and oogonia) which ultimately bear haploid gametes.

This represents the gametophytic or sexual phase in the life cycle. Haploid male and female gametes fuse to form a diploid zygote and this is the only diploid structure in the life cycle. The diploid nucleus of the zygote undergoes meiosis and then mitosis resulting into the formation of 8–32 haploid meiozoospores. The meiozoospore on germination gives rise to the haploid gametophytic plant. The life cycle of *Coleochaete* having a haploid phase represented by adult plant/thallus and a diploid phase which is only restricted to the zygote (zygotic meiosis) is described as haplontic.

9.7 Coleochaete *and the Origin of Land Plants*

Coleochaete is considered as one of the most advanced members of the green algae and is believed as progenitor of land plants because of the following reasons:

(i) Presence of phragmoplast
In *C. scutata*, a phragmoplast present during cytokinesis is similar to that of embryophytes (Marchant and Pickett-Heaps 1973).

(ii) Glycolate oxidase
Coleochaete produces glycolate oxidase as do land plants (Frederick et al. 1973).

(iii) Presence of MLS
In *C. scutata*, zoospores possess a multilayered structure (MLS) similar to those present in land plant spermatozoid (Graham and McBride 1979; Graham and Wedemayer 1984).

(iv) Retention of zygote
The zygote is retained on the parent plant and undergoes further development as seen in embryophytes.

(v) Presence of Transfer cells
In *C. orbicularis*, the zygote covering cells possess wall ingrowths resembling those of embryophytic placental transfer cells. These parenchymatous cells are supposed to function in short-distance transport of solutes from the parental thallus to the zygotes (Graham and Wilcox 1983).

10 Chara

10.1 Systematic Position

Fritsch (1935)	Bold and Wynne (1978)	Lee (2008)
Division – Algae	Division – Charophyta	Phylum – Chlorophyta
Class – Chlorophyceae	Class – Charophyceae	Class – Charophyceae
Order – Charales	Order – Charales	Order – Charales
Family– Characeae	Family – Characeae	Family – Characeae
Genus – *Chara*	Genus – *Chara*	Genus – *Chara*

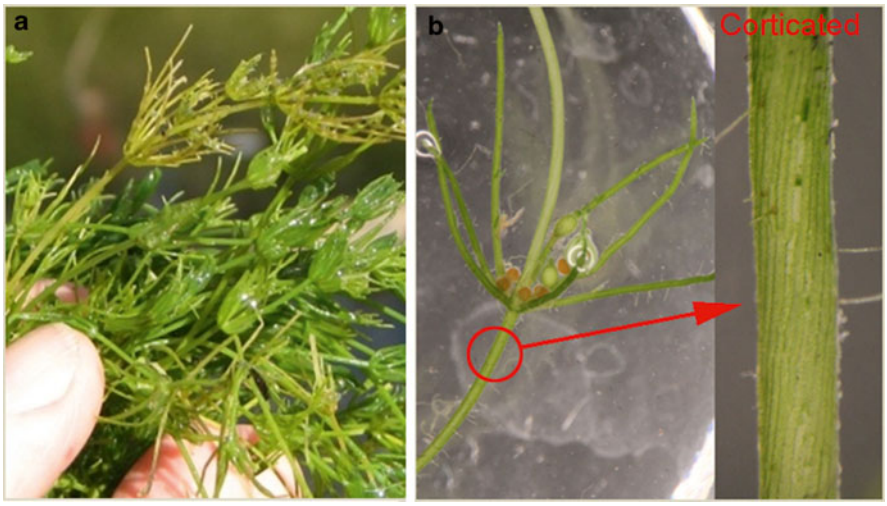

Fig. 26 *Chara* plant (Picture courtesy: (**a**) Michael P. Masser, Ph.D. Texas A&M University, College Station, TX. (**b**) Phil Novis, Allan Herbarium, Landcare Research New Zealand Ltd)

10.2 Occurrence

Chara is a submerged aquatic alga widely distributed in fresh, clear and still waters mostly in limestone areas. It usually remains attached to the soft, muddy bottom along the margins of pond or pool with the help of rhizoids. Several species of *Chara* occur in waters flowing over limestone rocks. Some species become abundantly encrusted with calcium carbonate, which gives them a white or pale green appearance (Grant 1990). *Chara* is represented by around 188 species in the world, of these *C. wallichii*, *C. corallina* and *C. zeylanica* are common. *Chara* thallus has leaf-like and stem-like structures (Fig. 26) and hence apparently resembles land plants.

10.3 Thallus Structure

The plant body is complex, macroscopic, much branched and is anchored to the substratum with the help of uniseriate filamentous rhizoids. The thallus reaches usually up to 20–30 cm in length and is differentiated into rhizoids and main axis. The rhizoids have oblique septa and are characterized by the presence of statoliths near the tip. Statoliths are specialized amyloplasts involved in the perception of gravity. The main axis is differentiated into nodes and internodes. The internode is a long, cylindrical, undivided cell ensheathed by a layer of cortical cells. The node consists of a central pair of cells surrounded by a peripheral group of 6–20 cells. From each node of the main axis arise four types of appendages:

(i) Branchlets or Primary Laterals

Branchlets are short branches which arise in whorls from the nodes of the central axis. They are characterized by limited growth and have been termed as primary laterals. These are green in colour. A branchlet or primary lateral also consists of a limited number of nodes and internodes. The primary laterals contain some unicellular, spine-like secondary laterals on their nodes. Nodes of primary laterals are characterized by the presence of sex organs. The branchlets develop from the peripheral cells of the node.

(ii) Axillary Branches or Branches of Unlimited Growth

These are the branches of unlimited growth. They bear a resemblance to the main axis in structure and are also called long laterals. They arise from some of the older nodes of the main axis. These are also differentiated into nodes and internodes and have the primary laterals in whorls on their nodes. The axillary branches arise from the primary intermodal cell situated below the basal node of the oldest branchlet.

(iii) Stipulodes

Two stipulodes develop at the base of each branchlet in most of the species. Stipulodes are unicellular outgrowths which arise from the basal node of each branchlet or primary lateral. Long intermodal cells of the axis, in most of the species of *Chara*, remain ensheathed or covered by a cortex.

(iv) Cortical Cells

Cortical cells consist of vertically elongated rows of cells (Fig. 26b). The intermodal cell remains ensheathed up to half of its length by a single layer of cortical cells developed from the upper node and remaining lower half by the cortical cells developed from the lower node. Each internode is thus covered by the cortical cells developed by the two adjacent nodes.

10.4 Cell Structure

The apical cell has a dense cytoplasm and a nucleus. The nodal cells have dense cytoplasm, having many discoid chloroplasts and a nucleus. The central vacuole is poorly developed in the nodal cell. In the intermodal cell, there is a large central vacuole and several nuclei and many discoid chloroplasts are present in the peripheral cytoplasm. Pyrenoids are absent. The cytoplasm exhibits streaming around the vacuole which is based on actin microfibril activity (Allen 1974; Palevitz and Hepler 1975; Williamson 1979, 1992). Immunolocalization studies have suggested that actin microtubules in the nodal and intermodal cells more closely resemble that in higher plant meristem cells (Braun and Wasteneys 1998). Ultrastructural observations of the nodal regions of *Chara* suggest that it has a parenchymatous tissue structure (Pickett-Heaps 1975; Cook et al. 1998).

10.5 Apical Growth of Thallus

The main axis and branches of unlimited growth grow by means of a dome shaped apical cell located at the tip. The apical cell divides parallel to its flat base and cuts off a derivative cell called basal cell. The basal cell also divides transversely into a lower internodal initial and an upper nodal initial. The lower internodal initial elongates without divisions and develops into internode.

The nodal initial divides vertically into two cells. Both the daughter cells again divide by divisions intersecting the first division. The successive divisions are in curved planes and intersecting resulting into the formation of a pair of central cells surrounded by 6–20 peripheral cells. Each peripheral cell divides to form an apical initial that produces the 'leaf' and a basal nodal cell of the primary lateral. Therefore, the number of leaves formed is equal to the number of peripheral cells.

10.6 Reproduction

Chara reproduces by vegetative and sexual methods. Asexual reproduction is completely absent.

10.6.1 Vegetative Reproduction

Chara reproduces vegetatively by the following means:

Bulbils: These are tuberous structures formed either on the rhizoids or on the nodes of the main axis.

Amylum stars: In some cases, an aggregate of cells looking like a star is developed on the lower nodes of the main axis. These star-shaped bodies are referred to as amylum stars or starch stars as they are profusely filled with starch.

Secondary protonema: *Chara* also reproduces by protonema-like outgrowths, produced from primary rhizoids or from the nodes of the old plants.

10.6.2 Sexual Reproduction

Sexual reproduction is oogamous. The sex organs are easily visible to the naked eye and have a complex structure. The male sex organ is a spherical, bright yellow or red structure commonly called the antheridium or globule (Fig. 27). Female sex organ is an oval, green structure called oogonium or nucule (Fig. 27). Most of the species are homothallic or monoecious but a few are also heterothallic or dioecious (*C. wallichii*). Homothallic species are protandrous and in these species the globule and nucule occur in pairs i.e., the nucule present above the globule on the adaxial side of the nodes of the primary laterals.

Fig. 27 Sex organs of *Chara*, the nucule present above the globule (Picture courtesy: Dr. Chris Carter)

Nucule Structure The initial of a nucule is the upper peripheral cell close to the antheridial initial. This initial divides by transverse divisions to form a row of three cells: (1) Lowermost or basal pedicel cell, (2) middle one is the nodal cell and (3) uppermost or terminal oogonial mother cell. The lowermost pedicel cell elongates without any further divisions and forms the pedicel of the oogonium. The middle nodal cell divides by vertical divisions and cuts off five peripheral cells or sheath initials surrounding a central cell. The oogonial mother cell extends vertically and divides by a transverse division to form a small stalk cell on the lower side and an upper oogonium that contains a single large uninucleate egg.

The five sheath initials elongate and divide transversely each forming a small terminal coronal cell and elongated tube cell. Thus the crown will have five coronal cells and five tube cells. The five tube cells elongate, thicken their walls and coil around the oogonium forming a sterile protective sheath. The oogonium accumulates a large amount of starch and oil as reserve foods.

Globule Structure The globule or antheridium occurs below the nucule. It develops from a peripheral cell at the node of the primary lateral. The initial of a globule first divides transversely and forms a small basal pedicel cell and a terminal antheridial mother cell. The antheridial cell divides by two divisions right angle to each other into a four-celled structure. All these cells divide transversely and form an eight celled structure. In this octad, each cell divides periclinally and forms a series of three cells, the outermost cell is the shield cell, the middle one is the manubrium and the innermost is primary capitulum cell. Each primary capitulum cell cuts off around six secondary capitula. The eight shield cells having their convex surface

outside expand laterally. Due to this expansion the shield cells and manubrial cells separate, forming cavities inside the globule. The eight manubrial cells elongate radially; however, the primary capitular cells remain together at the centre resulting into the enlargement of the globular cavity. The pedicel cell usually protrudes into the globular cavity. Each secondary capitulum cell gives rise to 2–4 spermatogenous or antheridial filaments. These filaments which are simple or branched fill up the cavities of the globule. Each antheridial filament consists of 100–200 cells. These cells function as spermatozoid mother cells and each gives rise to a single, narrow, spirally coiled biflagellate antherozoid or spermatozoid. When the antherozoids are mature, the shield cells fall apart from each other and the capitulate, antheridial filaments and manubria, are out in the open. Afterward, each antherozoid liberates through a pore in the antheridial wall. Thus, several antherozoids are released in the morning.

Fertilization In a mature nucule at the time of fertilization, the five tube cells separate from one another just below the coronary cells, resulting into the formation of five narrow slits. Several antherozoids may enter the nucule through these slits. One antherozoid penetrates through the gelatinized oogonial wall near the receptive spot and the male nucleus fuses with the egg nucleus. The zygote becomes an oospore after secreting a thick wall consisting of suberin and silicic acid. The oospore sinks to the bottom of the pond. It germinates after a dormant period.

Germination of Zygote The zygote nucleus migrates towards the apical pole and divides twice, of which the first is reductional division forming four haploid daughter nuclei. Hence the oospore is the only diploid structure in the life cycle of *Chara*. A cell wall is formed, asymmetrically dividing the oospore into a small distal uninucleate lenticular cell and a lower large basal cell containing three nuclei. These three nuclei in the basal cell generally degenerate later. The oogonial envelope bursts open at the apex and the lenticular cell gets exposed. Lenticular cell divides longitudinally into two cells: the rhizoidal initial and protonemal initial. The rhizoidal initial develops into the primary rhizoid. The protonemal initial develops into green filamentous primary protonema which is differentiated into nodes and internodes. Secondary rhizoids arise in a whorl at lowermost node of the primary protonema. The peripheral cells of the second node of primary protonema produce a whorl of appendages or lateral outgrowths, one of which develops into the *Chara* plant.

10.7 Life Cycle

The life cycle of *Chara* consists of both haploid and diploid phases. The branched multicellular plant is haploid. Sexual reproduction in *Chara* is strictly oogamous. Haploid gametes are formed in specialized complex structures called antheridia (globule) and oogonia (nucule). Most of the species are monoecious (homothallic)

but a few are dioecious (heterothallic). Antheridium has chains of colorless cells each one of which produces a single biflagellate sperm or antherozoid. Nucule or oogonium consists of a fertile cell (oogonium proper) surrounded by sterile cells (tube cells). Several antherozoids can enter the nucule but only one penetrates the upper portion of the oogonium through the gelatinized oogonial wall near the receptive spot. The haploid male nucleus travels down and fuses with the haploid egg nucleus forming a diploid zygote. Hence diploid phase is represented by zygote. The zygote develops a thick wall, and the oogonium gets abscised from the plant. In addition the inner walls of tube cells also get thickened and remain as spiral markings on the resting zygote. Zygote undergoes a period of dormancy, which is followed by meiosis. The diploid zygote divides meiotically and a cell wall is formed asymmetrically dividing the oospore into a small distal uninucleate lenticular cell and a lower large basal cell containing three nuclei. These three nuclei in the basal cell degenerate and the lenticular cell divides and develops into a juvenile plantlet of *Chara*. The evolutionarily huge steps forward with *Chara* are: the sterile jacket of cells which surround and protect the oogonium and the gametophyte which retains the zygote for a long time until it develops a thick wall and falls off to lie dormant (Bold et al. 1987). The life cycle of *Chara* having haploid *phases* represented by multicellular plant and gametes and a diploid phase which is only restricted to the zygote (zygotic meiosis) is described as haplontic.

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Brown Algae

Priyanka Verma, Alok Arun, and Dinabandhu Sahoo

1 Introduction

Brown algae (referred as Phaeophyceae) represent a diverse group of multicellular organisms that are usually found in marine habitats. Brown algae consist of approximately 1800 species that are grouped into 285 different genera (Silberfeld et al. 2010). Phylogenetically brown algae are grouped in the phylum Heterokonta (Baldauf 2003) that consist of both unicellular and multicellular organisms. Since brown algae are evolutionary independent as compared to plants, they show remarkable differences both in terms of metabolic activities and developmental processes (Charrier et al. 2008). Despite of the evolutionary distance, brown algae and plants share some similarities that include, but not restricted to, the ability of producing totipotent cells and intercellular plasmodesmatic connections that are essential for multicellular development (Bogaert et al. 2013). Unlike animals and plants, brown algae are poorly explored taxa at a scientific level. It is noteworthy to mention here that brown algae not only have immense economic importance but also can be used as excellent model organisms to study the unique features that members of brown algae have adapted during the course of evolution. In terms of complex multicellularity, brown algae represent one of the five major eukaryotic lineages that have evolved complex multicellularity (Cock et al. 2010). Emphasis has been laid on certain fields to exploit brown algae, for example fucoids, to study questions related to cell biology (Quatrano 1982) but a comprehensive understanding of the various metabolic, physiological and developmental processes occurring in brown algae at molecular level remains poorly understood.

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2 General Characteristics

- Brown algae contain only multicellular filamentous species with no reports of unicellular or colonial organisms found so far (Bold et al. 1987). The size of thalli may be microscopic or branched up to several meters long. The macroscopic thallus is usually differentiated into holdfast, stipe and blade. However, this differentiation of brown algae thallus may be the result of classification rather than a consequence of evolution, as all the groups hypothesized to be the closest relatives of the browns include single-celled or colonial form (Lee 2008).
- Brown algae are primarily found in marine habitat and brackish waters. According to Lee (2008) and Wehr (2002) fresh water species of brown algae are also found. Phaeophytes belonging to freshwater is mostly restricted to streams, rivers and littoral zone of lakes. Species such as *Heribaudiella fluviatilis*, may become dominate river flora but still their biology remains unexplored (Wehr 2002).
- The brown algae are characterised by brown colour that comes from a carotenoid pigment, fucoxanthin, present in their chloroplasts and in some species, various phaeophycean tannins. The chloroplasts also have chlorophylls *a*, *c*1, and *c*2, β-carotene, diatoxanthin, violaxanthin and large amounts of fucoxanthin.
- The chloroplasts found in brown algae are usually discoid in shape; their pyrenoid is stalked and protrudes from the chloroplast.
- The cell walls are composed of cellulose microfibrils, alginate and forms the structural component of the cell wall, together with amorphous mucilaginous matrix fraction and mucilaginous alginates.
- The spindle during mitosis does not persist long at telophase and as it collapses, the daughter nuclei which move back towards each other and lie close together. The spindle is formed inside the nucleus, between two polar pairs of centrioles, radiating out into the cytoplasm.
- Siliceous cysts and siliceous cell walls are absent in brown algae.
- The life cycle of sexually reproducing species is generally diplohaplontic, and can be either isomorphic or heteromorphic. The gametes are produced in unilocular and plurilocular sporangia.
- Flagellated cells of brown algae are reproductive in nature. There are no free living flagellates. The transition region lacks a transitional helix.
- The storage product is laminarin.

3 Geographical Distribution

The number of brown algae is arduous to evaluate with precision (Hoek et al. 2009). Albeit of intense cataloguing of brown alage, the recognition of their distribution is fragmented especially for freshwater species which show sparse diversity (Table 1).

Description of the number of genera and species of brown algae from freshwater vary among authors, largely due to lack of in-depth study. Four of the seven known

Table 1 Species of brown algae from freshwater environments

S.no	Freshwater species	Distribution
	Ectocarpales	
1	*Bodanella lauterbornii*	western Europe
2	*Ectocarpus siliculosus*	North America, Hopkins River, Australia
3	*Pleurocladia*	Lake Erken, Sweden
4	*Heribaudiella*	Europe, western North America, Japan, and China, the Mississippi River or south of Oregon, stream near Yellowknife, Northwest Territories
5	*Porterinema fluviatile*	freshwater sites in Europe
	Sphacelariales	
6	*Sphacelaria fluviatilis*,	Streams of south and central China as well as a small lake in Michigan.
7	*Sphacelaria lacustris*	western Lake Michigan

Adapted from Wehr (2002)

species from fresh waters have no identical species in marine environments hence showing no evidence of marine infiltration (Wehr 2002, 2010; Lee 2008).

Brown algal species exhibit marine littoral flora which is primarily dominant from sub-polar area to equator yet temperate areas shows significant diversity. Members of the genus fucaceae are quintessential of tidal zone in north-Atlantic temperate areas. Laminariales (also kown as kelps) are from sublittroral forests in temperate areas. Some tropical brown seaweed, such as taxon *Lobophora variegata*, grows at depths of 100 m. In the tropical, brown algal species such as *Sargassum* occur only as drift populations, whereas others like *Pilayella littoralis* can form extensive drift populations that contaminate beaches within Boston Bay (Vijayaraghavan and Kaur 1997).

4 Thallus Organization

Most of brown algae are *lithophytes*, which require stable hard substrata for attachment, and a number of the filamentous, smaller species are epiphytes. Unicellular, colonolial and unbranched filaments are absent in pheophyceae. The freshwater phaeophyta species are simply filamentous and smaller in size unlike their marine counterparts which have complex gigantic and bulky thalli (Fig. 1a–f).Their size ranging from small filamentous forms like *Ectocarpus* and *Hinskia*, which are few millimetres to massive intertidal weeds such as *Ascophyllum* and *Fucus*, to subtidal large kelps and the largest seaweed known *Macrocystis pyrifera* (Reviers et al. 2008). They have higher morphological and anatomical differentiation compared to the other algae. The size range vary greatly, from crustose form which may be 1–2 mm, macroscopic filmentous tufts 2–10 mm, subtidal kelp forests that might be as tall as 20–60 m (Wehr and Stein 1985; Bold and Wynne 1985).

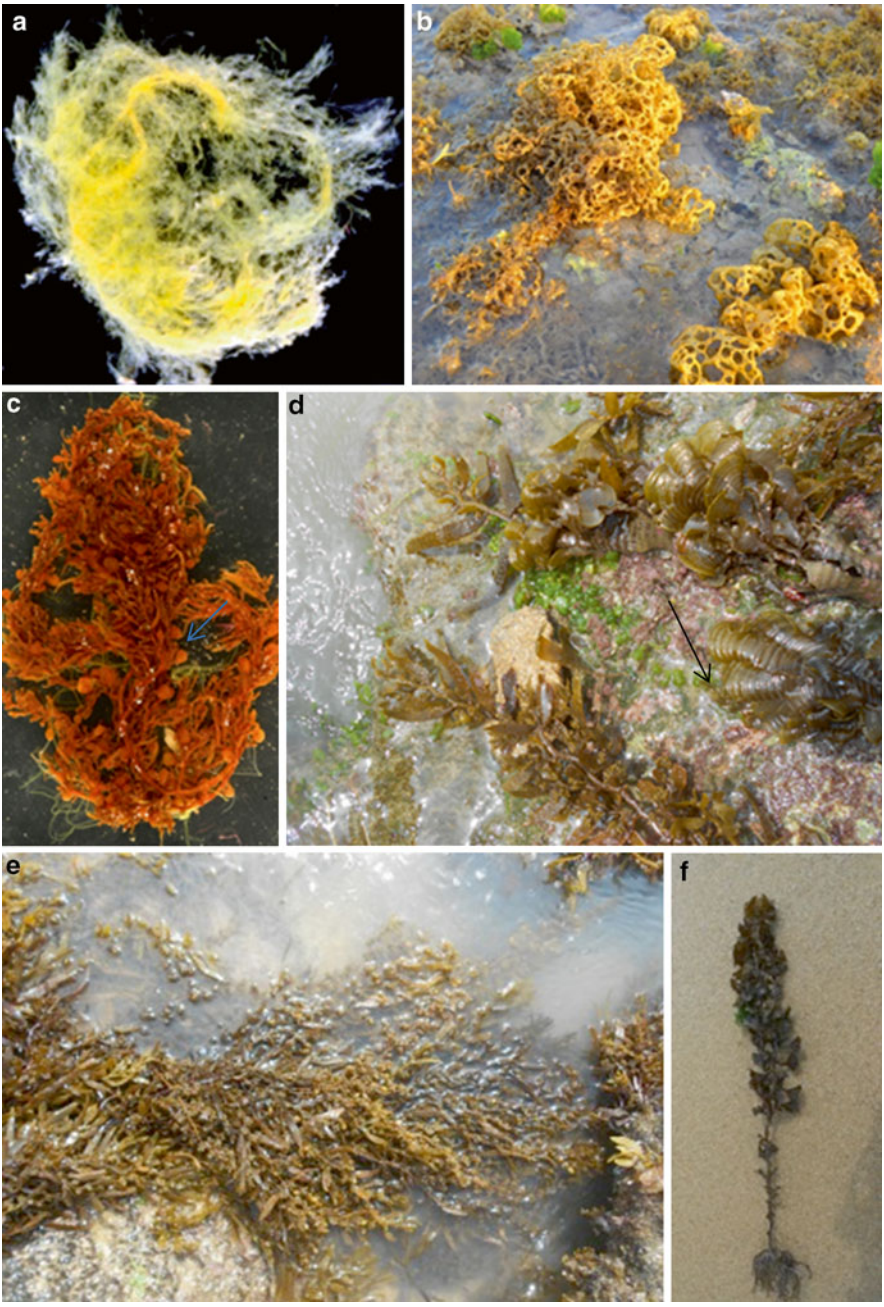

Fig. 1 (**a-f**) Morphology of various brown algae (**a**) A tuft of *Hincksiamitchelliae* (**b**) Field view of *Hydroclathrusclathratus* thalli showing hollow perforated net like structure. (**c**) Thallus *Sargassum*sp.showing spirally arranged branches, simple leaves and air bladder (*arrow*). (**d**) A field view of *Padina* sp. (*arrow*) growing on inter-tidal rock. (**e**) A field view of *Sargassum* (**f**) *Turbinaria* morphology

5 Cytology of Brown Algae

5.1 Cell Walls

The cell walls of brown algae are generally gelatinous and consist of two layers. Cellulose makes up the skeleton backbone but is present in small quantiites i.e. 1–8 % of dry weight (Fritsch 1977; Lee 2008). The main components of brown algal cell walls are anioinic polysaccrides such as Algin, Fucoidin and Fucin (Fritsch 1977). Alginic acid is β1,4 linked mannuronic acid units with uneven guluronic acid units attached through C-1 and C-4 linkages and makes the cell wall amorphous as well as mucilaginous to with stand long desiccation periods (Lee 2008). Fucoidin is α-1,2 linked sulfated-fucose units, with small amount of α-1,4 linked sulfated-fucose units. Fucin shows similarity to align and also contains sulphur. The average weight ratio for Alginates, fucoidans and cellulose is respectively 3:1:1. Brown algal cell walls also contain phlorotannins, which consist of halogenated, sulfated phenolic compounds (Michel et al 2010).Their quantities differ according to environmental conditions, species and various parts of thallus. *Padina* is the only species where deposition of calcium carbonates leading to calcification has been reported. Iron deposition has also been observed in *Padina* and *Sphacelariales* (Fritsch 1977). Plasmodesmeta or pores are present in cell walls and are bounded by plasmallema. In parenchymatous pheophyceae (Laminariales, Fucales, and Dictyotales) the pores are grouped into pit areas while in primitive parenchymatous plasmodesmata are scattered in the cell wall (Lee 2008).

5.2 Chloroplasts, Plastids, Pigments and Photosyntehsis

The chloroplasts of brown algae are usually discoid and surrounded by an envelope. The outer membranes of the chloroplast endoplasmic reticulum are continuous or discontinuous depending on the species. Microfibrils of DNA occuring in the plastid may be linear or circular attached to the thylakoid membranes (Lee 2008). The pigments are located in plastids lacking pyrenoid; their presence may also vary according to algal stage (Vijayaraghavan and Kaur 1997).They are present in sporolings but absent in spermatozoids, and zoospores. The chloroplasts also have chlorophylls *a*, *c*1, and *c*2, β-carotene, violaxanthin, diatoxanthin and large amounts of fucoxanthin. Pyrenoid is stalked and protrudes from the chloroplast. Laminarin is generally stored in endoplasmic reticulum outside the chloroplast. D-mannitol is accumulated through photosynthesis and is influenced by change in salinity as well as light conditions. According to Forster and Dring (1994) Inorganic carbon uptake and carbon fixation in presence of blue light is prevalent in pheophyceae with fucoids as an exception which have evolved a separate carbon-concentrating mechanism (Lee 2008).

5.3 Phlorotannins and Physodes

Presence of Physodes (fucosan granules) is one of the characteristic features of brown algae. In the meristmatic, photosynthetic and reproductive cells, cytoplasm a large number of colourless vesicles with highly refractive acidic fluid staining red with vanillin and hydrochloric acid are present. These are known as physodes, fucosan granules, fucosan vesicles (Vijayaraghavan and Kaur 1997). Physodes store phlorotannins which are formed by Golgi in the perinuclear area of the cell. These tannins are non-glycosidic, protiens which readily oxidizes in air forming phycophaein a black pigment giving members of pheophyceae their characteristic black colour. Their content varies from 1 to 15 % dry weight and is influenced by location in which it is growing. Main function of phlorotanin discourages grazing; absorb ultraviolet radiation, serving as a component of cell walls, as antifoulants and contribute towound plug development (Lee 2008).

6 Reproduction

Brown alga reproduces by vegetative, asexual and sexual methods of reproduction.

6.1 Vegetative Reproduction

Several species of brown algae show vegetative reproduction via fragmentation. In members of sphaecelariales propagules are found (Kumar and Singh 1971).

6.2 Asexual Reproduction

All brown algae reproduce asexually with exceptions of Tilopetridales, Dictyotales and Fucales (Kumar and Singh 1971). In ectocarpales and spherocarpales asexual reproduction occurs via biflagellate zoospores that develops in to reproductive organs called sporangia which could be unilocular (one-celled) or many cells plurilocular as observed in *Hinskia mitchelliae* Fig. 2a (many celled).Gametes can also reproduce parthenogenetically to form asexual progenies, for example in *Ectocarpus* (Peters et al. 2004, 2008). Asexual reproduction is absent in *Laminaria*.

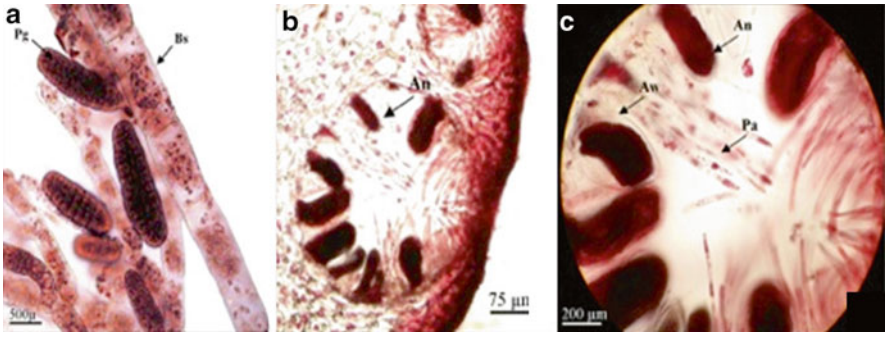

Fig. 2 (**a-c**) (**a**) Magnified view of *H. mitchelliae* showing barrel shaped cell (*Bs*) and plurilocular gamentangia (*Pg*). (b) Magnified view of *Hormophysa cuneiformis* receptacle showing antheridia (*An*) and paraphysis (*arrow*). (**c**) Enlarged view of receptacle showing Antheridia (*An*), Antheridial wall (*Aw*) and paraphysis (*Pa*)

6.3 Sexual Reproduction

In pheophyceae sexual reproduction takes place by the formation of flagellate gametes that are formed inside gametangia. Multicellular gametangia are formed only in some of the brown algae (Chapman 1910, 1970). The haploid thalli form ranges from isogamous (both male and female gametes exactly similar), anisogamous (female gamete larger than male) to oogamous (small flagellated male and large non-flagellated female gametes) (Bold 1978). The sexual reproduction is through fusion of flagellated male and female gametes or fusion of flagellated male and large non-flagellated female gametes (Fritsch 1977). The haploid (gametangial) and diploid (sporangial) thalli may be similar (isomorphic) as in Ectocarpales or different (heteromorphic) in appearance for example in Laminariales, or the gametangial generation may be extremely reduced (Fucales) (Kumar and Singh 1971).

Brown algal life cycle shows alteration of generations of haploid and diploid organisms. An example of life cycle of Ectocarpus has been described in detail in Sect. 7.1.1.

- Haploid gametophytes (n) give rise to haploid gametes by mitosis.
- male and female gametes (n) fusion give rise to zygote (2n) that forms diploid sporophyte
- The sporophyte (2n) produces meiospores (n) by meiosis which germinates and forms haploid gametophyte.

Brown algae life cycle may be isomorphic, heteromorphic and Diplontic.

Table 2 Various classifications of Brown Algae

Fritsch (1945)	Russell and Fletcher (1975)	Bold and Wynne (1985)	Lee (2008)	Reviers et al. (2008)
Ectocarpales	Ectocarpales	Ectocarpales	Dictyotales	Discosporangiales
Tilopteridales	Desmarestiales	Chordariales	Sphacelariales	Ishigeales
Cutleriales	Cutleriales	Cutleriales	Cutleriales	Petrodermatales
Sporochnales	Laminariales	Tilopteridales	Desmarestiales	Dictyotales
Desmarestiales	Sphacelariales	Sphacelariales	Ectocarpales	Sphacelariles
Laminariales	Dictyotales	Dictyotales	Laminariales	Onslowiales
Sphacelariales	Fucales	Sporochnales	Fucales	Syringodermatales
Dictyotales		Desmarestiales		Ascoseirales
Fucales		Dictyosiphonales		Nemodermatales
		Scytosiphonales		Fucales
		Laminariales		Tilopteidales
		Fucales		Related to Laminariales
		Durvillaeales		Laminariales
		Ascoseirales		Ectocarpales
				Scytothamnales
				Desmarestiales
				Sporochnales
				Ralfsiales

7 Classification of Brown Algae

Brown algae originated around 150–200 million years ago (Lee 2008). According to Rabenhorst (1864) brown algae were clubbed in class Melanophyceae, fucophyceae and pheophyceae which are most prevalent among these three (Reviers et al. 2008). Molecular techniques have greatly increased our understanding about brown algae and included many new family and orders (Table 2).

8 Life History

8.1 Ectocarpus

Phylum: Phaeophyta
Class: Phaeophyceae
Order: Ectocarpales
Family: Ectocarpaceae
Genus: Ectocarpus

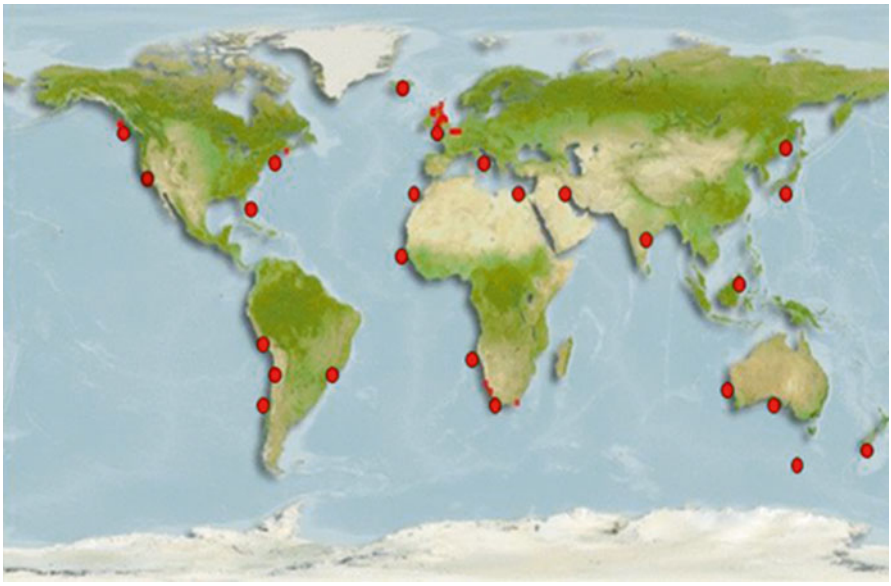

Fig. 3 Geographical distribution of *Ectocarpus*. *Red dots* represent the sites where species of *Ectocarpus* have been reported across the globe (Dittami 2009)

8.1.1 Habitat and Distribution

E. siliculosus and *E. fasciculatus* are the two most commonly known species of *Ectocarpus* for which a wealth of morphological, taxonomical and genomic information exist. *Ectocarpus siliculosus* is a cosmopolitan species found mostly in marine environment of temperate regions across the world (Fig. 3). Other species of *Ectocarpus* have been reported from different continents like South Africa, Asia and South America. *Ectocarpus siliculosus* has also been reported from a fresh water source in Australia (West and Kraft 1996) and in Germany (Geissler 1983). *Ectocarpus* can be commonly found as free-floating filaments in the intertidal zones to sublittoral zones on the shores. It can easily grow as epiphytes. The presence of *Ectocarpus* on various abiotic substrates makes it a very common fouling agent (Morris and Russel 1974). The widespread distribution of *Ectocarpus* across varying environmental conditions (for example presence in both high and low tides) makes *Ectocarpus* an excellent model system to study molecular mechanisms related to stress factors.

8.1.2 Life Cycle of Ectocarpus

The sexual life cycle of *Ectocarpus* (Fig. 4) consists of alternating heteromorphic gametophyte and sporophyte generations (Müller 1964; Peters et al. 2004, 2008). Both generations of the sexual life cycle are multicellular. It may be hard to

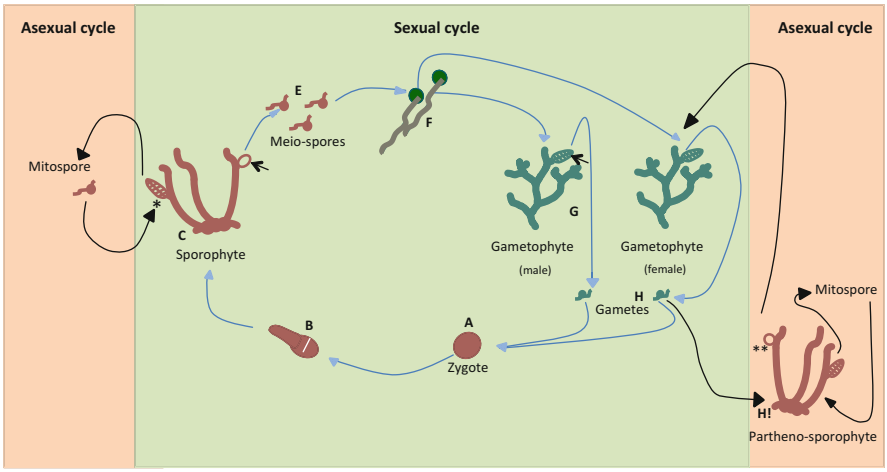

Fig. 4 Life cycle of *Ectocarpus*. The green area represents the sexual life cycle and the brown area represents the asexual life cycle. For details, see text (Adapted from Arun 2012)

distinguish between gametophyte and sporophyte in the field but they are easy to identify in cultures under laboratory conditions (Peters et al. 2004). Usually, when grown in petri dishes, gametophyte can be found freely floating whereas sporophytes form compact thalli that are attached to the substratum (Peters et al. 2008). Noteworthy is the ability to distinguish at the microscopic level the difference in the stages of early development of the gametophyte and the sporophyte generations.

The sexual life cycle in the sporophyte generation starts with the formation of a diploid zygote (Fig. 4a) that undergoes a bipolar germination to produce two germs tubes (Fig. 4b) that eventually forms the sporophyte. The initial cell division in the zygote is symmetric. Two kinds of filaments prostrate and upright are produced in the sporophytic generation. Two specialized reproductive structures; plurilocular and unilocular sporangia are produced on the upright filaments (Fig. 4c). A mitotic event in multi-chambered plurilocular sporangia releases mito-spores, which after their release, forms sporophyte (Fig. 4d). Unlike plurilocular sporangium, unilocular sporangium consists of single chamber that contain meiotically produced meio-spores (Fig. 4e) that give rise to multicellular gametophytes. Morphologically, it is hard to distinguish between a male and a female gametophyte. Like zygote, the meio-spores (Fig. 4e) undergo a bipolar germination but the initial cell division of meio-spore is asymmetric that produces different cell types (Fig. 4f). Only upright filaments have been observed in the gametophytic generation. Specialized reproductive structures called plurilocular gametangia are produced on the upright filaments of the gametophyte (Fig. 4g). Plurilocular gametangia produce male and female gametes (Fig. 4h) that are released in the surrounding marine water. Male and female gametes can be distinguished based on the behavior and physiology (Charrier et al. 2008). Unlike male gametes, female gametes settle quickly and release a sex pheromone that attracts male gametes. Once a male gamete fuses with

a female gamete, a diploid zygote is produced that marks the onset of the first diploid structure of the sporophyte generation.

Ectocarpus can also reproduce asexually using various methods: (1) Gametes that fail to find a suitable partner develop asexually into partheno-sporophytes (Fig. 4h!). A mature partheno-sporophyte produces unilocular (Fig. 4**) and plurilocular sporangia. (2) Mitotic events in plurilocular sporangium forms mito-spores that forms 'clones' of sporophytes (Fig. 4d*). (3) Sometimes, meio-spores released from unilocular sporangia can change their fate to develop into sporophytes. This particular phenomenon where a meio-spore develops into sporophytes is called heteroblasty and was first reported by Müller (1967) (Fig. 5).

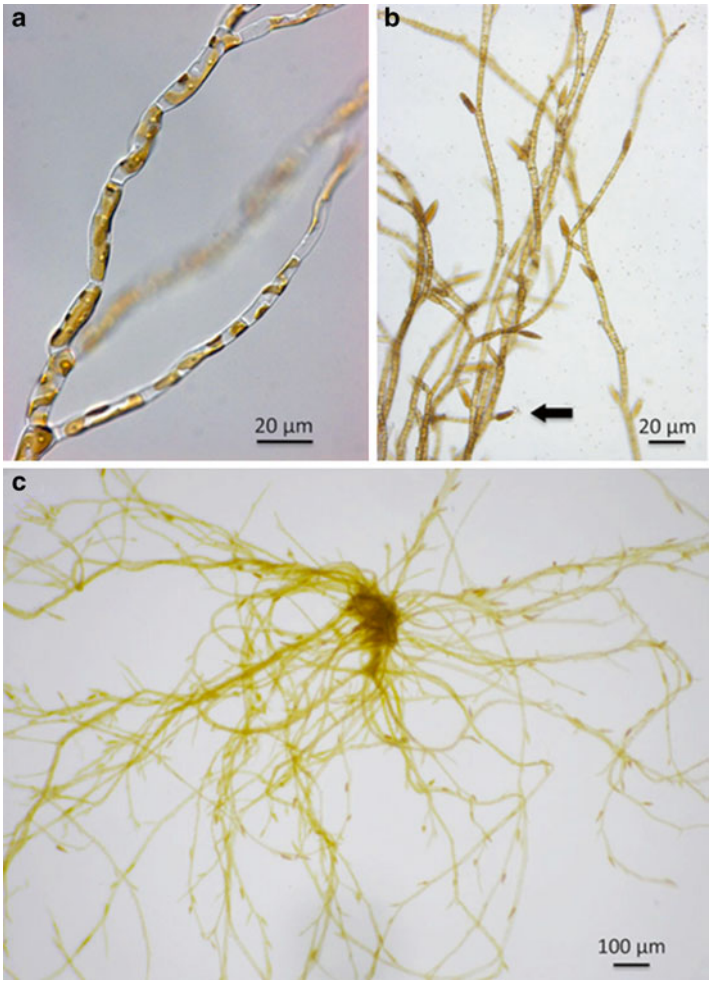

Fig. 5 ***Ectocarpus*** **in cultures**. (**a**) partheno-sporophyte filaments (**b**) gametophytes bearing plurilocular gametangia, (**c**) mature gametophyte filaments releasing gametes from plurilocular gametangia (*arrow*) (Adapted from Cock et al. 2012)

8.1.3 Ectocarpus: Emerging Model Organism

Ectocarpus has emerged as an excellent model system to study features like life cycles, metabolism and host parasite interactions. The key features that make *Ectocarpus* as a suitable model systems includes its very short life cycle that can be completed in laboratory in 3 months, establishment of axenic cultures, possibility to perform genetic crosses, availability of genome sequence (Cock et al. 2010), ease of raising mutants and other associated genomic tools including genetic maps (Heesch et al. 2010).

The presence of a variety of life cycles in brown algae makes them extremely interesting model systems to study the life cycle of organisms. Variability in life cycle of brown algae ranges from isomorphic haploid-diploid life cycle as observed in *Ectocarpus* (sporophyte and gametophyte generations are multicellular) to diploid life cycles (diploid generation is multicellular) as found in *Fucus* (Bell 1997). Unlike plants, we do not observe a clear trend of evolution of haploid-diploid life cycles towards diploid life cycles. The presence of various forms of haploid-diploid life cycles in brown algae may suggest that the haploid-diploid life cycles have evolved several times in brown algae. Further studies will be required to conclude if there was a common toolbox that gave rise to different life cycles in brown algae and/or in other eukaryotic lineages. Also, it would be interesting to know if there was common molecular machinery that controls both the sexual and asexual life cycle in *Ectocarpus*. The identification of two *Ectocarpus* life cycle mutants, *immediate upright* (*imm*) (Peters et al. 2008) and *ouroboros* (*oro*) (Coelho et al. 2011) that show partial and complete conversion, respectively, of the sporophyte generation into gametophyte generation lays the foundation for studying the molecular mechanism controlling haploid-diploid generations. *ORO* exhibits a new class of homeotic mutation wherein the mutation is not occurring at the level of organ but at the level of organism (Coelho et al. 2011). ORO appears to be a master regulator of life cycle process in *Ectocarpus* and hence identification of *ORO* and *IMM* genes will be important to reveal the control of life cycle at molecular level in a brown alga. Also, characterization of factors that contribute to the non-cell autonomous control of life cycle transitions in *Ectocarpus* (Arun et al. 2013) will enhance our understanding of life cycle regulation.

Brown algae show differences in the morphology of gametes and hence can be exploited to study factors that control the existence of various forms of gametes. Gametes can be isogamous (gametes of equal size) or anisogamous (gametes of unequal size) (Coelho et al. 2007). Also, gametes can differentiate into sperm cells and eggs (oogamy). In *Ectocarpus* a recent study has showed that there is a difference in the transcriptome profile between isogmaous gametes (Lipinska et al. 2013), which shows that despite morphological similarities in gametes differences exist at transcriptome level. The transcriptome study lays a good foundation for performing transcriptome studies in anisogamous and oogamous gametes to see if similar (or different pattern) pattern of transcript abundance exists in them. At a physiological level, *Ectocarpus* has been exploited to study processes like photosynthesis and majority of genes regulating photosynthesis in plants have been identified in *Ectocarpus*, which shows close similarities of brown lineage to green lineage. One

of the enzymes involved in mannitol biochemical pathway has been characterized both at molecular and biochemical level in *Ectocarpus* (Rousvoal et al. 2011). Such studies are one of the few reports of biochemical assays in brown algae. It will be interesting to see if biochemical profile of secondary metabolites of brown algae share close profile with metabolites produced from plants.

Ectocarpus is also interesting for virologists because a viral genome has been found associated with the nuclear genome of *Ectocarpus*. The genes of the viral genome are either suppressed or not active in different laboratory-tested stress conditions. Like other organisms, brown algae are also attacked by several pathogens, *Eurychasma dicksonii* (oomycete) being one of the abundant pathogenic stramenopiles. *Ectocarpus* has proved to be a good model system to study host-parasite interaction (Grenville-Biggs et al. 2011). It will be tempting to see if the mechanisms of host-parasite interactions are similar to other eukaryotic lineages.

However the extensive development of genomic and genetic tools to study and establish the multicellular brown algae *Ectocarpus* as a model organism during the last few years has opened numerous possibilities to explore brown algae.

8.2 Dictyota

Order: Dictyotales
Family: Dictyotaceae
Genus: *Dictyota*

8.2.1 Habitat and Distribution

Dictyota is foundrestricted to European Atlantic coasts and the Mediterranean Sea. It is found to be present from British Isles to various coasts in Europe (Portugal, Spain and France etc). *D. dichotoma* was most probably absent in the Indian Ocean and their presences in Pacific Ocean have also been questioned. Due to its morphological plasticity *D. dichotoma* geographical distribution has not been accurately determined. It has been found that distribution impacts life cycle strategies of, sexual vs. asexual cycle among *Dictyota* population (Tronholm et al. 2008).

8.2.2 Thallus Structure

Thallus is frondose, long and ribbon-like brown in colour. Thallus flattened, erects branched forming larger or smaller tufts and entangled masses (Fig. 6a). Branching is dichotomous and uniform (Fig. 6b). Fronds are narrow branched twisted and quite enlarged, wider and narrower towards extremities. Apical portion is acute and have entire margin (Verma 2010).The forked fronds of the branches are normally situated in the same plane which arises from the cylindrical rhizome, bearing single lenticular or epllitical apical cells (Fig. 6c).The mature thali has three layers, a large

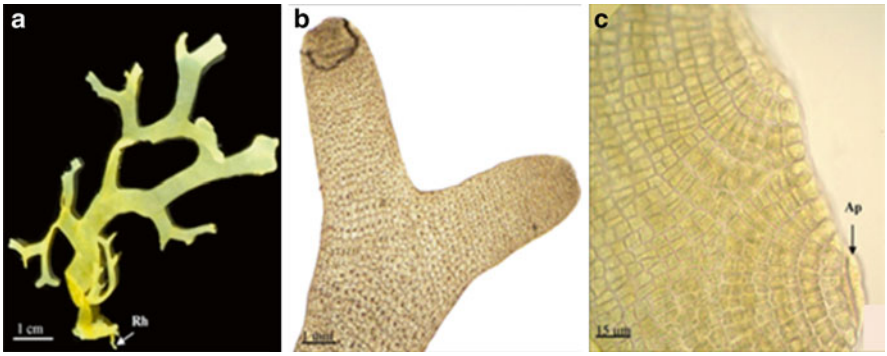

Fig. 6 (**a**-**c**) (**a**) Thalli of *Dictyota dichotoma* having erected ribbon like dichotomously branched with rhizoids (*arrow*). (**b**) Whole mount of *D. dichotoma* filament showing dichotomous branching (*arrow*) and entire margin. (**c**) Magnified view of apical portion of *D. dichotoma* showing lenticular or elliptical apical cell (*arrow*)

celled middle layer with few or no chloroplasts, surrounded on both sides by a layer of small cells densely packed with chloroplasts (Fritsch 1977). The thalli lack midrib.

8.2.3 Life Cycle of Dictyota

Life cycle of *Dictyota* has three overlapping generations with simultaneous sporophytic and gametophytic growth. Thallus has a life span of less than 3 months. Sporophytes are higher in number than gametophytes. Gametophytes form sex organs in projecting sori. The male and female sori are fan-shaped. They are bounded by sterile cell comprising of iridescent bodies. Antheridia develops from surface cells which further divides and redivides forming a pear-shaped sperm with a single, laterally inserted, tinsel flagellum and an anterior eyespot (Vijayaraghavan and Kaur 1997).

Female sori usually have eminent deep brown colour and appear as spots on both surfaces of the thallus (Phillips et al. 1990). Similar to anthredia they also develop suface cells. This surface cell divides to form a stalk cell and oogonia. Each oogonium contains one egg which gets released via gelatinized apex of the wall. Female sorus usually contains 25–50 oogonia arranged in rows with sterile oogonia at the margin. Fertilization is external in *Dictyota*. The zygote is formed through fertilization of egg. The egg develops to form sporophyte. The unfertilized eggs sporadically show normal development and usually terminates yet they can germinate parthenogenetically. At the thallus surface haploid aplanospores (tetraspores) are developed by sporophyte. Dictyotene a pheromone is secreted by egg to attract sperm (Lee 2008).Some members of the species are known to secrete terpenoids such as pachydictyol to inhibit grazing (Lee 2008).Factors such as development of apomeiotic tetraspores into new sporophytic thalli (apospory), non-random distri-

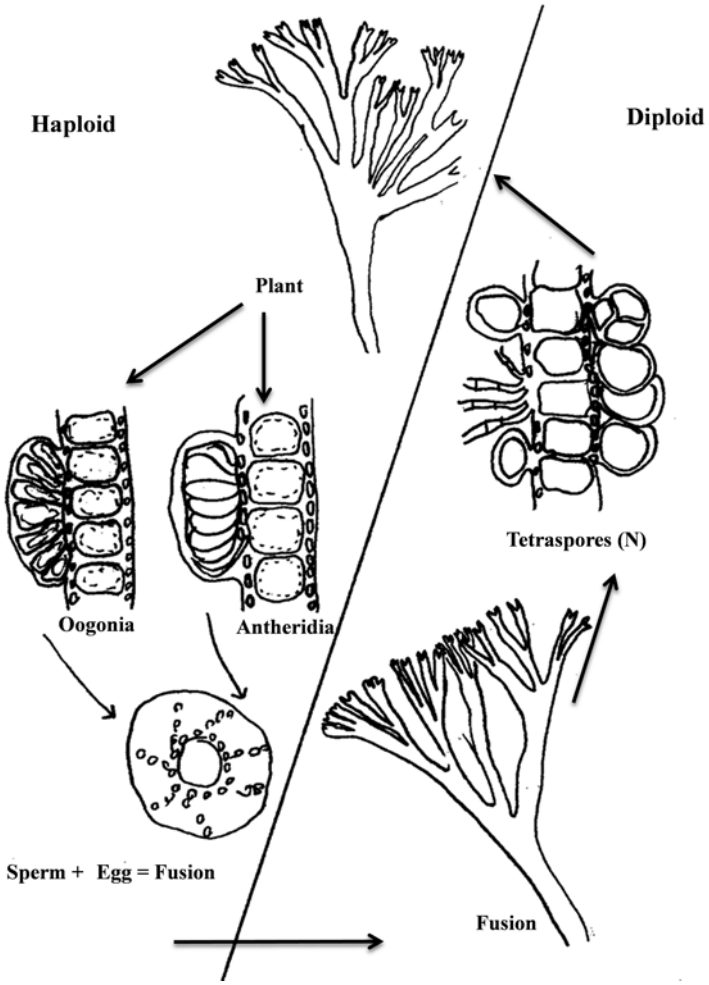

Fig. 7 Life cycle of *Dictyota dichotoma* (Adapted from Lee 2008)

bution patterns, greater longevity and vegetative reproduction of the sporophyte generation had led to sporophytic dominance (Tronholm et al. 2008) (Fig. 7).

8.3 Laminaria

Order: Laminariales
Family: Laminariaceae
Genus: *Laminaria*

8.3.1 Habitat and Distribution

Commonly known as Kelps, Laminariales include the most complex and largest brown algae. They are normally temprate water species. However they may grow vegetatively in warm waters. Primarily confined to the Northern Pacific, North and South Atlantic they form extensive sub-littoral kelp beds (Fritsch 1977; Vijayaraghavan and Kaur 1997; Lee 2008).*Laminaria* plants at times have prolonged stipes and a formidable blade area. Dense underwater forests of *Laminaria hyperborean* and *L. digitata* are found in northern Atlantic while Laminaria solid ungula produces dense growths in the Alaskan Beaufort Sea. There is a phase of fast growth from early winter to early summer and a phase of reduced growth during summer and autumn thus exhibiting a seasonal growth pattern (Lee 2008).

8.3.2 Thallus Structure

The morphology of thallus is differentiated into holdfast, stipe (stems) and lamina (blades). The holdfast is used to attach thalli to a substratum. They may be in the form of a solid disc or a branched cluster of cylindrical heptra. The stipe is smooth, round and thick arising from holdfast. The blade or laminas has rough and leathery texture and it arises terminally as a single blade with ruffled margines or a number of divided segments. Anatomical sections show tissue differentiating into epidermis, outer cortex, inner cortex and central medulla. *Laminaria* sporophytes are parenchymatous in nature and elongated by an intercalary meristem that is found at the base of the blade. A superficial *meristoderm* helps in expansion of *laminaria* diameter (Guiry and Guiry 2015).

8.3.3 Life Cycle of Laminaria

Asexual reproduction by means of spores is absent in *laminaria* (Vijayaraghavan and Kaur 1997). A typical Laminariales have diplo-haplontic life cycle (Fig. 8). They have sporophytic macrothalli followed by gameteophytic microthalli that undergoes oogamous sexual reproduction. Sori produce unilocular sporangia on both surfaces of blades. The sporangia in the sorus occur scattered with in paraphyses which are thick, sterile protective hair like structures. Each sporangiumhas haploid zoospores which evolve into dioecious heteromorphous microscopic gametophytes with oogamous reproduction. Male and female gametophytes are filamentous and males bear clusters of colorless one-celled antheridia at tips of branches each producing a single biflagellate spermatozoid while female gametophyte cell develop into a one-celled oogonium producing a single egg. After fertilization zygote is formed which germinates giving rise to a young sporophyte. Development of *Laminaria* is greatly influenced by seasonality, temperature, nutrients etc. (Lee 2008).

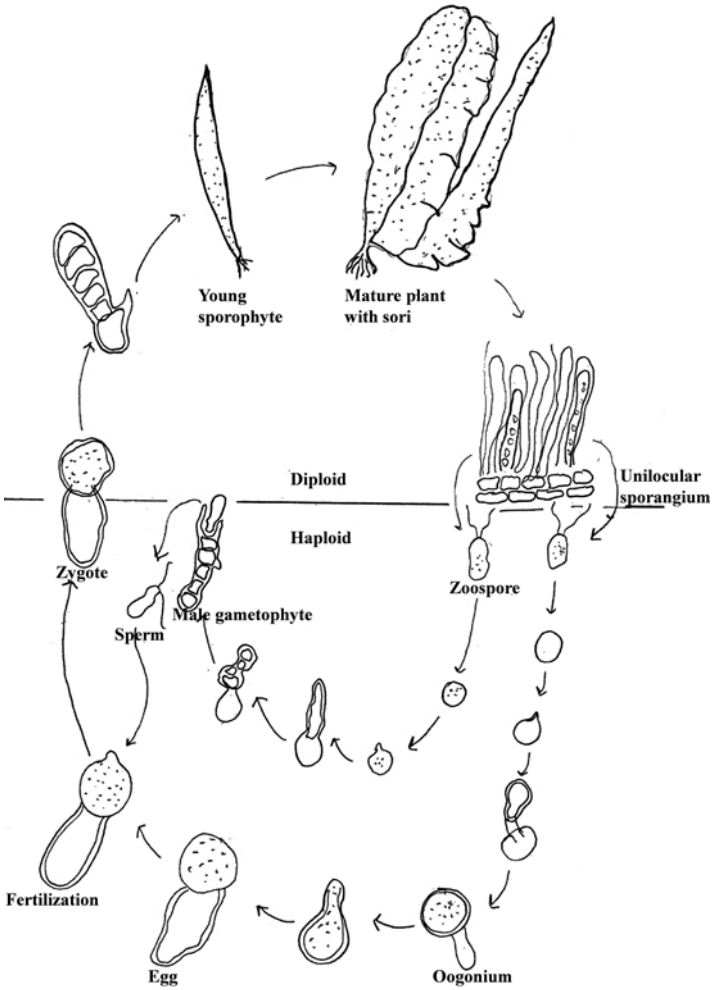

Fig. 8 Life cycle of *Laminaria* (Adapted from Lee 2008)

8.4 Sargassum

Order: Fucales
Family: Sargassaceae
Genus**:** *Sargassum*

8.4.1 Habitat and Distribution

Sargassum is distributed in tropical and subtropical waters ranging from mid-littoral to sub-littoral zones (Fig. 9).

Fig. 9 *Sargassum* in its natural habitat

8.4.2 Thallus Structure

Sargassum is usually dark brown olive or dark green in colour. Thallus resembles higher plants as it is diploid, perennial, erect and bushy with a bilateral or radial symmetry and may grow upto a length of 10–200 cm (Fig. 10a). Thallus is woody, tall with terete main axis (Verma 2010). The stipe may be short (a few cm high) or long (upto 100 cm or more), cylindrical or branched. The branching is monopodial. Branches of main axes are spirally arranged. Leaves are simple, linear and have acute apice. Air bladders (vesicles) are normally present. They are oval in shape attached to the algae with the help of stalk on the axis of the basal leaves of primary branches. These bladders are swollen, berry like, which keep the frond afloat by providing buoyancy (Vijayaraghavan and Kaur 1997).Transverse section of leaf shows cryptostomata with ostiole and protruding paraphyses (Fig. 11a). In young leaves cryptostomata are absent (Fig. 9b).Transverse section of thallus shows differentiation of tissues in to columnar assimilatory meristoderm (meristamatic in nature), several layered cortex made up of peripheral layers of compact, columnar thin-walled parenchymatous cells. Inner to cortex is the zone of medulla, which consists of narrow, elongated thick walled cells which serve the function of conduction. The medullary cells were elongated surrounded by polysaccharide material (Fig. 10c, d).

8.4.3 Life Cycle of *Sargassum*

Most of the species reproduce sexually except *S. natans* and *S. fluitans* which reproduce only by fragmentation (Lee 2008). The sex organs are produced inside special cavity called conceptacles a flask-like cavity. The Receptacles are stalked and may

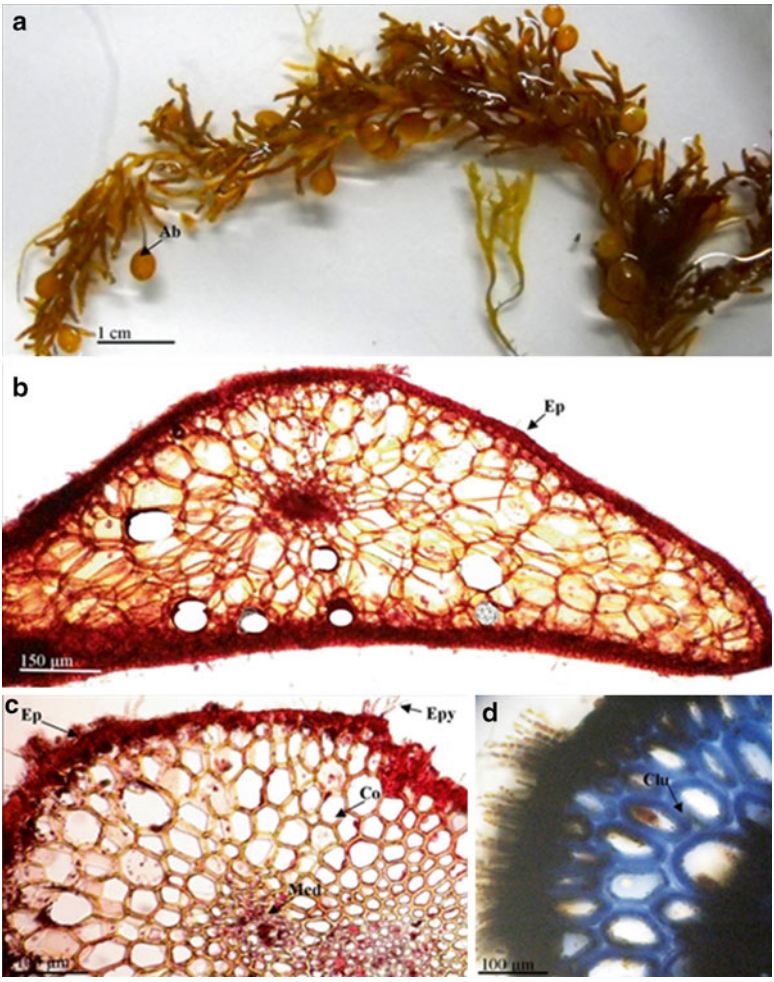

Fig. 10 (**a-d**).***Sargassum linearifolium*** **morphology and anatomy**. (Ab – Air bladder, Co – Cortex, Ep – Epidermis, Epy – Epiphytes, Med – medulla, IKI – Iodine-Potassium Iodide) (**a**) Thallus*S. Linearifolium* showing spirally arranged branches, simple leaves and air bladder. (**b**) T.S of *S. linearifolium* leaf showing epidermis, cortex and central medulla (*arrow*). (**c**) T.S of *S. linearifolium* stem showing epiphytes, epidermis, multi-layer cortex and central medulla (*arrow*). (**d**) T.S of *S. linearifolium* stem showing blue coloured cell wall with IKI indicating presence of cellulose (*arrow*)

be fertile or sterile (Fig. 11b, c). The conceptacles are found within small finger-like branchlets called receptacles. Embedded in the fertile, hermaphrodite receptacles are unisexual conceptacles which bear oogonia and antheridia. In case of dioecious forms the male receptacles are smooth and the female ones appear spinous. The conceptacles develop from a superficial meristodermal cell gradually becomes protubrent and is sunk down due to meristmatic activity of surrounding cells into a flask like cavity. By reaching at the base of this flask-like cavity the protubrent cell

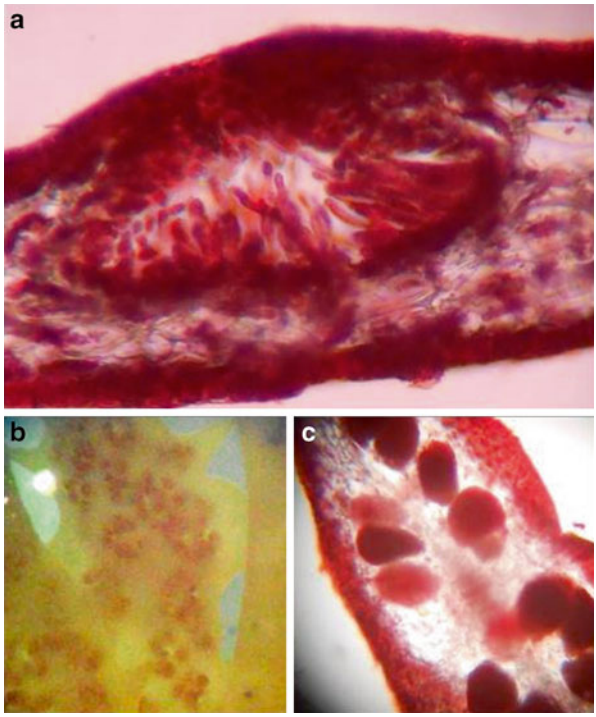

Fig. 11 (**a-c**): (**a**) T.S showing Cryptostomata on leaf. (**b**) Surface view of receptacles (**c**) L.S of receptacle showing Oogonia

divides transversally into an upper and lower basal cell layer. The basal cell divide anticlinaly and periclinally into two rows of curved cells. These layers represent fertile sheet. The antheridia and oogonia develop on this fertile sheet from the cells of the upper tier. The antheridia develops in large number with in each male conceptacle on the lower branches of paraphyses (Fritsch 1977; Vijayaraghavan and Kaur 1997; Lee 2008).The first antheridium arises from the tip of a 3-celled filament, which in turn are borne from a cell of the fertile layer region. The basal cell remains levelled with in the fertile layer. The upper or antheridial cell develops into an antheridium gives rise to 64 sperms. The middle one is known as stalk cell. The wall of the mature antheridium becomes mucilaginous. It breaks its connection from the paraphysis and comes out. The wall gets dissolved and releases the sperm in sea water. A sperm is a pear shaped biflagellate structure of pale brown colour. The flagella are unequal and inserted laterally (Uchida 1993).

Only a few oogonia are borne in a conceptacle and are from the cell of fertile layer at early stage during conceptacle development beds. The mature oogonium is globular or ellipsoidal in shape. The egg is liberated in water after the wall of oogonium ruptured. The sexual reproduction is oogamous and involves the fusion of motile male gamete with a non-motile ovum or female gamete. The oogonia attract a large number of sperm which are attracted through the anterior flagella and only one sperm penetrates the ovum. During favourable condition zygote germinates

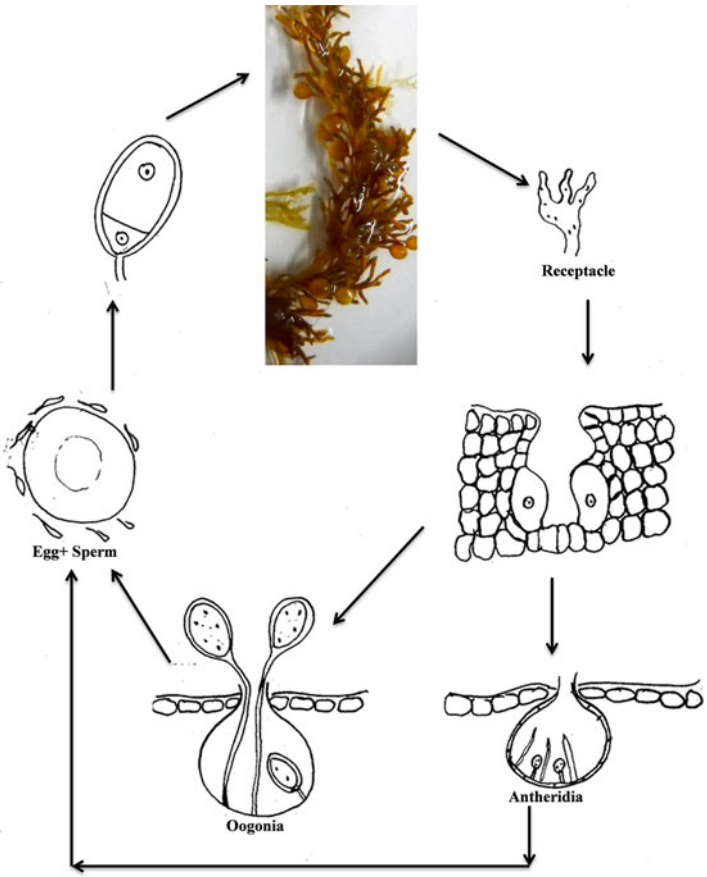

Fig. 12 Life cycle of *Sargassum*

while still surrounded by gelatinous sheath. The zygote germinates into a diploid sporophytic thallus (Fig. 12). (Fritsch 1977; Vijayaraghavan and Kaur 1997; Lee 2008)

9 Economic Importance of Brown Algae

Brown algae is being used for the purpose of food, feed and medicine in oriental (China, Japan, Korea, Malaysia and Philippines) as well as western countries (America, Australia, Europe, New Zealand, South Africa, and U.K) since ancient times. During the recent years algae has come up as a promising plant for the future because of its versatile usage and multi functional properties in terms of food, feed, energy, medicine, fertilizer and cosmetics.

9.1 Brown Algae as Food

Brown algae find its usage as food since ancient times. It is a very important part of diet in western countries (America, Australia, Europe, New Zealand, South Africa and U.K) as well as oriental countries (China, Japan, Korea, Malaysia and Philippines). In the coastal countries seaweeds are also popular as sea vegetables. Within brown seaweeds *Durvillaea, Ecklonia, Fucus, Hizikia, Lamianria* (kombu) and *Undaria* (wakame) are consumed as food (Chapman 1970 and Sahoo 2000). They are consumed raw or cooked as salads, soups, sushi, pickles, porridge, jelly, jam and toasted form while used as condiments in biscuits, snacks and tea etc. Seaweeds have gained popularity due to their high nutritive value. They are rich in protein, carbohydrate, vitamins, minerals and amino acids etc.

9.1.1 Animal Feed

Brown algae have been used as feed for a long time. According to Spolaore et al. (2006) about 5–10 % algae can be used safely as partial replacement for conventional proteins in poultry feed. They are also used to feed horses, cows and pigs. In maritime districts many seaweeds such as *Sargassum*, *Fucus*, *Laminaria*, *Ascophyllum*, *Alaria* also known as cow weed in Norway, *Chorda*, *Pelvetia*, *Hormosira* are also used as feed (Chapman 1970).

9.2 Industrial Usage

9.2.1 Algin and Alginates

The salts of algenic acid composed of L-guluronic acid monomers and D-guluronic acid polymers are known as alginates. Algin is the term used for products derived from alginic acid. Brown macroalge such as *Ascophyllum*, *Laminaria*, *Macrocystis*, *Durvellia*, *Sargassum*, *Cystoseira* and *Ecklonia* etc (Sahoo 2000; Radmer 1996 and Dhargalkar and Pereira 2005) are the major source of alginates. They are mainly used in food industry (frozen food, syrups, pastry, pudding, pies, salad and dressing, beer, milk shakes and meat flavor), paper industry (Verma 2010), explosives, polishes, ceramics, toys and various others due to their water holding, emulsifying, gelling and stabilizing properties (Sahoo 2000 and Radmer 1996). About half of the consumption of algin is in the making of ice cream and other dairy products. Also, the water-retaining properties of algin are utilized in a variety of ways in the baking industry, including the addition of algin to frostings to prevent undue drying. The colloidal nature of algin makes it useful as a suspending and emulsifying agent. In the rubber industry, it is used as a creaming and stabilizing agent in the processing of natural and synthetic rubber latex. When added to paints, alginates keep the pigments in suspension and make a product that can be brushed on a surface without

showing brush marks. Alginates are also used as suspending agents in a wide variety of pharmaceutical products.

9.3 Brown Algae as Fertilizer

Brown algae is been used as green manure. Easy availability, low cost, nutritional quality, rich micronutrients (Fe, Cu, Zn, B, Mn, Co and Mo), macronutrients (Ca, K and P), vitamins, growth hormones (auxins, betaines, cytokinins and gibberellins) and chelating property make brown seaweeds an excellent soil conditioner. Species of various seaweeds such as *Ascophyllum*, *Dictyopteris*, *Durvillaea, Fucus*, *Laminaria*, *Macrocystis*, *Padina* and *Sargassum* (Kumar and Sahoo 2011) are being used as a biofertilizer in many coastal countries like China, France, Ireland, Japan, Korea, Norway, Scotland and Philippines (Kumar et al. 2012).

9.4 Medicinal Properties

The first medicinal use of brown algae is as a goiter cure (through iodine) mentioned in sixteenth century literature in Li Shih-Chen's herbal *Pen Ts'ao Kang Mu* ("Outlines of Chinese Materia Medica"). *Aethiops vegetabilis* and kelp pills or kelp ash were being used in Europe for goiter treatment. *Laminaria* stipes was being used for the fistulae surgery while *Nereocystis*is being used for headache cure in Alaska. Sargassum and *Laminaria* are being used for the treatment of blood cleansing and easing blood pressure. Liquid extracted from ascophyllum is used for sprains and rheumatism treatments and on the other hand *Cutleria multifida* is used in stomach ulcer cures. (Vijayaraghavan and Kaur 1997). *Laminaria*, have reported anticoagulant activity (Wijesekaraa et al. 2011). Ethanolic extracts of *Fucusvesiculosus* has reported antioxidant activity (Zaragoza et al. 2008). Extract from *Fucus* brown seaweed are used for massages, impurity elimination and balancing skin pH (Dhargalkar and Pereira 2005).

9.5 Biofuel

According to Dhargalkar and Pereira (2005) biofuels supplies about 14 % of world energy needs. Due to high cost and limited availability of fossil fuel need for algal biofuel has increased tremendously. Algae can use water and carbon dioxide to produce biofuels (biodiesel, biogas and bioethanol). Microalgae play a major role in alternative biofuel production due to high lipid content. Microalgae produce 15–300 times oil compared to crop plants (Chisti 2007). Seaweed species such as *Sargassum* and *Laminaria* are being used for bioethanol production.

9.6 Aquaculture

According to Titlynova and Titlynova (2010) commercially cultivated most common brown seaweeds are *Cladosiphon, Laminaria, Saccharina* and *Undaria.* Over 4 million tons of kelps are harvested annually, mainly from mariculture in Asia (China, Japan, Korea) or from natural populations in Europe and North and South America (Lee 2008).

10 Conclusion

Brown algae have emerged as a valuable commercial resource. Utilization of brown algae have increased significantly in recent years, with the resultant increase in applied research in various fields; still their full agronomic and biotechnological potential is yet to be realized. Taxonomic studies for creation of biodiversity database on algae, *in-situ* conservation of germplasm and establishment of herbaria for macroalgae are long sought for. To utilize algal resources in a sustainable manner as well as conservation of these resources is a prerequisite. Unplanned and continuous harvesting of these seaweeds from their natural habitats resulted in depletion of standing crop. It is important to take a multidisciplinary approach to the research and commercial relevance of algae in aquaculture, algae based products as well as reconnaissance of new business opportunities for algae based technologies.

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Red Algae

Arunjit Mayanglambam and Dinabandhu Sahoo

1 Introduction

The biodiversity of algae is large but difficult to determine, mainly because of the limited biogeographic inventories worldwide (Norton et al. 1996). Bold and Wynne (1985) noted that the morphological diversity and cytology of algae make them difficult to clearly define. Seaweeds are categorized on the basis of multicellular nature and its mode of attachment to substrata, which are primarily found in the Divisions Chlorophyta, Phaeophyta, and Rhodophyta. The differences between divisions of seaweeds are evident when comparing the photosynthetic pigments, reserve foods, cell wall, mitosis, flagellar construction, morphology, and life histories.

The red algae comprise approximately 6,500–10,000 species (Woelkerling 1990) and form a unique intriguing and intrinsically interesting group of plants that need critical study. Majority of red algal species are marine with about 3 % (150 species from 20 genera) being freshwater (Sheath 1984). Features of red algae include eukaryotic cells, a complete lack of flagellar structures, food reserves of floridean starch, which is an amylopectin (α 1–4 main chain, (β 1–6 side chain glucans), the presence of phycoblins, chloroplasts without stacked thylakoids, and no external endoplasmic reticulum.

The fresh water species are found either in fast flowing streams (*Batrachospermum*, *Lemanea*) or grow in stagnant water with sufficient aeration (*Compsopogon*, *Asterocytis*). Amongst marine red algae, larger and fleshy forms occur in cool temperate areas whereas species found in the tropical seas are mostly small and

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filamentous. The members of Rhodophyceae have ability to live at greater depths in the ocean than the members of other algal group. The red algae also exhibit a high degree of epiphytism and parasitism with considerable specificity. The epiphytic species possess normal form and pigmentation, but parasitic species show a great reduction in their form and pigmentation. *Polysiphonia lanosa* (host- *Ascophyllum nodosum*), *Ceramium condicola* (host- *Codium fragile*) are some parasitic species while species of *Ceratocolax*, *Lithothamnium* are epiphytic on other red algae.

2 General Characters

1. The name 'red algae' is given to these plants because of the excess and dominance of pigments r-phycoerythrin and r-phycocyanin which hide the green colour of Chlorophyll. Chlorophyll-b which is replaced by the chlorophyll-d. The chief xanthophyll is taraxanthin.
2. Red algae are characterized by absence of flagellated motile stages.
3. The main food storage products are in the form of floridean starch, floridoside and mannoglycerate.
4. In addition to the cellulose and pectin, chief characteristic components of the cell walls are polysulphate esters. Cell wall consists of rigid components such as microfibrils and a mucilaginous matrix. Agar and carrageenan are two red algal polysaccharides that are widely used as gelling and thickening agents in the food and pharmaceutical industries are found only in some red algal species.
5. Though some genera such as *Gigartina* and *Schizymenia* may reach upto 1 m (Smith 1944) and 2 m (Abbott 1967), respectively, the red algae do not generally attain the size as large as brown algae.
6. Except a few unicellular forms (*Porphyridium*), the great majority of red algae are filamentous (*Goniotrichum, Gracilaria, Kappaphycus*), pseudoparenchymatous (*Dumontia* and *Helminthocladia*) or parenchymatous (*Porphyra*) forms.
7. In most multicellular genera the vegetative cells are interconnected by cytoplasmic connection or pit connections. The pit-connection serves as the passage through which the cytoplasmic materials flow between the adjacent cells.
8. Sexual reproduction is highly specialized and it is of advanced oogamous type.
9. Male sex organs are known as spermatangia. In each spermatangium develops a single, non-flagellated, non-motile male gamete known as spermatium.
10. Female sex organs are known as carpogonia. Each carpogonium is generally a flask-shaped body, having a long neck-like structure called as trichogyne.
11. During fertilization the non-motile male gamete are taken upto the trichogyne with the help of water.
12. Post-fertilization stages are also highly elaborate and characteristic of the red algae. Though they exist in many variations, many gominoblast filaments develop from the fertilized carpogonium in many cases. Tip cells of these filaments start to function as carposporangia. In each carposporangium develops a carpospores. This entire structure represents the carposporophyte.

13. Higher forms (*Polysiphonia*) also produce tetraspores and tetrasporophyte.
14. Most of the members show diphasic or triphasic life-cycles.

3 Occurrence

The occurrence of red algae varies from genus to genus and their growth forms can be divided into three major types i.e. aquatic forms, terrestrial forms, epiphytic and parasitic forms.

3.1 Aquatic Form

The aquatic form can be divided into two broad classes namely fresh and marine water forms. Fresh water forms are found in ponds, pools, lakes, rivers etc. These can grow either in flowing streams *Lemanea* (Fig. 1a), *Batrachospermum* or in stagnant water (*Compsopogon*). A few species of *Gracilaria* can adapt on brackish water (Fig. 1b). While the marine forms, *Kappaphycus* sp. (Fig. 1c) grows in sea water. Most of the members of Rhodophyceae belong to marine forms. They can grow in the intertidal and sublittoral regions.

3.2 Terrestrial Forms

Some red algal species are found in terrestrial habitats like soils, rocks etc. Unicellular red alga *Porphyridium* is found in moist soil. Moreover the terrestrial types are largely characterized by relatively simple construction and simple mode of reproduction.

3.3 Parasitic, Epiphytic and Other Forms

Members of red algae exhibit a high degree of epiphytism and parasitism. The epiphytic species maintained normal form and pigmentations but parasitic species show a great reduction in their form and pigmentation. The growth of some red algae is accompanied by the occurrence of other species and competes for their growth. It has been observed that *Kappaphycus* cultivation is mostly hindered by the occurrence of *Neosiphonia* sp. (Fig. 2a). In case of parasitism, *Gelidiocolax* in host plant *Gelidium* sp., (Fig. 2b). Other parasitic forms are *Polysiphonia* species which grows on *Ascophyllum nodusum*, *Ceramium* sp. in host *Codium fragile* and *Ceratolax* sp. on *Phyllophora* sp. etc. Some red algae *Corallina* sp. (Fig. 2c) etc. also exist in coralline form.

Fig. 1 (**a**) Freshwater aquatic habitat, *Lemanea* sp. on substratum (**b**) Brackish water habitat showing *Gracilaria* (*arrow*) and other macroalgae. (**c**) Marine habitat showing morphotype of *Kappaphycus alvarezii*

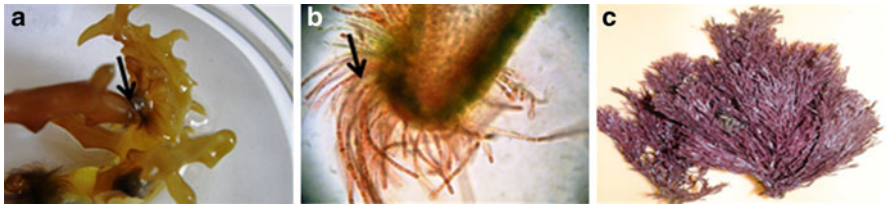

Fig. 2 (**a**) *Kappaphycus alvarezii* growth is accompanied by epiphytic *Neosiphonia* sp. (*arrow*) (Mayanglambam and Sahoo 2014b) (**b**) *Gelidiocolax* sp. in host *Gelidium* sp. (*arrow*) showing parasitic form (**c**). *Corallina* sp. showing coralline form

4 Classification

The main points of difference between the two groups (Bangiophycedae and Floridiophycedae) that are often stressed are: (1) the Bangiophycedae are either unicellular or filamentous or expanded, without pit-connections between cells. In Floridiophycedae, cells of the thallus are always connected by pit-connection of a characteristics structure and this may be primary or secondary. The thallus is basically filamentous, cell division more or less restricted to the apical cells of the filament whereas those of Bangiophycedae shows diffused growth. (2) Sexual reproduction is well established only in a few genera of Bangiophycedae. Where known, the female reproductive cell is a structurally unmodified cell of the thallus. In the Floridiophycedae, the female reproductive cell, the carpogonium is distinctive in structure. (3) Cells in Bangiophycedae are usually with a single chromatophore and generally without an evident central vacuole. While in Floridiophycedae usually cells are vacuolate with one or more chromatophore. (4) No gonimoblast filaments are produced in Bangiophycedae, the zygote dividing directly to form either one or more carpospores. In the Floridiophycedae, the zygote, either directly or through auxiliary cells, gives rise to gonimoblast filaments from which carposporangia are produced, the division of the zygote being generally mitotic, free living gametophytes alternating with free living tetrasporophytes. Fritsch (1945), on the basis of thallus structure and post-fertilization changes, classified Rhodophyceae into two subclasses:

Class Rhodophyceae	(e) Solieriaceae
Sub-class Bangioideae	(f) Rissoellaceae
Order (i) Bangiales	(g) Hypneaceae
Family (a) Bangiaceae	(h) Plocamiaceae
(b) Porphyridiaceae	(i) Sphaerococcaceae
Sub-class Florideae	(j) Gracilariaceae
Orders (ii) Nemalionales	(k) Mychodeaceae
Family(a) Achrochaetaceae	(l) Acrotylaceae
(b) Batrachospermaceae	(m) Phyllophoraceae
(c) Lemaneaceae	(n) Gigartinaceae
(d) Naccariaceae	**(vi) Rhodymeniales**
(e) Bonnemaisoniaceae	**Family** (a) Champiaceae
(f) Thoreaceae	(b) Rhodymeniaceae
(g) Helminthocladiaceae	**(vii) Ceramiales**
(h) Chaetangiaceae	**Family** (a) Ceramiaceae
(iii) Gelidiales	(b) Delesseriaceae
Family (a) Gelidiaceae	**Sub-family** (a) Delesserieae
(iv) Cryptonemiales	(b) Nitophylleae
Family (a) Gloeosiphoniaceae	(c) Sarcomenieae
(b) Endocladiaceae	(c) Rhodomelaceae
(c) Callymeniaceae	**Sub-family** (a) Polysiphonieae
(d) Grateloupiaceae	(b) Lophothaliaeae
(e) Dumontiaceae	(c) Bostrychieae
(f) Cruoriaceae	(d) Rhodomeleae
(g) Rhizophyllidaceae	(e) Chondrieae
(h) Squamariaceae	(f) Laurencieae
(i) Corallinaceae	(g) Pterosiphonieae
(j) Choreocolaceae	(h) Herposiphonieae
(v) Gigartinales	(i) Lophosiphonieae
Family (a) Calosiphoniaceae	(j) Polyzonieae
(b) Nemastomaceae	(k) Amansieae
(c) Sebdeniaceae	(d) Dayaceae
(d) Furcellariaceae	

5 Thallus Organisation

Most of the red algal thalli are beautiful, soft and slimy. The thallus ranges from simple unicellular to complex multiaxial form. Thalli are usually multicellular, filamentous or non-filamentous, cylindrical or flattened or foliaceous, branched or unbranched, with or without having differences between the superficial and deep-lying cells. Filamentous thalli are simple or branched, uni- or multiseriate, often heterotrichous. The growth in Bangiophycidae is generally intercalary or diffuse

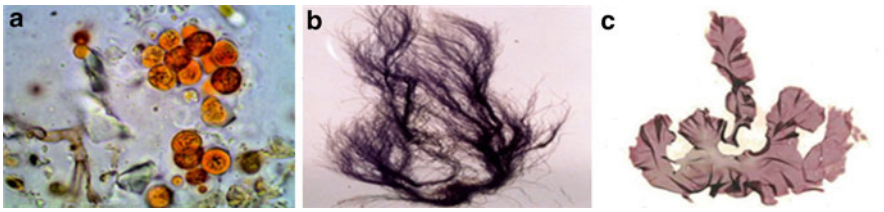

Fig. 3 (**a**) Unicellar forms *Porphyridium* sp. (Image copyright © www.aquaportal.com) (**b**) Filamentous form *Polysiphonia* sp. (**c**) Parenchymatous form *Porphyra* sp.

except in *Compsopogon*, where as the growth is apical. Morphological forms of the group ranged from the unicellular species such as, *Porphyridium* sp. (Fig. 3a) to the filamentous types such as *Polysiphonia* species (Fig. 3b). Other unicellular forms are *Chroothece*, *Rhodosorus*, *Rhodospora* while some other filamentous forms are *Goniotrichum*, *Bangia*, *Rhodochaete*. The thalli of some red algae such as *Porphyra* sp. (Fig. 3c) are parenchymatous in nature.

In case of floridiophycidae the thalli are mostly filamentous. Simple uniseriate branched filament is seen only in two families (Acrochaetiaceae and Batrachospermaceae). Majority of forms have more massive thalli with a plectenchymatous development and the filamentous nature is not apparent. Thalli of the later type fall under two main categories (1) wherein the close juxtaposition of the branch system of a single main axial filament has resulted in a more or less pseudoparenchymatous thallus (the uniaxial or central filament type) example *Batrachospermum* sp. (Fig. 4a) and *Gracilaria* sp. (Fig. 4b). (2) Where there is juxtaposition of a number of axial filaments so that the central axis consists of a strand of filaments whose numerous branches form a more or less compact cortex, the multiaxial or fountain type, *Kappaphycus* sp. (Fig. 4c).

6 Cell Structure

A typical red algal cell is truly eukaryotic in structure Fig. 5. It has a definite cell wall, one or more plastids, with or without pyrenoid, nuclei and other cell organelles such as golgi apparatus, ribosome, mitochondria, endoplasmic reticulum, phycobilisomes, dictyosomes, membrane bound vesicles etc.

6.1 Cell Wall

The cell wall in most of the red algae shows two distinct layers under the optical microscope. Electron microscopic studies in *Gracilaria corticata* reveals cells having thick cell walls that have microfibrils arranged in three regions – (1) the innermost electron-dense, (2) middle electron-translucent and (3) outermost electron

Fig. 4 Range of thallus structure in floridiophycidae showing (**a**). Uniaxial form *Batrachospermum* sp. (Image copyright © www.macrography.net) (**b**) *Gracilaria* sp. (**c**) Multiaxial form *Kappaphycus* sp. (Mayanglambam and Sahoo 2014a)

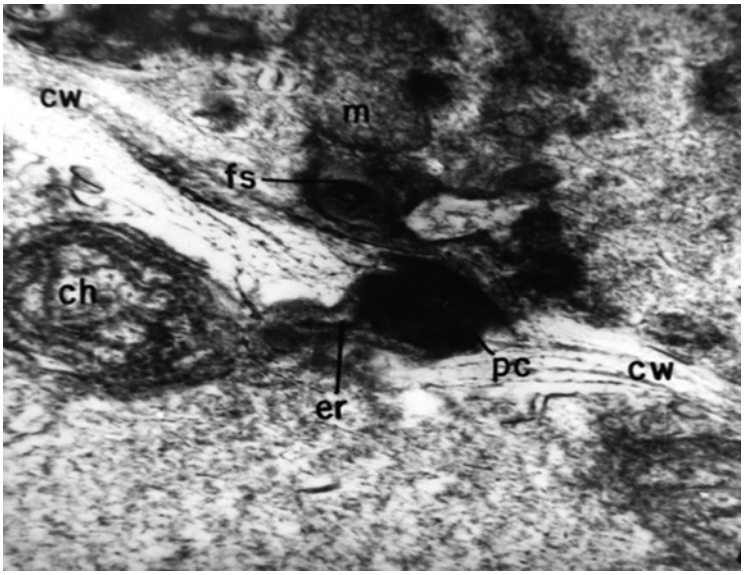

Fig. 5 Electron micrograph of *Coelarthrum opuntia* showing cell wall (*cw*) microfibrils, Endoplasmic reticulum (*er*), mitochondria (*m*), chloroplast (*ch*) and floridean starch (*fs*) associated with pit-connection (*pc*)

dense regions (Fig. 6a, b). In all the three regions, microfibrils are parallelly arranged. The cells are connected through distinct pit-connections and their cytoplasm show large starch grains (Fig. 6a). Extracellular matrix consists of cellulose microfibrillar network and amorphous matrix of cellulose, galactans, and mucilage. Components of mucilages are polymers of D-xylose, glucose, glucoronic acid and galactose, which are produced in large golgi vesicles. The inner layer is mainly cellulose microfibrils while the outer is electron dense and fibrillar materials are embedded in an amorphous substance (Cole et al. 1985). Cellulose is considered to be absent in some red alage such as *Porphyra* and *Bangia*. Crystalline material of calcium composition is mainly deposited in the matrix of the cell wall (Bailey and Bisalputra 1970). Calcification is characteristic of cell walls of members within the Corallinales, with its crystalline form being calcite instead of the aragonite of

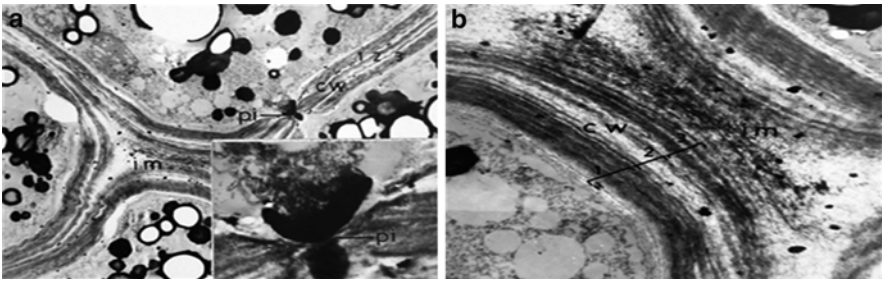

Fig. 6 (**a**) and (**b**) Cell wall electron micrograph of *Gracilaria corticata* showing microfibrils arranged in three regions – (*1*) the innermost electron-dense, (*2*) middle electron-translucent and (*3*) outermost electron dense regions. In all the three regions, microfibrils are parallelly arranged. The cells are connected through pit-connections (*inset*) and their cytoplasm show large starch grains

calcified green algae. The process of calcification is linked to carbon fixation, with uptake of CO_2 from cell walls resulting in an increase in pH and an increase CO_3^{-2} ions during photosynthesis. The increase in carbonate results in a precipitation of $CaCO_3$. Coralline red algae play a critical role in coral reef development via their primary productivity, cementation of coral reef rubble, and serving as a source of sediment.

6.2 Chloroplast

Chloroplast is the most distinctive features of red algae; the most apparent is the thallus colour. The chloroplasts are enclosed by a double membrane. This characteristic colour of the red algae is due to the presence of water soluble phycobilin pigments. These phycobiliproteins are arranged in large pigment protein complex called phycobilisomes which are attached to the stromal surface of the photosynthetic membrane. The red algae typically have unstacked, evenly spaced thyllakoid. The chloroplast in red algae varies in morphology from stellate to discoid to highly lobed structure. Plastids are present in all the red algae even in the colourless parasitic forms have plastids with very little thyllakoids (Goff 1982). In *Griffithsia pacifica* (Florideaphyceae) chloroplast with unstacked photosynthetic lamellae (Fig. 7a, b) bearing granules, called phycobilisomes composed of water soluble phycobiliprotein accessory pigments (Pueschel 2000). Cytoplasm shows chloroplast and starch grains which is a reserve and deposited free in the cytoplasm. Pyrenoids are present in some members of Bangiophytes and Nemaliales.

However when a plastid divides unevenly by detachment of arms of stellate plastids, pyrenoids developed *de novo* in smaller plastids. This type is seen in the germinating carpospores of *Porphyra* (Pueschel and Cole 1985).

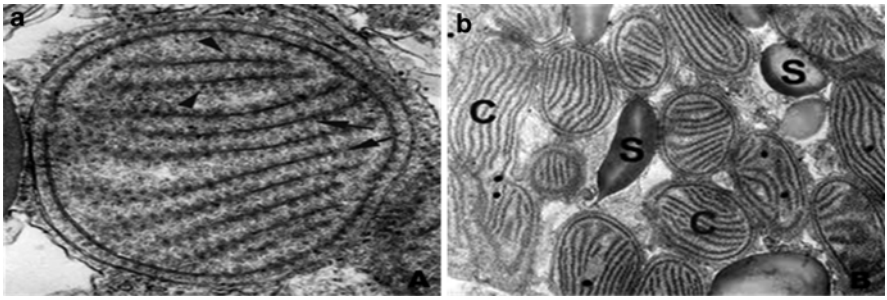

Fig. 7 *Griffithsia pacifica* (**a**) Electron micrograph showing a chloroplast with unstacked photosynthetic lamellae (*arrows*) bearing granules, called phycobilosomes (*arrowheads*) (**b**) Electron micrograph showing cytoplasm with numerous chloroplasts and starch. (*C* Chloroplast, *S* Starch) (Image courtesy© 2000, C.M. Pueschel)

6.3 Nucleus

Cells may be uninucleate and multinucleate in red algae. Single nucleus is present in the cells of Bangiophycidae and some members of Florideophycidae. In general the nucleus of most red algae is small. The nuclear size varies from species to species in different tissues of the same species at different times of year and under different environmental conditions. The smallest nuclei occur in some Cryptonemiales and Gigartinales while the largest occur in some Ceramiales (Magne 1964). Electron micrograph shows prominent nuclei with electron dense nucleoplasm and nucleolus. The nucleus appears irregular or stellate and compressed due to compact arrangement of many perinuclear chloroplast and floridean starch granules (Fig. 8a). There is evolutionary significance that the centrioles and flagella are absent in red algae. Other organelles such as microbodies are apparent in red algae but catalase localization which is an important feature of microbodies in higher plants and animals has not been reported.

6.4 Mitochondria, Golgi Bodies, Endoplasmic Reticulum

Mitochondria are long, slender, double membrane where the inner membrane are folded to form tubular cristae. The position of mitochondria inside the cells varies from species to species. In *Rhodella reticulata*, the mitochondria are present mainly in the outer regions of the cell while in *Bangia* the mitochondria are situated close to dictyosomes. Mitochondrial genophore are attached in specialized region of crista (Fig. 8c).

In red algae Golgi bodies are generally composed of 4–5 stacked cisternae. In *Bangia* the *cis* face of Golgi bodies coexist with other cell organelles such as Endoplasmic Reticulum and Mitochondria (Gantt and Conti 1965). In *Rhodella* species the cis Golgi are associated closely with nucleus (Fig. 8b). The golgi appa-

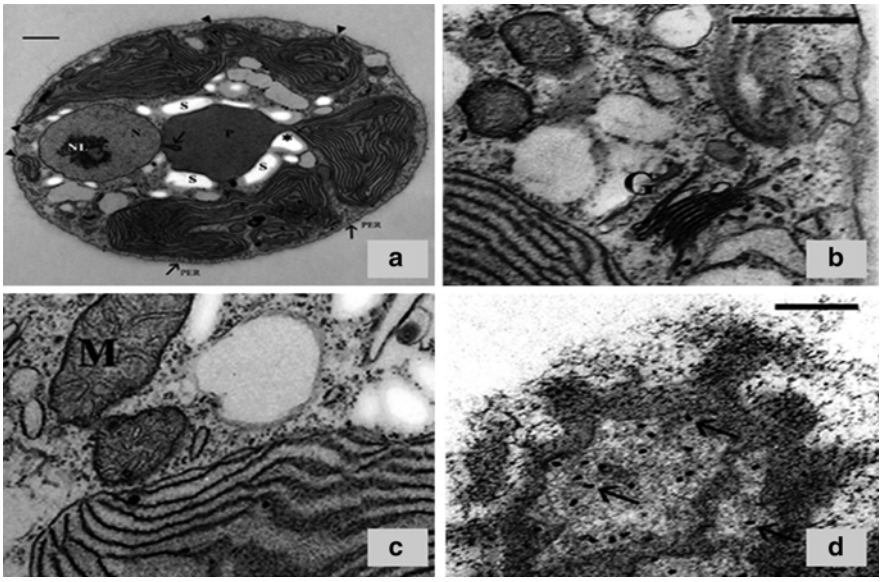

Fig. 8 *Rhodella violacea* ultrastructure. (**a**) The nucleus is peripheral with a centrally located nucleolus. A nuclear extension (*arrow*) projects into the adjacent pyrenoid which is surrounded by starch grains. The pyrenoid lacks thylakoids and is seen connected to one of the chloroplast lobes (*asterisk*). (**b**) A Golgi body is near the cell membrane (*arrow*). (**c**) Double membrane Mitochondria (**d**) Tubules of the peripheral ER system (*arrows*) are seen in cross section. (*Scale bars* represent: (**a**) 1.0 μm, (**b**)–(**c**) 0.5 μm; (**d**) 0.1 μm) (Scott et al. 2011). *N* Nucleus, *NL* Nucleolus, *P* Pyrenoid, *S* Starch grains, *ER* Endoplasmic reticulum, *PER* Peripheral Endoplasmic reticulum, *G* Golgi body

ratus in red algae appears to function principally by cisternal progression. Endoplasmic reticulum of both rough and smooth form is present in the cell periphery and around the nucleus of red algae (Fig. 8a). The endoplasmic reticulum of some members *Porphyridium* (Gantt and Conti 1965; Gantt et al. 1968), *Rhodella* (Deason et al. 1983; Evans 1970) and *Flintiella* (Scott 1986), a cisternal network is found beneath the plasmalemma. In rhizoidal cells of parasitic red alga, ER forms direct connections with the plasmalemma (Goff 1982). The endoplasmic reticulum is interconnected with the cell membrane by short tubules (Fig. 8d).

6.5 Pit Connections and Pit Plugs

Pit-connection is one of the most distinguished features of the red algae. Pit-connections are membrane bounded plugs containing granular material fitting into the pores of the septum. A dome shaped membrane enclosed structure fits around the plug called the plug cap or cap membrane (Fig. 9a). Some members of bangiophyceae have complete and incomplete septation depending on the stage of the life history. When the septation is incomplete, a connection called pit connection

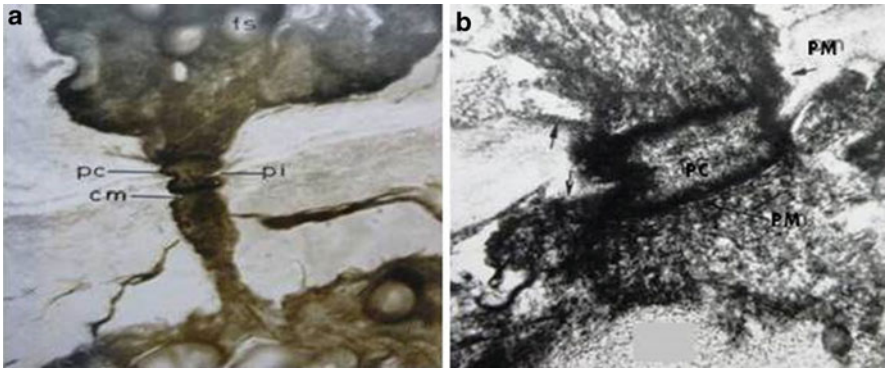

Fig. 9 (**a**) The thallus of *Gracilaria corticata* showing epidermal and cortical cells interface show pit-connection with prominent lenticular plug-core that is constricted in the middle. The plug-core is surrounded by a cap-membrane which is in continuity with plasmamembrane. (**b**) Electron micrograph showing a distinct cap-membrane surrounding the plug core in *Gracilaria corticata* (*pc* plug-core, *pi* pit-connection, *cm* cap-membrane, *fs* floridean starch, *PM* Plasmamembrane)

maintains the contact between the daughter cells. Pit connection can be divided into primary pit connections if they joined between kindred cells, cells formed by division of common parent cells and secondary pit connection in which the connections occurred between the non kindred cells.

Pit connection does not allow cytoplasmic continuity because just after the furrowing ceases, a plug is deposited in the septal aperture (Pueschel and Magne 1987). The ultra structure of red algal pit plugs shows a relative large core called the "plug core" which is bracketed by a pair of thin caps (Fig. 9b). This plug core and plug cap differ only in their contents especially proteins and polysaccharides. Degeneration of pit plugs occurs when one of the connected cells dies (Pueschel and Vander Meer 1985). The pit connection is considered as the only means of symplastic communication in red algae. Thus pit connection helps in transport and cellular communication in the cells.

7 Reproduction

In red algae, reproduction takes place by vegetative, asexual or sexual modes. Vegetative propagation is confined to the unicellular members of the Porphyridiales, although in filamentous members of the other orders, fragments of the algae may grow into new thalli. While in multicellular forms both asexual and sexual reproduction is more common.

7.1 Vegetative Reproduction

Multicellular asexual structures termed 'gemmae' are reported from the freshwater species *Hildenbrandia rivularis*. Propagules resembling spermatangial branches occur on male, female and tetrasporangial branches of *Polysiphonia ferulacea*. Hook like propagules (tendrils) and stellate propagules are found in *Hypnea muciformis* and *H. valentiae* respectively.

7.2 Asexual Reproduction

Asexual reproduction is by endospores which are produced in varying numbers by special sporangia. In conformity with the number of spores produced which are of different types based on the nature of cells from which they are produced, these sporangia are called as monosporangia, bisporangia, tetrasporangia and polysporangia. These are haploid spores (monospore, bispore, neutral spore, carpospores, tetraspore and polyspores) produced on diploid thalli or diploid parts of haploid thalli. Their formation is preceded by a reduction division in normally an enlarged, differentiated cell called the sporangium.

7.3 Sexual Reproduction

Sexual reproduction in red algae is oogamous where the male gamete 'spermatium' is nonflagellate and the female cells 'carpogonium' is a flask shaped cell with a neck like protuberance called trichogyne. Carpogonia are produced near the apices of the branches. The carpogonial branch is a two-celled structure consisting of carpogonium cell having trichogyne and a hypogonous cell borne on a big supporting cell. The hypogonous cell is lateral in position and remains attached to carpogonium and supporting cell through pit connection. The carpogonium is conical in shape, bears an elongate and swollen trichogyne (Fig. 10a). The egg nucleus is restricted to the basal portion. In the Bangioideae carpogonia are sessile but in Florideae the carpogonia are stalked and produced terminally on a special branch, the carpogonial filament. The male spermatangial conceptacles are present in the subcortical region. The spermatangial conceptacle is irregularly ovoid in shape and they are surrounded by thick mucilaginous covering giving a swollen appearance to the epidermis (Fig. 10b).

Water motion plays an important role in the fertilization of red algae. The wall of both spermatium and trichogyne are dissolved at the point of contact and spermatial contents with the male nucleus pass down the trichogyne into the carpogonium to fuse with the female nucleus there. The zygote does not divide directly into spore as in Bangiophycedae, but produces sporogenous filaments, the Gonimoblast fila-

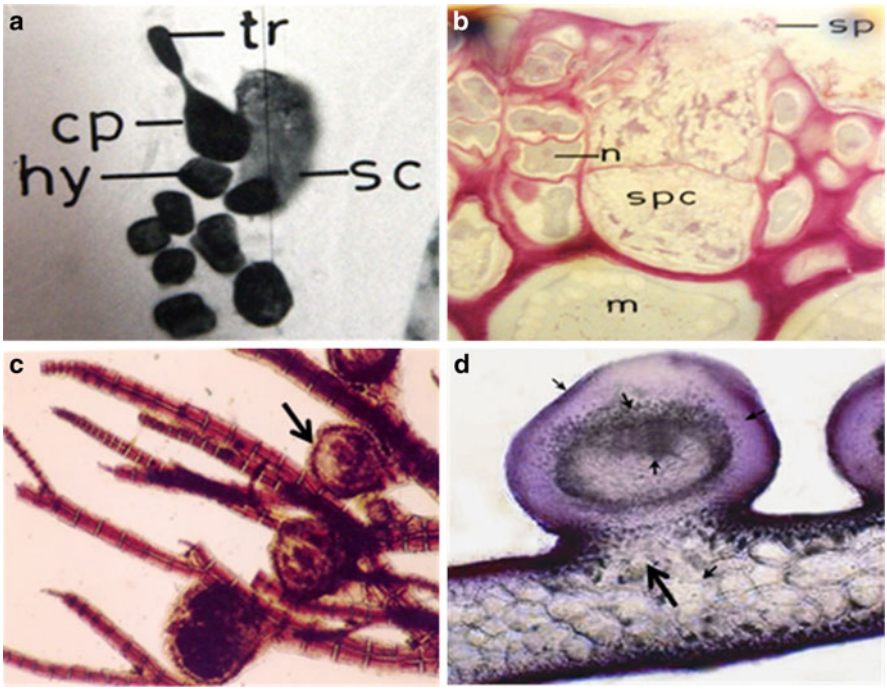

Fig. 10 (**a**) Four-celled carpogonial branch arising from the stellate supporting cell. The carpogonium has an intensely stained basal region and a long trichogyne in *Gracilaria coticata*. (**b**) Spermatangial conceptacles are surrounded by elongate and ovoid cortical cells. The release of spermatium is marked by lysis of outermost tangential epidermal wall. (**c**) and (**d**) Cystocarps on the *Polysiphonia* sp. and *Gracilaria* sp. respectively (*arrow*). (*cp* carpogonium, *hy* hypogonous cell, *sc* supporting cell, *spc* spermatangial conceptacles, *n* nucleus, *m* medulla, *tr* trichogyne)

ments. The terminal cell of these filaments developed into carposporangia, each of which produces a single spore, the carpospores. In the higher red algae the sporogenous (Gonimoblast) filaments do not arise directly from the fertilized carpogonium but from special "auxiliary cells" which may be situated in the carpogonial branch itself or may be present in other independent branch system. Except in most primitive forms, the developing gonimoblast is protected by an envelope of loosely interwoven sterile branches or by a compact cellular wall, the "Pericarp". The resultant fruitification is termed as 'cystocarp' (Fig. 10c, d).

The production of sex organ and its fertilization is uniform throughout the rhodophyta but in case of Florideae a post fertilization changes resulting in the production of carposporophyte which is parasitic on the gametophyte is observed. There is a distinct stage in the post fertilization activities such as the place of formation of gonimoblast filaments either from fertilized carpogonium or from generative auxiliary cell, the position and the time of formation (before or after fertilization of auxiliary cell) are so significant that they have provided the main basis for the classification of florideae into various orders. But in case of Bangiodeae no new genera-

tion like carposporophyte is produced but zygote divides directly into carpospores which later germinate to form conchocelis phase as common in *Porphyra*.

8 Spores Types in Rhodophyta

8.1 Monosporangia

In monosporangia a single large uninucleate spore called monospore is formed either on haploid or diploid plants. They are not derived from goniblast tissue and are always mitotic in nature. They are found in members of *Compsopogon* and *Batrachospermum* etc. A range of monosporangial types is found in bangiophycideae where monospores are formed from a differentiated sporangium by division of the sporangial cell. The sporangial cell usually divides into two unequal parts, the larger parts often remain sterile and the smaller differentiating into a spore. The division wall is often oblique and curved and the contents of the two cells are frequently different. The larger cell may be subsequently enlarged and repeat spore formation. Such spores are formed in *Erythrotrichia*, *Porphyropsis*, *Porphyra*. In another monospores type, spores are formed from entire contents of undifferentiated vegetative cells which function as sporangia forming a single spore. Such spores are found in *Goniotrichum*, *Phragmonema* and *Bangia*.

8.2 Neutral Spores

Neutral spores are those spores that are not formed in sporangia but are formed by direct division of vegetative cells. They are diploid in nature and on germination give rise to sporophytic plants. They are common in sub-class bangioideae.

8.3 Carposporangia

In carposporangia carpospores are formed from the zygote. In bangioideae carpospores are formed directly from the reduction division of the zygote nucleus. The nuclei passed into the goniblast initial which later developed into gonimoblast filaments where the terminal cells of each filament bear a single haploid carpospores. In other genera, *Polysiphonia* the zygote nucleus directly developes into carpospores. Such spores on germination give rise diploid sporophytic plants.

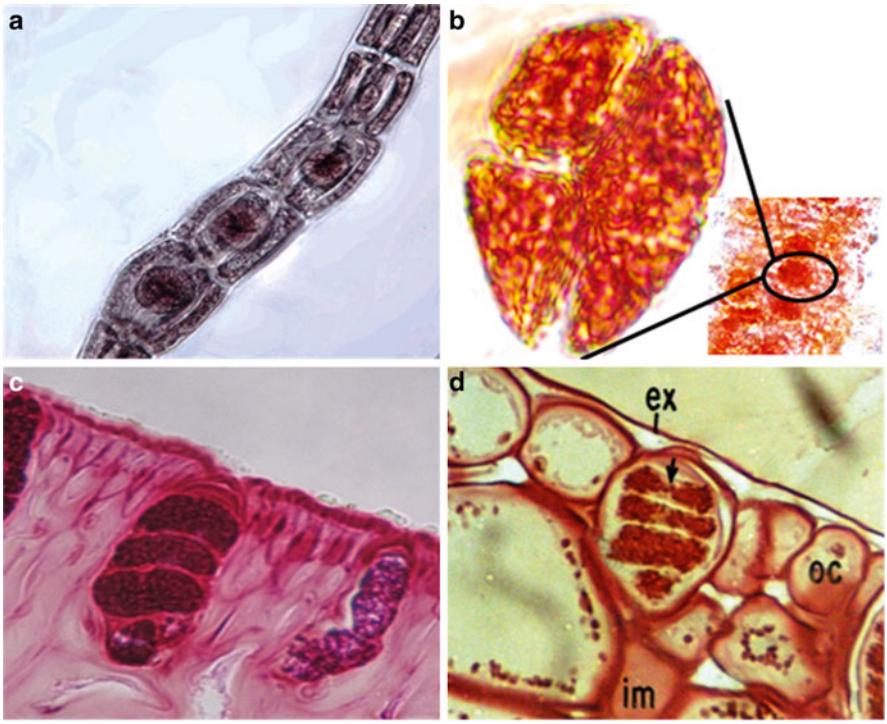

Fig. 11 Different forms of tetrasporangia commonly found in Rhodophyta. (**a**) Tetrahedral type in *Polysiphonia* sp. (**b**) Cruciate type in *Gracilaria* sp. (**c**) and (**d**) Zonnate type in *Kappaphycus alvarezii* and *Coelarthrum opuntia* respectively (*ex* extracellular mucilage, *oc* outer cortex, *im* inter cellular mucilage

8.4 Tetrasporangia

Tetrasporangia give rise to four uninucleate equal size spores called tetraspores. They are symmetrically cleaved and are characteristics of florideophycideae. Tetrasporangia are commonly found on free living diploid thalli. Sporangia with four spores formed by means of two or three cleavages generally give rise to three basic types of tetraspore: cruciate, zonnate and tetrahedral. In tertrahedral type of tetrasporangia the two divisions are oriented in such a manner as to form a group of four tetrahedrally dispose spores.

This type tetrasporagia occur in all families of Rhodimeniales and Ceramiales (Fig. 11a). A cruciate type of tetrasporangia is the one where the two divisions of the cells are at right angle to each other. This type tetrasporangium is found of orders of florideophycideae and seems to be the most primitive of all sporangial types (Fig. 11b) (Guiry and Irvine 1989). Zonate type tetrasporangia are the one where there are three parallel transverse divisions. Two types zonnately divided tetrasporangia were observed in florideophycideae. The first division and the most common type is successive zonate where a median division occur initially followed

by two roughly parallel divisions at equal distance at both side. The second type of division is found only in Corallinales where the cleavage are all initiated at the same time and this type is referred as simultaneous zonate (Fig. 11c, d) (Guiry 1978).

8.5 Conchosporangia

In the conchocelis phase of *Bangia* and *Porphyra* species Conchosporangia are formed in irregularly shaped conchosporangial branches. Conchosporangia showed rectangular outlines, strong pigmentation, size and thickness of the cell walls. Conchosporangia form a single uninucleate spore and are group together in structure called as sporangial branches.

8.6 Zygotosporangia

Zygotosporangia are those sporangia form within the fertilized carpogonium from the zygote or by direct division of zygote. In the zygotosporangia of *Bangia* and *Porphyra* formation of zygotospore takes place by parenchymatous cell division of the zygote.

8.7 Polysporangia

Polysporangia are found only in the Rhodemeniales and Ceramiales. Drew (1937) stated the term polysporangia as those sporangia with more than four meiotic spores formed in place of tetrasporangia. This polysporangia with spores in multiples of four have been reported in about 13 genera of Cerameceae and in a genus of Champiceae. Westbrook (1930) and Guiry and Irvine (1989) observed both tetrahedrally divided tetrasporangia and polysporangia with eight spores occurring on the same thallus in *Compsothamnion* sp. Different forms of sporangia found in rhodophyta with differential cleavages and arrangement of nuclei are shown in (Fig. 12).

9 Types of Life Cycle

Biphasic life cycle occurs in evolutionary early species (Fig. 13a). Triphasic life cycle is unique to evolutionary young red algae (Fig. 13b). Life cycle can change in some species, e.g. *Porphyra*: monospore, aplanospores, gametophyte (sexual reproduction) the pattern of life cycle in Bangiophycideae showed asexual spore formation as the chief mode of production of successive generations of individuals.

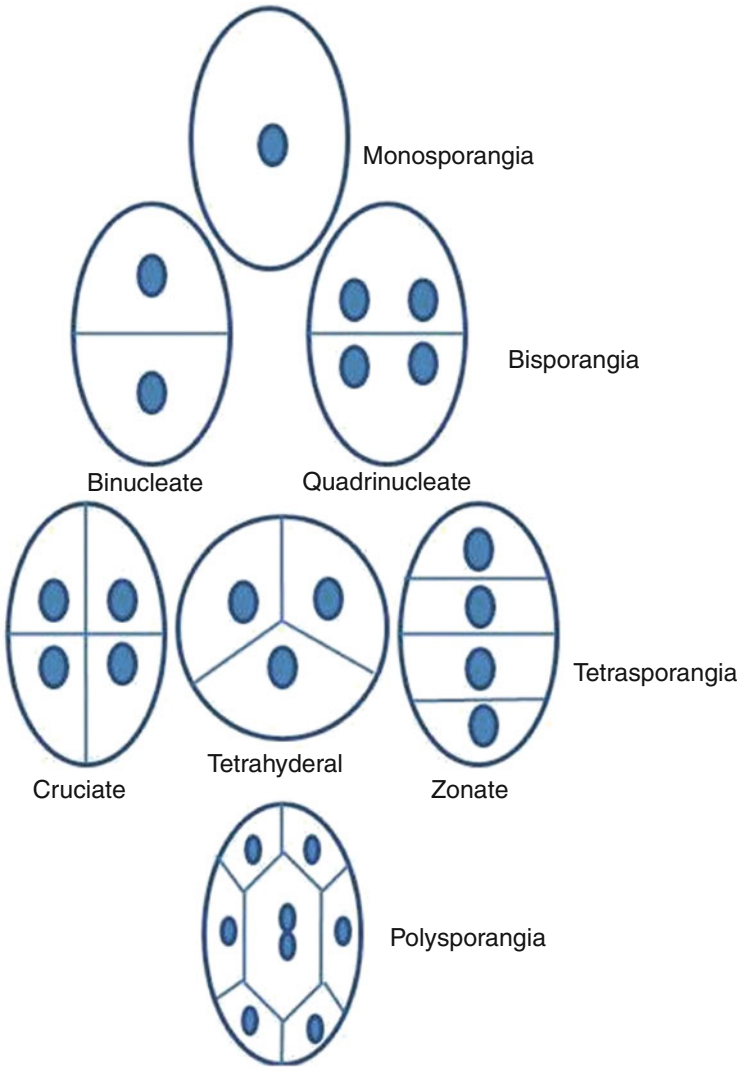

Fig. 12 Cleavage and arrangement of nuclei of some sporangial types in Rhodophyta (Guiry 1978)

From the available knowledge regarding pattern of life cycle in Bangiophycidae, *Bangia* and *Porphyra* show biphasic life cycle (Fig. 13a) (Cole and Conway 1980).

In Floridiophycidae only two basic life history patterns are recognizable. The first one is a cycle in which a haploid gametophyte produces a diploid carposporophyte which in turn give rise to a diploid tetrasporophyte and the second one is a cycle in which a haploid gametophyte alternates with a diploid carposporophyte producing meiotic tetraspore. An important feature of the red algal life history is that while the carposporophyte is always dependent on the gametophyte from which

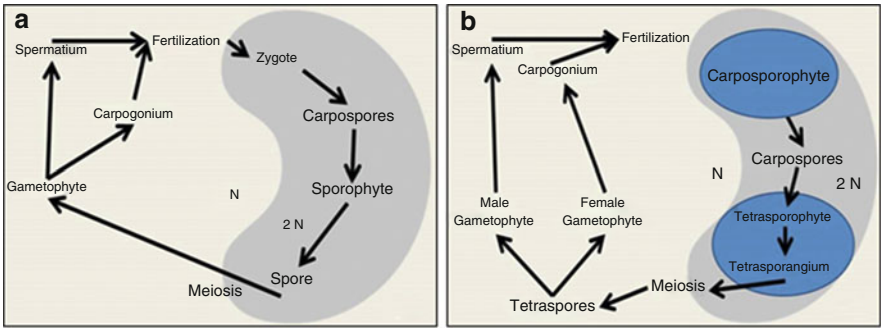

Fig. 13 Diagrammatic representation of (**a**) Diphasic and (**b**). Triphasic life cycle in Rhodophyta

it develops, the tetrasporophyte is with a few notable exceptions, quite independent. The first pattern appears to be the most common among the Floridiophycidae (Fig. 15).

9.1 Polysiphonia *Type*

This type is very common and has three phases (triphasic) (Fig. 15), haploid gametophyte, diploid carposporophyte and diploid tetrasporophyte. The gametophyte and tetrasporophyte are morphological identical and independent and the carposporophyte is parasitic on its gametophyte. Example: *Polysiphonia* sp.

9.2 Asparagopsis *Type*

In *Asparagopsis* type the life cycle has three phases, haploid gametophyte, diploid carposporophyte and diploid tetrasporophyte. The gametophyte and tetrasporophyte are dissimilar in size. The tetrasporophytes are larger than the gametophytes. Example: *Bonnemaisonia hamifera* and *Asparagopsis* sp.

9.3 Liagora *Type*

In Liagora type there is a large haploid gametophyte generation and small diploid carposporophyte generation. Carposporophyte produces tetraspores by meiosis and monospores by mitosis. Example: *Liagora* sp.

9.4 Hildenbrandia *Type*

In this type sexual reproductive organs are not recorded. Here tetrasporophyte generation is repeated in the life history. The plant is diploid and its tetraspores are considered to be produced by apomeiosis. This kind of life history is monophasic where the plant reproduced by the formation of diploid spores (Chihara 1975). Example: *Rhodochorton* sp.

9.5 Palmaria *Type*

The tetraspores from a tetrasporophyte develop into female gametophytes which are extremely small and microscopic even upon maturing or into male gametophytes which are similar in size and habit to macroscopic tetrasporophytes. This type of life history is diphasic: the gametophytes, although their male and female plants are much different in size, alternate with tetrasporophytes, lacking a carposporophyte completely, although having a sexual process. Example: *Palmaria palmata*.

10 Life Cycle of *Porphyra*

10.1 Systematic Position

Class: Rhodophyceae
Order: Bangiales
Family: Bangiaceae
Genus: *Porphyra*

10.2 Habitat

Porphyra grows attached to rocks in intertidal and sublittoral zones in shore from polar to tropical seas. Commonly species appear in winter or summer annuals. Around 133 species of *Porphyra* have been reported from various part of the world, which includes 28 species from Japan, 30 from North Atlantic coasts of Europe and America and 27 species from the Pacific coast of Canada and United States (Yoshida et al. 1977). Although seven species have been reported from the Indian coast, these are not being exploited commercially (Sahoo et al. 2001). *Porphyra* sp. have capacity to face desiccation, so they can survive in the highest, driest reaches of the intertidal zone.

10.3 Structure of the Thallus

Outline of *Porphyra* blades may be vary from circular to linear in nature, and from a few centimetres to over a metre in length. Variable colour ranges from rose-pink in entirely submerged species, to multiple mottled reds, yellows, browns and greens in intertidal species. Plants are membranous, mono or distromatic; blades arising singly from a basal disc-shaped holdfast composed of rhizoids, or several blades arising in clusters from common multicellular spherical or hemispherical cushion like basal portion; blades sessile or stipitate or umbelicate, margins entire or deeply laciniate, provide with microscopic spinulose process in some species edges often conspicuously ruffled; cells embedded in a colourless gelatinous matrix formed by gelatinization of the thick cell walls, each cell with one or two stellate chromatophores with single central pyrenoids.

10.4 Reproduction

Heteromorphic life cycle of *Porphyra* species consist a macroscopic foliose thallus, gametophytic phase and a filamentous sporophyte called conchocelis phase. This diploid conchocelis phase in the life cycle was earlier thought to be *Conchocelis rosea*, a shell boring organism. However, it was Drew in 1949 who demonstrated in culture that *P. umbilicalis* (L.) Kütz had a diploid conchocelis phase (Drew 1956). Until this landmark work concholeis was considered as an independent organism. These findings completely revolutionized the *Porphyra* industry in Japan and subsequently throughout Asia (Sahoo et al. 2002).

Reproduction in *Porphyra* takes place both by sexually and asexually. Sexual reproduction occurs mainly by differentiation of vegetative cells into carpogonia (Fig. 13h) and colourless spermatangia. Spermatia (Fig. 13g) and carpogonia are formed in packets at the blade margins. After fertilization, the carposporogonia (Fig. 13i) divides to form packets of spores called zygotospores (Fig. 13h) (carpospores). Mature "carpospores" are freed from the carposporangium from the margins of the blade. Carpospores germinates to form diploid conchocelis filaments (Fig. 13l), in natural conditions only those carpospores that manage to germinate on, and subsequently penetrate, this substrate normally mollusc shells (Fig. 13m) avoid getting eaten by snails and other small marine grazers. Zygotospores germinate producing filamentous conchocelis phase which can survive in adverse environmental conditions and later give rise to conchosporangia (Fig. 13n) and conchospores under suitable conditions. This conchospores germinate to young thalli, thus completing the life cycle (Fig. 13).

In case of asexual reproduction vegetative cells directly formed the spores called archeospores (Fig. 13e) which can directly germinate to thallus (Nelson et al. 1999). Besides these two modes of reproduction, *Porphyra* also reproduces by endosporangia or endospores which give raise to thallus Nelson and Knight 1995. In some species, like *P. hawkes* monospores produced at thallus margins reproduce asexu-

ally which germinate directly into new plants. In short *Porphyra* produces three types of spores namely archeospores, zygotospores and conchospores. The archeospores are haploid which directly germinate and divide to give rise to thallus. On the other hand, the zygotospores and conchospores are diploid and zygotospores give rise to conchocelis (sporophyte) and the conchospores which undergo meiosis to give rise to haploid individual thalli.

11 Life cycle of *Polysiphonia*

11.1 Systematic Position

Class: Rhodophyceae
Order: Ceramiales
Family: Rhodomelaceae
Genus: *Polysiphonia*

11.2 Habitat

Polysiphonia is a marine alga. It is widely distributed in the coastal area in the littoral zone. Most of the species of *Polysiphonia* prefer still waters and appear brownish-red to purple-red. The species are either annual or perennial and the thallus shows bushy appearance. Most of the 150 species of the alga occurs in the eastern coast of North America and around the Great Britain. In India, it is found along the western coast. A few species are also found in Pacific Ocean. Some species are epiphytic, lithophytic or semi-parasitic. *P. ferulaceae* is epiphytic on *Gelidium* sp. which is another red alga. Many species grow on the rocks. *P. fastigata* is a semi-parasite on *Ascophyllum* sp. *P. ureceolata* is a epiphytic on *Laminania*.

11.3 Structure of the Thallus

The species of *Polysiphonia* are freely branched with polysiphonous construction. The thallus of the alga is basically filamentous. Due to repeated branching the thallus appears as a bushy structure (Fig. 14d). The thallus is attached to a substratum by means of holdfast. The plants attain a height of a few to several centimetres. The thallus is hetrotrichous. It is differentiated into two types of branches-prostrate and vertical.

Prostrate branches are spread out on the substratum to which they are attached with the help of unseptate unicellular rhizoids. The rhizoids are flattened structures for better anchorage and are termed haptera. The rhizoids arise from the peripheral cells of the branch. The erect vertical filaments arise from the upwards tips of the

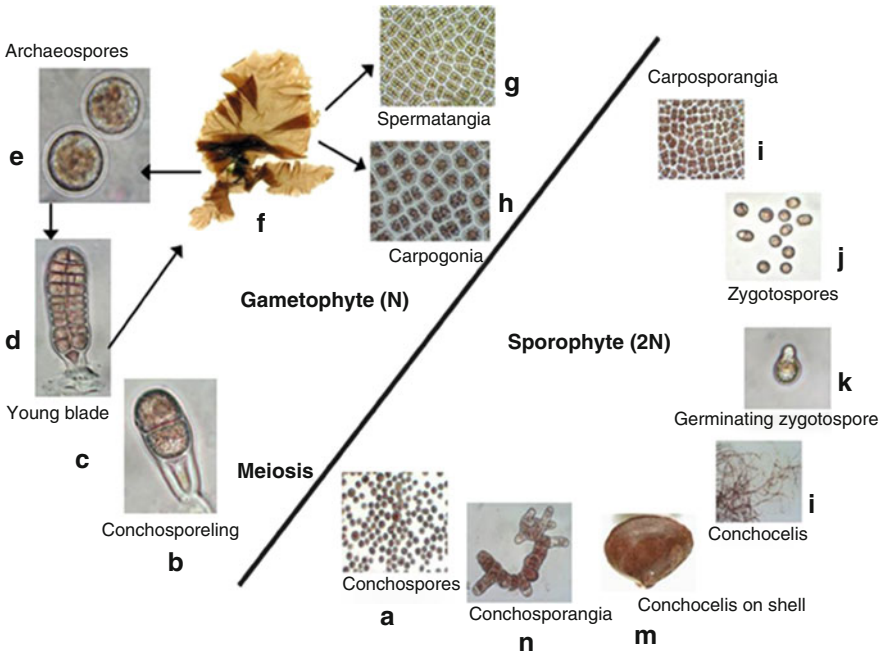

Fig. 14 *Porphyra* life history illustrating different stages of development (Reproduced from Sahoo et al. 2002)

prostrate filaments, or from its any other part. The filaments consist of elongated siphon like cells arranged above one another in vertical rows and hence the name *Polysiphonia*. The dome-shaped apical cell cuts off segments proximally which produces lateral branches before dividing longitudinally into central and pericentral cells. In *Polysiphonia*, the pericentral cells are of the same length as the axial cells, so that the primary cortication of the axial filament is carried out by a smaller number of cells. The number of pericentral cells varies from 4 to 24, and their number and arrangement is of taxonomic importance. The young thallus of the alga in only 6–7 cells in height and are uniaxial. The young plants grow by means of an apical cell. In older parts of the thallus the pericentral cells undergo pericinal divisions to form a multilayered parenchymatous cortex. The cells of the cortex are smaller than the pericentral cells. The central siphons as well as the pericentral siphons are interconnected by means of pit-connections. The primary pit connections between these siphons are developed at the time of cell divisions. The Secondary pit connections develop between pericentral cells and cortical cells.

In several species of *Polysiphonia* two types of branches arise from the main axis on the lateral sides just 2–5 cells behind the apical cell. The branches are short and long. The short branches are limited growth and are termed trichoblasts. They are uniseriate *i.e.*, monosiphonous. They are arranged spirally around the main filament. In the perennial species they fall off annually before the onset of the winter season. The trichoblasts are forked in a dichotomous manner. The long branches

arise sometimes directly from the central siphon but more often from the basal cell of the trichoblasts. The basal cell of a trichoblast cuts off a small lateral protuberance by means of an oblique septum. This outgrowth acts as an apical all for the growth of the long branch, which has a row of central siphons surrounded by pericentral cells.

11.4 Reproduction

Polysiphonia has a well developed triphasic life cycle (Fig. 15). It has the gametophytic generation alternating with not one but two sporophytic generations which are called carposporophyte and tetrasporophyte (Fig. 15a, h). The life cycle is, therefore, termed diplobiontic. Unlike the vast majority of plants which have heteromorphic alternation of generation *Polysiphonia* has isomorphic alternation of generation.

11.5 Gametophyte

Sexual reproduction in *Polysiphonia* is oogamous. The male and the female gametophytes are morphologically similar. The male reproductive structure is known as spermatangia or antheridia (Fig. 15e). They are very small dot like structures arranged compactly in large numbers around central siphons of the fertile trichoblast. The fertile trichoblast is branched in a dichotomous manner. The cells of the trichoblast except the two lowermost cells of the fertile branch cut off a variable number of pericentral cells. The pericentral cells cut off one or more cells which act as the antheridial or spermatangial mother cells. They produce two or four spermatangia so that a cluster of spermatangia get compactly arranged to give a cone like appearance. A spermtangium is a minute unhinucleate one-celled structure. It is almost spherical in shape and is rich in protoplasm. The wall of the spermatangium is three layered. It contains a single non- motile spermatium. After liberation the spermatangium floats in water and fertilizes the egg if it comes in contact with a carpogonium.

The female sex organ *Polysiphonia* is known as carpogonium. The carpogonium is developed terminally on a four-celled carpogonial branch (Fig. 15f) located on the fertile female trichoblast. The carpogonium has a long neck, the trichogyne and the swollen base, below which a supporting cell is situated (Fig. 15f). The female trichoblast, develops from the central siphon of the filament. The lowermost two cells of the female trichoblast gives raise a ring of pericentral cells. One of the pericentral cells on the adaxial side produces a supporting cell which gives rise to the carpogonial filament or procarp. The supporting cell cuts off a small initial cell which divides to produce a four-celled carpogonial filament. The uppermost cell of this filament becomes a carpogonium. The carpogonium is a flask-shaped structure

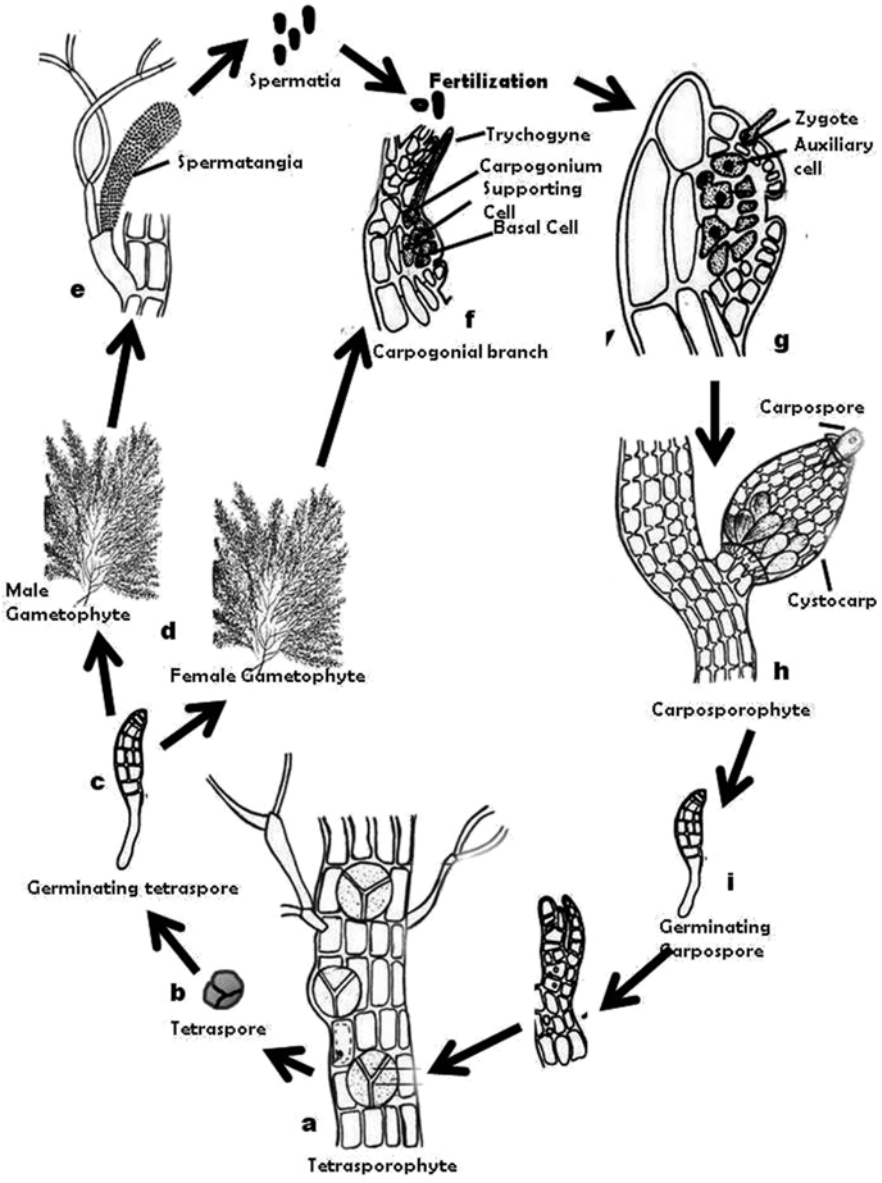

Fig. 15 *Polysiphonia* life history illustrating different stages of development

with a swollen egg containing a basal part and a tubular elongated trichogyne. Meanwhile the supporting cell cuts of basal sterile filament initial and a lateral sterile filament initial. The lateral sterile initial divides to produce a two- celled lateral sterile filament.

11.6 Fertilization

The trichogyne of the carpogonium is the receptive part of the spermatium. The spermatium is carried by water to the tip of the trichogyne to which it attaches. The walls of the spermatium and the trichogyne dissolve at the point of their contact creating a pore like structure for the entry of the male nucleus. The nucleus of the spermatium comes to lie near the egg for sometime after which they fuse together to form a diploid zygote (Fig. 15g).

11.7 Post-fertilization Changes

The basal sterile initial divides to form a two-celled filament whereas lateral sterile initial develops into 4–10 celled filaments. The supporting cell now cuts off on the upper side an auxiliary cell towards the carpogonium. A tubular connection now develops between carpogonial base and the auxiliary cell. The nucleus of the auxiliary cell degenerates. The zygote nucleus of the carpogonium divides into two diploid nuclei out of which one migrates into the auxiliary cell through the tubular connection. The pericentral cells grow in the form of an envelope around the developing sporophytic structures meanwhile the carpogonium degenerates. A small protuberance grows out from the auxiliary cell which is termed gonimoblast initial. The surviving diploid nucleus of the auxiliary cell divides into two diploid nuclei. One of them migrates into the gonimoblast initial. Several such gonimoblast initials arise in this manner. Each initial as a result of mitotic divisions gives rise to gonimoblast filaments. The terminal cell of these filament enlarge to become carposporangia, which contain a single diploid carpospores. Meanwhile the auxiliary cell fuses with the supporting cell as well as with the cells of the sterile filaments to form an irregular structure called the placental cell. The gonimoblast filaments appear to arise from this large placental cell. The pericentral cells of the tricoblast adjacent to the supporting cell divide and grow into a two layered protective sheath called pericarp with an ostiole at the tip (Fig. 15h).

11.8 Carposporophyte

The entire structure consisting of placental cell, gonimoblast filaments, carposporangia and the surrounding pericap is termed cystocarp. The diploid portion of the cystocarp represents the carposporophyte (Fig. 15h). The naked diploid carpospore after release from the cystocarp germinates on a suitable substratum by first secreting a wall around itself. It undergoes an unequal mitotic division to form a smaller lower cell and a larger upper cell (Fig. 15i). They divide again to form a four-celled filament. The lower most cell colourless and is termed rhizoidal cell. The filament grows into central siphons by means of the uppermost dome shaped apical cell.

The new filament of *Polysiphonia* is diploid and is known as tetrasporophyte (Fig. 15a). Morphologically it resembles the gametophytic *Polysiphonia* plant and therefore, the plants shows isomorphic alternation of generation.

11.9 Tetrasporophyte

Tetrasporophyte has the same organisation of siphons as that of the gametophyte. It produces several tetrasporangia which are named so because of the presence of spores in tetrads (Fig. 15b). The tetrasporangia develop from the pericentral cells. The pericentral cells meant for becoming transporangia are smaller in size than the other cells of the tier. Only one of the pericentral cells of a tier develops into a tetra sporangium. This cells divide vertically into two halves- an outer half and an inner half. The inner undergoes a transverse division to form a lower stalk cell an upper tetrasporangium. The outer half divides to form two cover cells. Every tier of the tetrasporophyte has a single transporangium. The diploid nuclei of the transporangia undergo meiosis to produce four haploid spores which arranged in a tetrahedral manner. Finally tetraspore will lead to gametophytic phase thus completing the triphasic life cycle.

12 Red Algae Demand and Uses

The demand for seaweeds and their products are enormous and are increasing with annual growth of 7.4 %. As per FAO, the total value of farmed aquatic algae in 2010 is estimated at US$5.7 billion. The world production of common seaweed has increased several folds. The production increased from 3.8 million tonnes in 1990 to 19 million tonnes in 2010 (FAO 2012). Presently 221 species of seaweeds are utilized commercially which include 125 for food, 110 for phycocolloid production (Sahoo 2000). Seaweeds which abundantly grow in the seawater have emerged as a major group of plants for CO_2 Sequestration in the ocean ecosystem (Elangbam et al. 2013; Sahoo et al. 2012). Seaweed contain interesting group of

polysaccharides technically called as phycocolloids which include agar, carrageenan and alginates. Since these phycocolloids have special properties as stabilizers and stiffeners, they are widely used in food, cosmetics, pharmaceuticals, paper production and biotechnology industries (Sahoo and Yarish 2005). The production of carrageenan sale volume increased from 42,000 tonnes in 1999 to 50,000 tonnes in the year 2009. At the same time the market sale value of carrageenan have increased from US$291 million in 1999 to US$527 million in 2009 (Bixler and Porse 2011). Although there are seven types of carrageenan, only three types, *kappa* (κ), *iota* (ι) and *lambda* (λ) are commercially important, out of which *kappa*-carrageenan contributes to 80 % of world carrageenan production. *Kappa-carrageenan* is produced from *Eucheuma cottonii* (*Kappaphycus alvarezii*), *iota*-carrageenan from *Eucheuma spinosum* (*E. denticulatum*) and *lambda*-carrageenan is obtained from different species of *Gigartina* and *Chondrus* genera. Among these only *K. alvarezii* and *E. denticulatum* are cultivated in large scale in tropical waters while *Chondrus crispus*, *Irridea*, and *Hypnea* are being harvested from the wild source.

Presently 160,000 tonnes of *K. alvarezii* (*kappa*-carrageenan) and 23,000 tonnes of *E. denticulatum* (*iota*-carrageenan) are produced from the culture in 2009. Since the demand of carrageenan has been increasing steadily over the years, the production of *K. alvarezii* and *E. denticulatum* have also increased to 5.7 million tonnes in FAO (2012). The red agarophytes of the genus *Gracilaria*, called "Ogonori" in Japan (296,000 tons), and the red *Porphyra* spp. valuable as food alga "Nori" (1.6 million tons) are particularly important. Other species like *Palmaria*, *Chondrus*, or the green *Ulva*, etc. are produced to a minor extent (FAO 2011).

13 General Uses

- Primary Producers
- Algae are the main Oxygen producers in aquatic areas. They are also useful in decreasing water pollution by realizing Oxygen. Marine primary producers contribute at least 50 % of the world's carbon fixation and may account for as much as 71 % of all carbon storage (Chung et al. 2011).
- Algae as food
- Algae species are used as food in several countries and in several forms. Algae species have proteins, vitamins (A, B, C and E), lipids, and minerals. *Laminaria* species is the important edible seaweed in Japan and the food item 'Kombu' is prepared from it. 'Aonori' from *Monostroma*; 'Asakusa Nori' from *Porphyra* are prepared in different countries. *Porphyra* has 35 % protein, 45 % carbohydrates, Vitamins B and C and Niacin. *Nostoc* is used as food material in South America.
- Many red algae are used as the basic raw material for extraction of gel-like non-nitrogenous extract. This material is mainly used in preparation of microbial culture media. Some of the important Agar sources are – *Gelidium*, *Gracilaria*, *Pterocladia*, *Gigartina*, *Ceramium*, *Chondrus*, etc.

- Agar is made from seaweed and is used in a wide range of applications: in food products (such as frozen foods, bakery icings, meringues, dessert gels, candies and fruit juices), industry uses (like paper sizing/coating, adhesives, textile printing/dyeing, castings, impressions), in biological culture media, in molecular biology (more specifically agarose, used for separation methods) and in the medical/pharmaceutical field (to produce bulking agents, laxatives, suppositories, capsules, tablets and anticoagulants) (Sahoo 2000).
- Carrageenans have some distinct properties, thus used in gelation, emulsification and protein stabilization etc. Carrageenans occupies vital position in food industry due to their excellent physical functional properties such as thickening, gelling, stabilizing and have been used to improve the texture of cottage cheese, puddings, dairy desserts, to control the viscosity and as binders in the meat-processing industry (Sahoo 2000).
- Carrageenans have wide application in multiple non-food products, such as pharmaceutical, cosmetics, printing and textile formulations. Carrageenan stabilizes toothpaste preparation, absorb body fluids when formulated in wound dressings and interact with human keratin to give soft skin. They have proved to be useful as tableting excipients due to the good compatibility, high robustness and persistent viscoelasticity of the tablet during compression (Campo et al. 2009).

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Diatoms: Yellow or Golden Brown Algae

Savindra Kumar, Pooja Baweja, and Dinabandhu Sahoo

1 Introduction

Diatoms, the members of class Bacillariophyceae are grouped under planktonic algae, as mostly they are free floating microorganism of marine or freshwater environment. Many diatoms live epiphytically on the surface of aquatic organisms including plants, molluscs, turtles, fishes, seaweeds etc. When under water, we are always surrounded with diatoms, but because they are at or below the limit of resolution of naked eye, we can literally swim through them and never know that they are present in surroundings (Nagy 2011). Diatoms are supposed to be originated in late Permian era (about 200 years ago) possibly from Chrysophyceae or Bolidophyceae (Guillou et al. 1999; Lee 2008). Diatoms are considered as the primary producers and thus contribute around 20–25 % of total global primary productivity (Hoek et al. 2009). Diatoms share some features that are typical of the heterokontophyta such as typical tripartite mastigonemes on flagellum, face of Golgi body, continuous chloroplast endoplasmic reticulum with nucleus, presence of periplastidial reticulum, presence of girdle lamellae in chloroplast, presence of chlorophyll *a* and c_2. Diatoms have some unique characteristic features which separate them from other Heterokonts such as:

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D. Sahoo, J. Seckbach (eds.), *The Algae World*, Cellular Origin, Life
in Extreme Habitats and Astrobiology 26, DOI 10.1007/978-94-017-7321-8_8

- Marine and freshwater distribution
- Unicellular or colonial protists consisting of two overlapping halves
- Unique silica containing cell wall
- Male gametes are only flagellated stage in centric diatoms
- Lack of transitional helix in flagellum
- Chloroplast usually golden brown (due to fucoxanthin)
- Ring shaped chloroplast DNA
- Open mitosis
- Diplontic life cycle in sexually reproducible species

2 Classification

At present there are over 500 genera of diatoms (350 living and 150 extinct), with around 17,000 species (12,000 living and 5000 extinct) (Hoek et al. 2009; Williams and Kociolek 2011). Diatoms were sought to be unique group of algae by earlier scientist and they were placed as a distinct group by Harvey (1836), Eichler (1883), Engler and Prantle (1912). It was Antonie won Leewenhoek (1703) who recorded the appearance of diatoms, and considered them as animals. He named diatoms as "Animalcule" (The little animal) (Williams and Kociolek 2011). Linnaeus also considered them as animal and placed them under "Vermes", in Zoophyta in the 12th edition of *Systema naturae* (1767). Carl Adolph Agardh gave a comprehensive diatom classification in *Systema Algarum* (1824) and later after few years in *Conspectus criticus diatoma cerum* (1830–1832) in *Sytema Algarum* Agardh classified diatoms in one order with 50 species.

Friedrich Traugott Kützing (1843) classified diatoms into two groups: Diatomaceae Liberae and Diatomaceae Inclusae with 16 and 10 genera respectively. Pfitzer (1871) classified diatoms into two divisions Coccochromaticae and Placochromaticae with two subdivisions each. The classification of Pfitzer was based on the structure of valve rather than contents of frustule (Williams and Kociolek 2011).

Petit (1877a, b), followed the classification of diatoms as proposed by Pfitzer and named his two subdivisions as families. H. L. Smith (1872) classified the diatoms based on the presence of raphe on the valve into three tribes with 15 families and 110 genera. This classification was adopted by Henri Van Heurck (1878). Franz Schütt (1896) classified diatoms into two groups Centricae and Pennatae and his classification has been proposed in Engler and Prantl's *Die naturlichen Pflanzenfamilien*. With many such classification diatoms are sought to be a unique group of algae by earlier scientist and has always got recognitions as distinct group. Pascher (1931) kept diatoms under division Chrysophyta along with Crysophyceae. Fritsch (1935), proposed the most acceptable and comprehensive diatoms classification in which he kept all the diatoms under class Bacillariophyceae with two taxonomic groups Centrales and Pennales. G. M. Smith (1955) and Prescott (1969) kept Class Bacillariophyceae along with Chrysophyceae and Xanthophyceae in the division Chrysophyta as there are many important similarities between the two

groups such as (1) olive green to brown colour chloroplast (2) presence of chlorophyll *a* and *c* with fucoxanthin and acetylenic carotenoids (3) oil and chrysolaminarin storage food (4) pleuronematic flagellum of gametes and (5) silicification of cell wall (Chapman and Chapman 1981). Bold and Wynne (1985) include three more classes namely Prymnesiophyceae, Eustigmatophyceae, Raphidophyceae along with Chrysophyceae, Xanthophyceae and Bacillariophyceae in division Chrysophyta. In comparison to other major algal groups classification of diatoms is relatively easy to investigate, because the silica shells bear characteristic patterns of pores and ornamentation. Fritsch (1935), Smith (1955), Prescott (1969), Morris (1971), Bold and Wynne (1985), and Hoek et al. (2009) divided class Bacillariophyceae into two orders namely Centrales and Pennales. A simple way of distinguishing between these two orders is that former one has pill boxes like shape and later one has shape of date boxes (Chapman 1962). Bold and Wynne (1985) further divided these orders in to following subgroups:

- **Order-Centrales**
- Valve arrangement is usually to a central point on the valve (centric or radial) or to 2, 3 or more points (gonioid) which resulted into biangular, triangular or polygonal valve is evident
  - Suborder: Coscinodiscineae
    Valve disciform, flat or convex, without prominent processes or intercalary bands

    Melosiraceae e.g. *Melosira*, *Stephanopyxis*
    Coscinodiscaceae e.g. *Coscinodiscus*
    Asterolampraceae e.g. *Asteromphalus*
    Heliopeltaceae e.g. *Actinoptychus*
    Thalassiosiraceae e.g. *Cyclotella*, *Planktoniella*, *Skeletonema*, *Stephanodiscus*, *Thalassiosira*
  - Suborder: Biddulphiineae
    Valve bipolar or multipolar, angles of valves provided with spines, elevations, ocelli, or other projections

    Eupodiscaceae e.g. *Eucampia*, *Odontella*, *Triceratium*, *Zygoceros*
    Lithodesmiaceae e.g. *Ditylum*, *Lithodesmium*, *Streptotheca*
    Stictodiscaceae e.g. *Stictodiscus*
    Biddulphiaceae e.g. *Biddulphia*
  - Suborder:Rhizosoleniineae

    Frustule long, cylindrical; many intercalary bands present

    Rhizosoleniaceae e.g. *Guinardia*, *Rhizosolenia*
- **Order-Pennales**
  Structure of the valve is arranged in reference to a central line (pennate) or in reference to a point not on the valve surface (trellisoid); raphe or a hyaline field in the axial are present

– Suborder: Araphidineae
  Hyaline field present in the axial area of the valve

  Fragilariaceae e.g. *Asterionella*, *Climacosphenia*, *Fragilaria*, *Grammatophora*, *Rhabdonema*, *Licmophora*, *Striatella*, *Subsilicea*, *Synedra*, *Tabellaria*

– Suborder: Raphidiodineae
  Rudimentary raphe present at ends of valve

  Eunotiaceae e.g. *Eunotia*

– Suborder: Biraphidineae
  Fully developed raphe present on both valves

  Naviculaceae e.g. *Amphipleura*, *Berkeleya*, *Caloneis*, *Diploneis*, *Frustulia*, *Gyrosigma*, *Navicula*, *Pinnularia*, *Pleurosigma*, *Stauroneis*
  Cymbellaceae e.g. *Cymbella*, *Gomphoneis*, *Gomphonema*, *Phaeodactylum*
  Entomoneidaceae e.g. *Entomoneis*
  Bacillariiaceae e.g. *Bacillaria*, *Cylindrotheca*, *Hantzschia*, *Nitzschia*
  Surirellaceae e.g. *Campylodiscus*, *Cymatopleura*, *Surirella*

– Suborder: Monoraphidineae
  Raphe present on one valve; hyaline field present on the araphid valve

  Achanthaceae e.g. *Achnanthes*, *Cocconeis*

Lee (2008) proposed a different classification system of diatoms based on following characteristics and divided class Bacillariophyceae in to two orders.

S. no.	Characteristics	Order-Biddulphiales or centrals	Order-Bacillariales or pennales
1	Ornamentation	Radial or gonoid	Pennate or trellisoid
2	Chloroplasts	Many	One or two
3	Raphe	No raphe	Possibly present with gliding
4	Spermatozoids	Motile with a single tinsel flagellum	No flagellated spermatozoids
5	Sexual reproduction	Oogamous type	By conjugation

Although a recent account of the diatoms recognizes many more classes and orders: there the diatoms (as division Bacillariophyceae) are subdivided into three classes, Fragilariophyceae (pennate diatoms without a raphe system), Bacillariophyceae (pennate diatoms with a raphe system) and Coscinodiscophyceae (the centric diatoms) (Hoek et al. 2009). Recently Williams (2007) and Williams and Kociolek (2011) gave detailed history of diatom's classification in which they have discussed almost all the proposed classifications for diatoms till now.

3 Occurrence

Diatoms are extremely abundant, cosmopolitan which comprises the main component of aquatic systems, especially marine waters. They are widespread and can be present in terrestrial systems as well as in aquatic habitats as free living photosynthetic autotrophs, colorless heterotrophs, or photosynthetic symbionts. Diatoms are enormously successful organisms as judged by their adaptability, distribution, biomass and relative antiquity to survive in extreme conditions such as hot springs in Kuril and Sakhalin Island or extreme low pH lakes in Western Pomerania (NW Poland) (Nikulina and Kociolek 2011; Witkowski et al. 2011). Diatoms are largely responsible for the very high primary productivity in temperate or cold parts of the oceans where pennales mostly represent benthic forms and centrales are planktonics, which forms the basic food of aquatic animals. Many diatoms grow as epiphytes on the thalli of other algae or stems and leaves and other parts of the higher plants. Diatoms are also found as facultative heterotrophs (e.g. *Nitzschia*), or as soil diatoms, as endosymbionts diatoms (Hoek et al. 2009). Diatoms are greatly influenced by water salinity and therefore marine and freshwater forms of diatoms are strikingly different (Potapova 2011).

3.1 Freshwater and Marine Diatoms

- Common marine centric diatoms: *Thalassiosira*, *Chaetoceros*, *Skeletonema*, *Triceratium*, *Asteromphalus*, *Rhizosolenia*, *Biddulphia*,
- Common freshwater centric diatoms: *Cyclotella*, *Stephanodiscus*, *Melosira*
- Common marine pennate diatoms: *Licmophora*
- Common freshwater or marine pennate diatoms: *Navicula*, *Nitzschia*, *Pseudonitzschia*, *Synedra*, *Cocconeis*, *Gyrosigma*, *Rhopalodia*
- Common primarily freshwater pennate diatoms: *Cymbella*, *Epithemia*, *Pinnularia*, *Gomphonema*, *Stauroneis*, *Cymatopleura*, *Fragilaria*, *Tabellaria*, *Asterionella*

3.2 Epizoic and Epiphytic Diatoms

Diatoms are found attached to a number of diverse animal groups such as ciliates, copepods, cladocera, barnacles, hydroids, krill, bryozoans, whales and porpoises, diving birds etc. Some of the common epizoic diatoms are *Cocconeis*, *Bennettella*, *Epipellis*, *Melosira*, *Falcula* etc. All types of aquatic vegetation (algae or higher plants, attached or free floating) can provide habitat for epiphytic diatoms. Some of the common epiphytic diatoms are *Hyalodiscus*, *Licmophora*, *Cocconeis*, *Campyloneis*, *Leminicola*, *Achnanthes*, *Gomphonema*, *Fragilaria* etc. (Tiffany 2011).

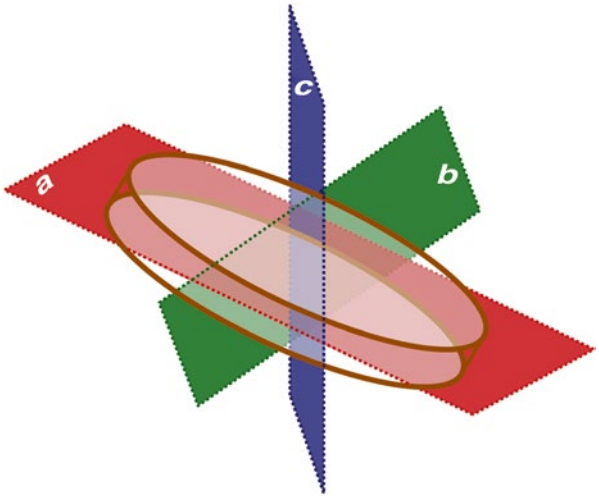

Fig. 1 Axes and planes of symmetry in a pennate diatoms. (**a**) Apical axis; (**b**) Transapical axis; (**c**) Pervalvar axis (Adopted from Bold and Wynne 1985)

4 Morphology and Ultrastructure

Diatoms are unicellular, colonial or filamentous in organization. Two major series may be distinguished on the basis of their symmetry and other features: the pennate and centric diatoms (Alexopoulos and Bold 1971). With a few exceptions, such as *Phaeodactylum tricornutum* and endosymbiotic species, all diatoms are recognized by the possession of distinctive, essentially bipartite, variously perforated and ornamented, silica cell walls (frustules) that enclose the eukaryotic protoplast (Cox 2011). Diatoms are essentially unicellular, although chains of cells and colonial aggregation may occur. The vegetative cell of mostly diatoms can be regarded as having either bilateral or radial symmetry. Any diatoms cell with bilateral symmetry (pennate diatoms) can be positioned along three axes (Fig. 1): the apical axis, the pervalvar axis and the transapical axis (Bold and Wynne 1985). Diatom cells always divide along with the apical axis or valvar plane (Hoek et al. 2009).

4.1 Frustule and Raphe System

The key distinguishing feature of the diatoms is their ability to secrete an external wall, (frustule), which is composed of quartzite (95 %) or hydrated amorphous silica along with small amounts of aluminum, magnesium, iron, and titanium (Prescott 1969; Lee 2008). Frustule of diatoms composed of overlapping halves like that of a close fitting container with one larger valve (**epivalve**) and the other slightly smaller

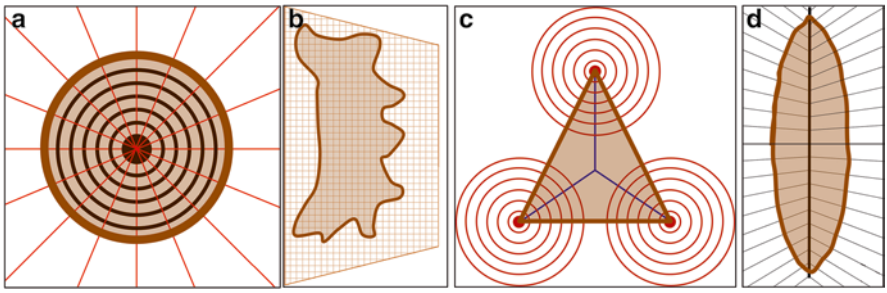

Fig. 2 The basic patterns of ornamentation in the Bacillariophyceae. (**a**) Centric and radial e.g. *Coscinodiscus* (**b**) Trellisoid, e.g. *Eunotia* (**c**) Gonoid, e.g. *Triceratium* (**d**) Pennate e.g. *Navicula* (Adopted from Lee 2008)

valve (**hypovalve**). **Girdle** is located between these two valves, which can be subdivided into two overlapping portion: the **epicingulum** and the **hypocingulum**. The epivalve and epicingulum makes **epitheca** and hypovalve and hypocingulum makes **hypotheca** (Bold and Wynne 1985). Under microscope, diatom's shell can be oriented either in valve view or girdle view (Fig. 2). Simple girdle consist of two cingula only whereas additional pieces of intercalary bands also found in compound girdle (Bold and Wynne 1985). There may be one, two, or more additional intercalary bands besides the girdle that are inserted between the two wall sections (Prescott 1969). The additions of these intercalary bands also help in growth of diatoms along the pervalvar axis. A certain regular patterns in the siliceous material of the frustule leave the wall or frustule ornamented into four types: Centric and radial, Trellisoid, Gonoid and Pennate as shown in Fig. 2 (Lee 2008):

Raphe system (a longitudinal slit in the theca) found in many pennate diatoms is an opening or fissure running along the apical axis (Bold and Wynne 1985). It is supposed that raphe is involved in diatom's locomotion because raphe less diatoms always move sluggishly. Raphe-bearing diatoms are also known as **raphid diatoms**. Each raphe also divides the cell in two equal parts by the central nodule which terminates at polar nodule (Lee 2008). In cross section the raphe is sometime shaped like a "V" because slitting of the raphe as in *Pinnularia* (Hoek et al. 2009). Electron microscopical studies suggest that raphe system is also associated with several other cytoplasmic structures. A pseudoraphe, an unornamented area running down the center of the valve is present in those pennate diatoms, where raphe is absent (Fig. 3). Apart from the raphe, there are two other types of wall perforations found in diatoms (1) the simple pore or hole, and (2) the more complex loculus or areola (Lee 2008).

Areolae are complex structures and have silicified layer, a sieve membrane which is also known as pore membrane or velum or cribrum or rica also found at one end (inside/outside) of the loculus (Lee 2008; Graham and Wilcox 2000). This membrane resembles honeycomb. Some processes or extensions found on colony forming diatom's valve to maintain contact between themselves. These extensions have different names such as **cornutate**, **strutted**, **spinulae**, **awns** or **setae** (Lee 2008).

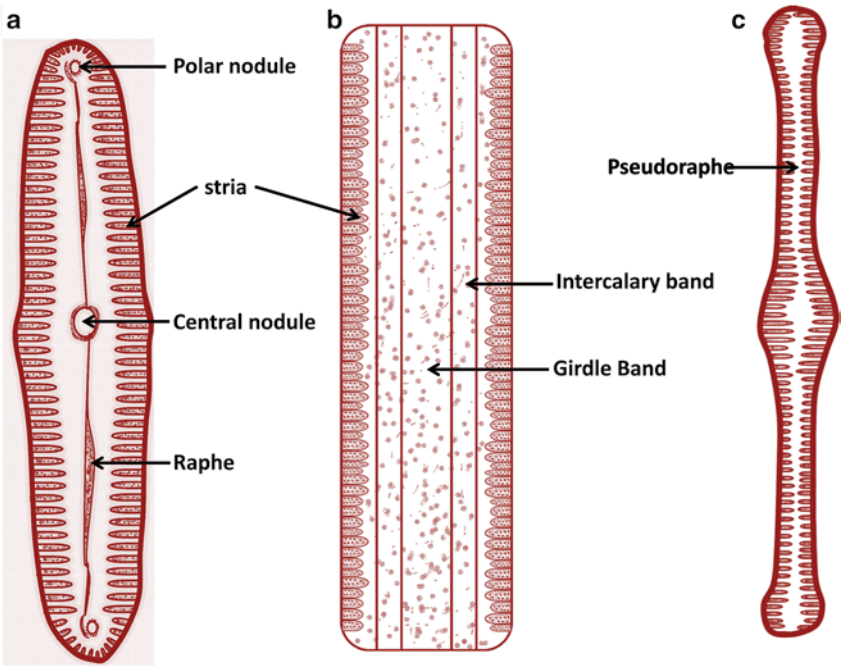

Fig. 3 (**a**) Valve view of *Pinnularia* with a raphe system, (**b**) Girdle view of *Pinnularia* (**c**) *Tabellaria* with a pseudoraphe system (Adopted from Lee 2008)

4.2 Extracellular Mucilage

In some diatoms, extracellular mucilage or slime pores (Mucilage secreting pores) are also reported. The extracellular mucilage aggregates are of five types namely tubes, pad, stalks, fibrils and adhering films (Fig. 4). According to some phycologists mucilaginous secretions help in diatom's movement and attachment.

4.3 Protoplast

Protoplast is present within the cell wall and in a photosynthetic diatom, it comprises of a nucleus, mitochondria, endoplasmic reticulum, Golgi bodies, chloroplast with or without pyrenoids. One or two large central vacuoles are often present. The position of nucleus varies in both centrales and pennales diatoms.

The nucleus may often be present in the central portion of the vacuole interrupted by a broad band of cytoplasm in some pennales or araphid diatoms (Smith 1955)

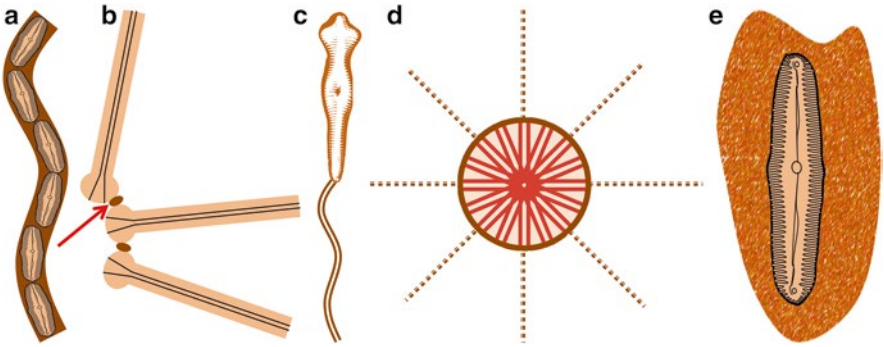

Fig. 4 Extracellular mucilage in diatoms. (**a**) Tube of *Navicula*, (**b**) Pads of *Asterionella*, (**c**) Stalk of *Gomphonema*, (**d**) Fibrils of a centric diatoms and (**e**) Adhering film of a pennate diatoms (Modified from Hoagland et al. 1993 and Lee 2008)

and raphid diatoms or it may be positioned against the girdle in centrales (Pickett-Heaps et al. 1988). In most of the raphid diatoms nucleus is always positioned at one side of the cell or oscillates across the cell with subsequent mitosis (Mann 1982a, b).

Numerous Golgi bodies surrounded by vesicles are present in protoplast. Golgi bodies in some centric diatoms are closely associated with endoplasmic reticulum and mitochondria and forms the Golgi-Endoplasmic Reticulum-Mitochondria complex (Medlin et al. 2000). Mitochondria are dispersed throughout the protoplast.

4.4 Plastids and Storage Food

Diatoms chloroplast has the same structure as in other typical members of the Heterokontophyta. Chloroplast remains surrounded by two membrane and outer membrane of chloroplast endoplasmic reticulum being continuous with the outer membrane of the nuclear membrane. Pyrenoids remain centrally placed in chloroplast. Chloroplasts are either brownish or greenish in colour and contains chlorophyll *a*, c_1 and c_2 along with fucoxanthin (carotenoid) which give golden brown colour to diatoms. Chlorophyll *b* is never found in diatoms. Some diatoms (**apochlorotic diatoms**) live on decaying marine vegetation and the mucilages of seaweeds (Lee 2008). These diatoms have no visual plastids and thus become facultative heterotrophs (Hoek et al. 2009).

Chrysolaminarin (β-1,3-linked glucan) is the main storage product of diatoms, which is located in vesicles in the diatoms (Hoek et al. 2009). Diatoms also contain unique 4α-methyl sterols, such as 4-desmethylsterol and cholesterol (Granum and Myklestad 2001; Lee 2008).

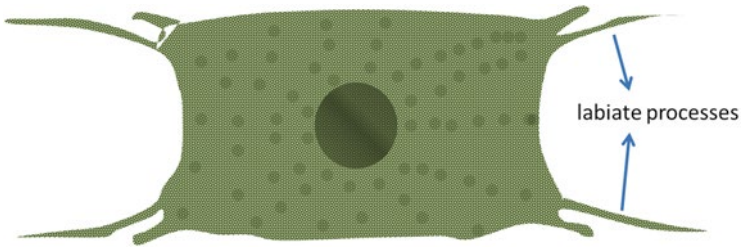

Fig. 5 Diagram of side view of *Odontella* showing the location of the labiate processes (After Pickett-Heaps et al. 1986 and Lee 2008)

5 Motility

Diatoms do not move in a continuous straight line but in a series of curves and zigzags. One of the most spectacular patterns of motility is seen in *Bacillaria paxillifera*, a colony of pennate cells that continuously line up and then synchronously slide apart with precise rhythmic patterns (Bold and Wynne 1985). A number of hypothetical mechanisms have been proposed to explain diatom motility (Graham and Wilcox 2000). Pennate diatoms with a raphe system and centric diatoms with labiate processes can glide over the surface of a substrate which leaves a mucilaginous trail in their wake (Fig. 5). Labiate processes projects inward, terminating a flattened tube with a longitudinal slit surrounded by two liplike edges (Bold and Wynne 1985). These processes may be one to many. Raphe in pennate diatoms determine path of gliding which can be straight (e.g. *Navicula*), curved (e.g. *Amphora*) or curved with two different radii (e.g. *Nitzschia*). Some diatoms also exhibit alternate movement (backward and forward) at an interval of a minute. The contact of raphe with surface also helps in diatom's gliding. If girdle contacts the substrate, a mucilaginous tether from the raphe near the central nodule attaches to the substratum and the cell pulls itself onto a valve containing a raphe using the tether (Lee 2008). Bundles of actin microfilaments parallel to raphe are found in gliding pennate diatoms. Labiate processes of some centric diatoms have a pore (slit like opening in rimoportula tube which is an elongate tube through the cell wall) in the center and secrete mucilage which helps in movement of centric diatoms (Hoek et al. 2009). According to Pickett-Heaps et al. (1988) raphe of pennate diatoms evolved from labiate processes of some centric diatoms.

6 Formation of the Cell Wall

Diatoms cell wall is an amorphous and non-crystal structure, consisting of polymerized silicic acid, along with protein, polysaccharides and lipids (Hoek et al. 2009). During cell division of diatoms which is a normal method of reproduction, the valve of the parent cell becomes the epitheca of the two daughter cells. Each daughter cell

then produces a new hypotheca (Lee 2008). Therefore, one daughter cell is of the same size as the parent cell, and the other is smaller. To overcome this uptake of silica is confined prior to the separation of the two daughter cells and there is a great requirement of silicon, if cell division is to take place. Silicon metabolism in diatoms has been the focus of research interest (Bold and Wynne 1985). Solid silica dissociates to produce silicic acid (97 % of dissolved silicon of oceans and 23 % of freshwater) under water:

$$SiO_2(\text{Solid}) + 2H_2O = {}^{\text{Si}(OH_4)}_{\text{Silicic acid}}$$

Most of the $Si(OH)_4$ is present as ionized silicic acid

$$Si(OH)_4 + H_2O = SiO(OH)_3^- + H^-$$

At pH less than 9 this silicic acid gets auto-polymerized to form amorphous silica, main form of silicon in diatom cell walls. With the help of a transport protein, silica is taken up into the diatom cell by active transport system. Epitheca is pushed away from hypotheca before cell or nuclear division. The silicalemma or membrane of the silica deposition vesicles formed by the fusion of translucent vesicles produced by golgi body beneath invagination of the plasmalemma during cell division (Li et al. 1989). Packaging of silicon is accomplished by golgi into vesicles with the help of microfilaments in the cytoplasm (Lee 2008). These vesicles determine the final form of the silicified frustule. In the form of 30–50 nm diameter spheres, silica remain deposited as amorphous form in diatoms (Crawford et al. 2001). Silaffin peptide determine the size of silica sphere and controls the frustule ornamentation. Frustulins (a glycoprotein) is also associated with the silica deposition vesicle. After complete deposition of silica inner membrane of the silicalemma becomes the plasmalemma of the daughter cell (Lee 2008). According to Bidle and Azam (1999) this silica usually dissolves after death of diatom cell.

7 Reproduction

The diatoms have a complex life cycle, as in other unicellular microalgae, diatoms also has two main distinct but interconnected phases-vegetative phase and a sexual phase. Vegetative phase includes mitosis and increase in cell number, whereas sexual phase includes meiosis and genetic recombination. Diatoms have a peculiar property in their life cycle, i.e. the diatoms cell progressively reduce in size after each mitotic division (Round et al. 1990). In such diatoms, the sexual phase is the only way to avoid death and reduction in number. Although, some diatom species evolve by enlargement in size to escape extreme miniaturization (Chepurnov et al. 2004).

7.1 *Vegetative Phase*

7.1.1 Cell Division

This is the commonest method of reproduction and usually cell division occurs at night. When a cell has to undergo division, protoplast expands, losing the connections between the overlapping thecae. The nuclear division takes place mitotically along with the division of cytoplasm in a longitudinal axis. Before the division of cytoplasm the cell organelles also duplicates. Sometimes spindles are formed by the centrosomes during nuclear division. After division each half is present either in epitheca or hypotheca, with opposite sides naked. A new hypotheca forms in each half of divided cell, thus reducing the size of one cell (Figs. 6 and 7).

7.1.2 Resting Spore Formation

Under stress conditions the cell wall of some diatoms such as *Ditylum*, become thick, thus forming **dormant** or **resting spores** which can survive unfavourable period and divide on the onset of favourable period. During resting spore formation

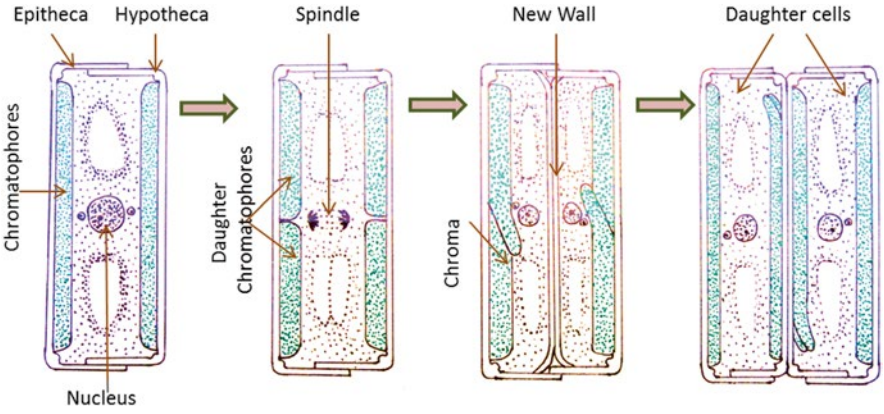

Fig. 6 Cell division in a pennate diatom

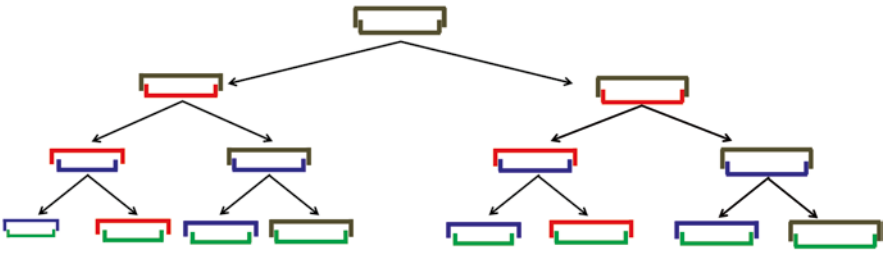

Fig. 7 Process of cell division in diatoms showing continuous reduction in cell size

the protoplasm of the parental frustule darkens, contracts and develops a very heavy siliceous wall of its own. The cytoplasmic volume of these resting cells decreases due to the loss of vacuoles and their contents along with reduction in numbers of mitochondria and formation of large lipid bodies. Resting spores show sharp reduction in photosynthesis and respiration process. After formation the resting spore sinks to bottom (Bold and Wynne 1985). During germination, the resting spore produces a number of fine protoplasmic strands, which radiate in all directions (Lee 2008). Compared to auxospores formation (sometime once in more than 2 years) resting spores formation is more frequent in diatoms. Light, temperature, and salinity are comparatively stable parameter in sea thus nutrient depletion is main stress shock for resting spores formation which normally occurs after diatoms bloom (Lee 2008). In freshwater there is frequent environmental changes than marine waters which makes it more suitable for resting spore formation. Compared to pennate diatoms resting spore formation is far more common in centric diatoms.

7.2 Sexual Reproduction or Auxospores Formation

Compared to resting spores formation of auxospores is quite rare which is formed by the fusion of gametes. According to Edlund and Stoermer (1997) sexual reproduction or auxospore formation occur only in two conditions: (1) if cell reach one third of their maximum size and (2) during suitable environmental condition such as temperature, light, nutrients, trace metals, organic growth factors, and osmolarity (Lee 2008). Contrary to other algal groups diatoms undergo sexual reproduction for restoration of cell size. In centric and gonoid diatoms only male gametes bear flagella (motile) and female gamete act as egg cell (non-motile). On the other hand in pennate and trellisoid diatoms gametes are flagella-less. The fusion of gametes results into the zygote which is also known as "Auxospore". It is larger than the vegetative cells and is surrounded by a two piece silicified wall called "perizonium", which can be smooth or ornamented. The perizonium is secreted by the zygotic protoplast and it replace the thin zygotic membrane, when a zygote grows. When perizonium covers the auxospores which is a growth spore, it photosynthesis and grow rapidly to attain the size greater than the vegetative cell from which it has been formed. After this frustule is secreted, a new diatom is formed. Auxospores develop into three types' namely isodiametric auxospores (e.g. *Melosira*, *Coscinodiscus*, *Stephanopyxis*), properizonial[1] auxospores (e.g. *Chaetoceros*) and perizonial auxospores (e.g. *Navicula*, *Pseudo-nitzschia*). Isodiametric and properizonial auxospores are common in centric diatoms whereas perizonial auxospores are formed in pennate diatoms (Lee 2008). Isodiametric auxospores are normally attached to the parent valves during maturation. The immature properizonial auxospore splits and

[1] The term perizonial derived from perizonium. Perizonium is an inner silicified membrane within which the initial cell is formed. In the great majority of centric diatoms only one membrane or fertilization membrane or perizonium remain (Bold and Wynne 1985).

bands (also known as properizonium) are produced from an asymmetrical auxospore. On the other hand in perizonial auxospores immature globular auxospores has scales embedded in a primary wall and the primary auxospores wall divide into two equal halves. Auxospore formation vary considerably in Centrales and Pennales diatoms as discussed below.

7.2.1 Auxospore Formation in Pennales

Mostly the pennate diatoms are monoecious (homothallic) and rarely dioecious (heterothallis) for e.g. *Rhabdonema adriaticum* and *Navicula halophila*. In pennales auxospores are either formed mostly by union between iso and anisogametes, or oogamy and rarely by autogamy or parthenogenesis.

- **Fusion of iso or anisogametes**: The fusion of isogametes or anisogametes either forms one auxospore from two cells or two auxospores from two cells

  A. Formation of single auxospores from fusion of two cells: It is common in *Surirella saxonica*, *Cocconeis* species. During conjugation two diatoms (sister or non-sister cells) come close and lie next to each other either by side to side or end to end (Fig. 8). Once they are close, get eneveloped by common mucilage secreted by them. The first meiotic division takes place in diploid nuclei of both cells, but there is immediate degeneration of one sister nuclei in each cell. There may be degeneration of one nucleus after each division (*Cocconeis pediculus*) or three haploid nuclei degenerate after the completion of meiosis. In each sister cell (Geitler 1927; Karsten 1899), resulting into

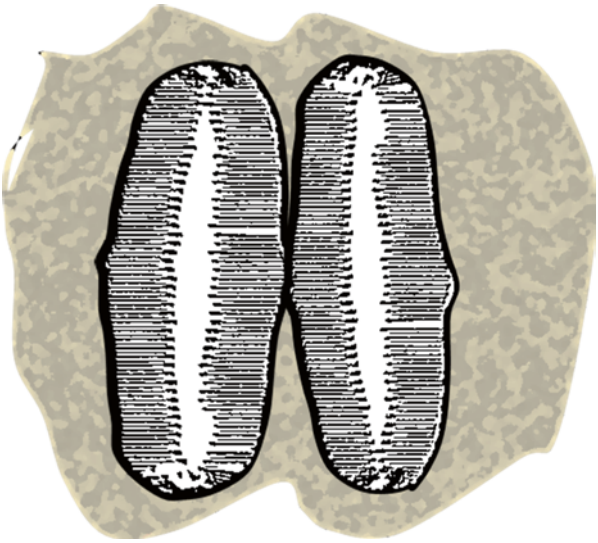

Fig. 8 Two fusing diatoms lying close to each other in a common mucilaginous envelope

one haploid nuclei in each cell. Protoplast of which finally behaves as a haploid gamete. It escapes from the frustule. Since, the liberated gametes are identical are called Isogammetes. The two isogametes show amoeboid movement, escape from the parent frustule and fuse immediately in between the empty parent cells, forming a diploid zygote. The zygote enlarge, (more in longitudinal plane) and functions as an auxospores. Later, it secretes a fresh frustule within the perizonium, thus producing a new vegetative cell resembling parent cell both in size and construction.

B. Formation of two auxospores from fusion of two cells: In Pennale diatoms, this is the commonest method, e.g. *Cymbella lanceolata*. During this, the two diminutive diatoms of same species come together and secrete a mucilaginous envelop around them. The diploid nucleus of each cell divides meiotically and forms four haploid nuclei, two of which degenerate. The protoplast of sustaining cells now divides into two equal or unequal daughters either longitudinally or transversely, which functions as a gamete. Each gamete thus contains one large haploid nucleus (functional) and one degenerated haploid nucleus. The two gametes formed by a cell may be of equal or unequal in size depending on whether the division is equal or unequal in parent protoplast. The two gametes produced by a cell normally unite with the gamete produced from the other cell and many a times physiological anisogamy is present (Smith 1955). The gametic union occurs by either of the following ways:

(i) Both the gametes are amoeboid, alike and are isogametes. They unite midway between the parent frustule and the two zygote are formed outside the parent frustule and this is the most common method e.g. *Navicula radiosa*

(ii) Either of the gamete is active or amoeboid and the other one is passive or immobile. This type of condition is observed either in two gametes of same cell, or one parent cell produces two mobile gametes and other parent cell produce two immobile gametes (e.g. *Navicula halophila*). This condition represents physiological anisogamy.

The mobile gametes, move out of the parent frustule and creep inside the other parent cell to fuse with the passive gametes of other cell and produces two zygotes in one cell. They remain dormant for some time and later enlarge parallel to the longitudinal axes of the parent frustule to form an auxospores. The auxospores is considerable larger than the parent cell and is enclosed by a membrane called perizonium, secreted by the either auxospores or it may be a ruminant of zygotic membrane. A new frustule is secreted inside the perizonium.

(iii) In the third method, the two gametes are morphologically alike and have functional disparity. One of the gamete produced in parent frustule is smaller and active (amoeboid), while the other one is bigger and passive. Such type of gametes represents physiological anisogamy as the division of protoplast to form gametes is asymmetrical resulting into two gametes of unequal size with different functions. The amoeboid

gamete from each parent frustule will come out, creep into the other parent frustule and will fuse with its passive counterpart e.g. *Cymbella lanceolata*, *Gomphonema parvulum*. By this method each cell have one zygote.

- **Auxospore formation by oogamy** (von Stosch 1958): Such type of auxospores formation is reported in dioecious diatoms example *Rhabdonema adriaticum*. One bigger diatom cell acts as female egg cell or oogonium. The diploid nucleus of oogonium undergoes meiosis and produces four haploid nuclei. Later the protoplast cleaves into a large functional ovum with two nuclei out of which one degenerates on maturity. The functional ovum occupies the upper third part of the female cell. Spermatogonia are formed in distinct male cells, which are smaller and numerous in number. Each spermatogonium nucleus undergoes meiosis, followed by cleavage of parent protoplast into two globular, naked, non-flagellated microgametes. Out of two microgametes one is fertile and other aborts. On liberation, the spermatogonium, carried away by water current to the oogonium and both of them get adhered because of mucilaginous pads. Only the male nucleus enters the oogonium for fertilization. The diploid zygote thus formed matures into an auxospores as usual.
- **Auxospore formation by autogamy**: During autogamy, the diatom (e.g. *Amphora normani*) secretes thick mucilage, undergoes meiosis and forms four haploid nuclei, two of which degenerate. Two remaining haploid nuclei fuse immediately forming a diploid nucleus (autogamy). The protoplast with new diploid nucleus comes out from the old frustule and develops into a new auxospore.
- **Auxospore formation by parthenogenesis**: During the process auxospore formation does not involve sexual reproduction and meiosis is replaced by mitotic division resulting into a diploid daughter cells. It has been reported in *Rhabdonema adriaticum*, *Navicula*, *Nitzschia*, *Cocconeis placentula* (Geitler 1973). During parthenogenesis the protoplast of a quiescent cell comes out and directly grows into an auxospore without any nuclear change or in many cases somatic division takes place in parent cell producing two daughters, each growing into an auxospore (Fig. 9).

7.2.2 Auxospore Formation in the Centrales

In centric diatoms sexual reproduction and auxospore formation are commonly occur by oogamy or autogamy. Isogamy and anisogamy are not common in centrales. A single auxospore is formed by a single individual which may be an old cell or a newly divided cell.

- **Auxospore formation by oogamy**: The sexual mode of auxospore formation is common in many centric diatoms, where different diploid cells act as male or female gametangia. The male diatom cell or antheridia, may be a modified

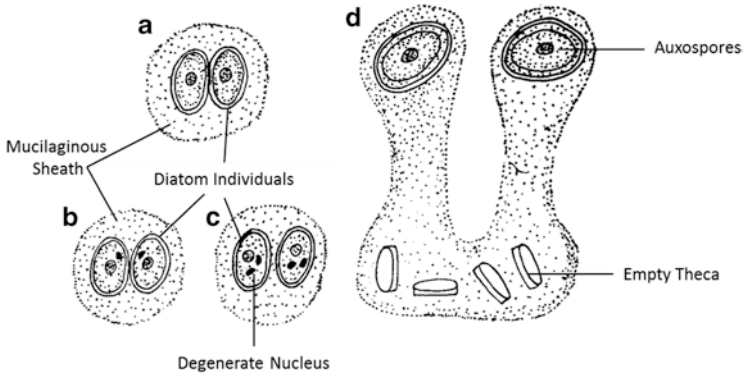

Fig. 9 Auxospore formation by parthenogenesis or apogamy in *Cocconeis placentula* var. *lineata*. Nuclear division in B and C are mitotic (Adopted from Reddy 2001)

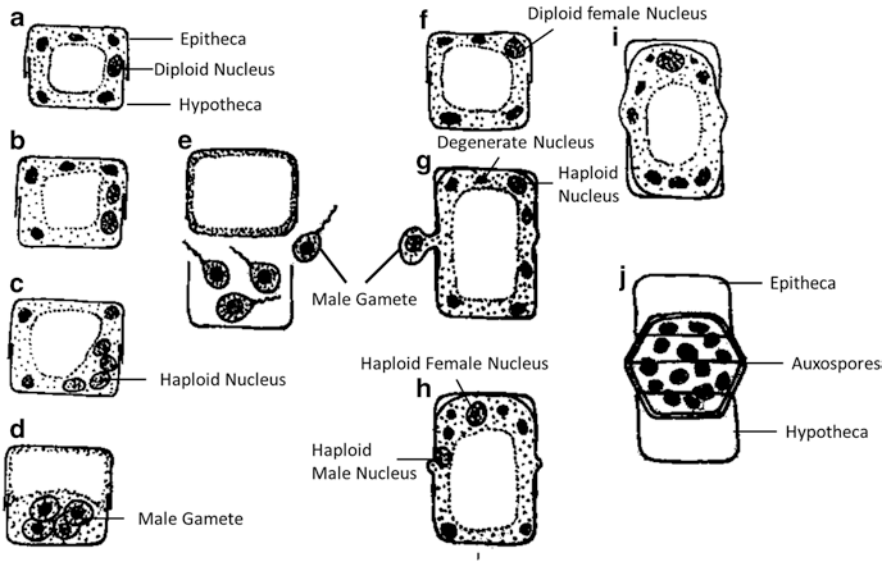

Fig. 10 Auxospore formation by oogamy in the distinct male and female centric diatom *Melosira varlans* (Adopted from Reddy 2001)

vegetative cell (*Cyclotella* species) or may be formed by division of a vegetative cell (*Melosira* species) (Fig. 10). Each antheridium or spermatogonium produces numerous sperm cells which vary from specie to species. There number varies from 4, 8, 16, 32, 64 or it may even reach 128 which is a rare phenomenon. Cell which act as an antheridium undergoes meiosis and produces haploid spermatozoids, which were earlier considered to be asexual in nature and was termed microspores. These spermatozoids are motile and have a single tinsel flagellum.

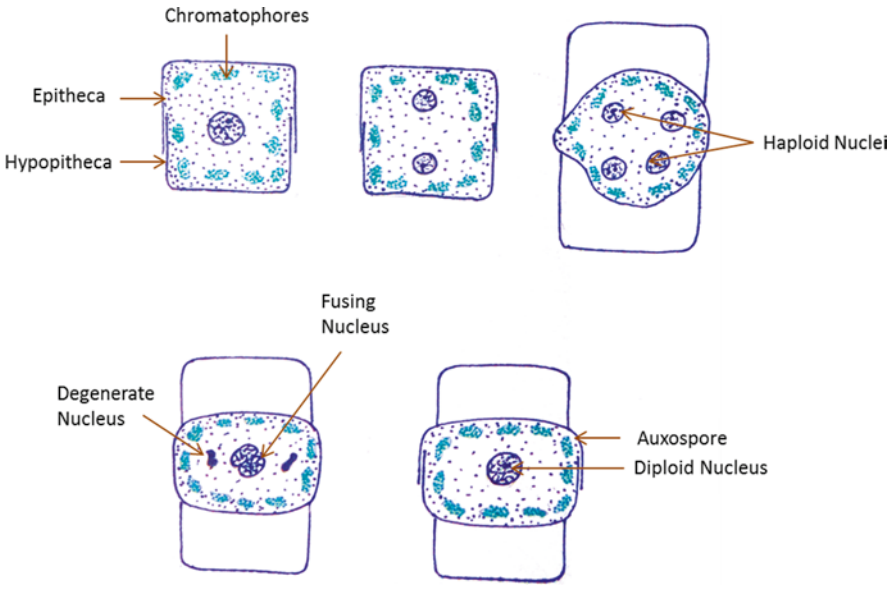

Fig. 11 Auxospore formation by autogamy in the centric diatom *Cyclotella meneghiniana* (Adopted from Reddy 2001)

Both uniflagellate and biflagellate sperms has been observed experimentally in culture of *Cyclotella meneghiniana* and *C. cryptica* by Schultz and Trainor (1970). The female gamete or egg is produced singly in an oogonium. The female cell undergoes meiosis and produces four haploid nuclei of which three nuclei disintegrates and one surviving nucleus along with undivided protoplast function as an egg. The spermatozoids get released from antheridium by the separation of frustule and theca. They swim and search egg. Both spermatozoid and egg cells get attached by mucilaginous fibres resulting into a zygote. After fertilization the zygote (diploid) gets released from the parent frustule and develops into an auxospore. The auxospore increases in size, its diploid nucleus divides into two by mitosis and only one persist. The auxospore with diploid surviving nucleus secretes a frustule inside the perizonium which called as "First ling Cell". This is a new diatom individual.

- **Auxospore formation by autogamy**: During autogamy, the protoplast of the diploid cell secretes a mucilaginous covering, forcing apart the theca, and nucleus undergoes meiosis resulting into four haploid nuclei. Out of four newly formed nuclei only two survive and fuse with each other thus resulting into a diploid nuclei. This is known as autogamy. After the formation of diploid nucleus, the protoplast escape from the parent frustule, and develops into an auxospore by forming a fresh frustule within the perizonium (Fig. 11).

8 Defense Against Predation

Some diatoms (e.g. *Phaeodactylum*, *Skeletonema*) release specific chemicals to defend themselves from many predators such as copepods, sea urchins, polychaetes, and ascidians. These chemicals include highly unsaturated fatty acids such as eicosanoic acid, which is only toxic after the unsaturated fatty acid it's released into the environment and conversion into an aldehyde (Lee 2008). Diatoms spine which help to kept diatoms at upper layer of oceans or lakes also discourage grazing by herbivorous zooplankton (Hoek et al. 2009).

9 Economic Importance

9.1 Diatomaceous Earth

In some parts where diatoms are largely responsible for the very high primary productivity and are found in abundance, the death of diatoms and there sinking to the bottom of these areas results in the accumulation of diatom oozes. Normally the frustules of diatoms are dissolved by bacterial degradation but under certain circumstances, the frustules remain intact and accumulate at the bottom of the water. These large fossil deposits from past geological period (but not older than Cretaceous era) are known as "diatomite" or "diatomaceous earth" or "kieselguhr" (Lee 2008; Hoek et al. 2009). This diatomaceous earth is being used by several industries such as:

- Toothpaste
- Metal polishes
- As an absorbent for liquid nitroglycerin to make dynamite that could be transported with comparative safety
- Filtration of liquids, especially sugar refineries
- As insulation of boilers, blast furnaces, and other places where a high temperature is maintained

9.2 Diatoms as Indicators

Diatoms are also being used as indicators of water quality and pollution tolerance (Bold and Wynne 1985). Many heavy metals have inhibitory effect on diatoms such as Cu, Zi and Ge can inhibit silicon metabolism whereas Hg, Cd, and Pb inhibit cell division. On the other hand Cr, Ni, and Se have inhibitory effect only at very high concentration (>1 μM). Due to high sensitivity copper is normally being used to control diatom blooms. Concentrations of germanium dioxide (GeO_2) above 1.5 mg liter $^{-1}$ will specifically suppress the growth of diatoms except those which have little or no silicified wall such as *Phaeodactylum tricornutum* (Lee 2008).

9.3 Health Effects

Some diatoms show shellfish poisoning such as *Nitzschia*, *Pseudonitzschia*, and *Amphora* (Lee 2008). Memory loss (amnesia), abdominal cramps, vomiting, disorientation, and even death can be seen in human and other animals after subsequent ingestion of shellfish (Lee 2008).

10 Adaptation of Diatoms

Main problem which is faced by heavy silica cell walled diatoms during photosynthesis is to remain in the uppermost layers of lakes or oceans to get enough light (Morris 1971). To overcome this problem several marine diatoms such as *Ditylum* reduce their density and become more buoyant by excluding heavy ions from their cell sap. Some diatoms increased their surface area by setae (*Chaetoceros*) or by cells shaped as discs (*Coscinodiscus*), ribbons (*Fragilaria*) or elongate forms (*Rhizosolenia*) (Lee 2008). On the other hand many diatoms bear long spines, horns, setae or other protrusions such as in *Thalassiosira* that result in a greater surface area relative to the volume (Bold and Wynne 1985; Hoek et al. 2009). Some diatoms (e.g. *Ditylum*) change rate of fat deposition with light period to remain floating.

Diatoms living at great water depth which receive only blue-green light have a "switch on mechanism" to capture maximum light and increased fixation of carbon dioxide (Lee 2008). Many diatoms such as *Rhizosolenia* and *Hemiaulus* also remain as symbionts with nitrogen-fixing bacteria and cyanobacteria. *Rhizosolenia* can sink (negatively buoyant) after nitrogen deprivation but can move up (positive buoyant) after taking up nutrients (Lee 2008).

11 Phylogeny

In terms of evolutionary diversification, the diatoms have been widely successful (Graham and Wilcox 2000). First diatoms were discovered at the beginning of eighteenth century but lack of advance microscopic technology it took almost one century for their detailed study (Mann and Evans 2007). Because of the siliceous nature of the diatom cell walls, well preserved fossil are available. The centrales have been reported from the Jurassic and the Pennales from early Tertiary (Kumar and Singh 1971). The group of diatom is one of the rare groups in which no organism regarded previously as diatoms have been not to be diatoms. However there are some unconventional representatives too such as shell-less endosymbionts of dinoflagellates (Mann and Evans 2007). Well before the molecular techniques, transmission electron microscopy and basic biochemical techniques had shown long before that diatoms belong to heterokonts, together with Phaeophyceae, Crysophyceae,

Xanthophyceae, Eustigmatophyceae, Oomycetes and some other classes of autotrophic and heterotrophic protists (Mann and Evans 2007). Molecular sequences information revealed two lineage or clades. Clade 1 consist of Centric diatoms, many of which may possess peripheral rings of tube-like structure (labiate processes or rimoportulae. Clade 2 includes centric diatoms having central labiate processes or fultoportula and pennate diatoms (Graham and Wilcox 2000). Fossil records suggest that centric diatoms evolved before pennate diatoms (Round et al. 1990).

Acknowledgment The authors are grateful for the support provided by Mrs. Meena Malhotra, Department of Botany. Maitreyi College, University of Delhi for sketches.

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Xanthophyceae, Euglenophyceae and Dinophyceae

Dinabandhu Sahoo and Savindra Kumar

1 Xanthophyceae (Yellow Green Algae)

1.1 Introduction

Xanthophyceae (Xanthophytes or Xanthophyceans) are also known as Tribophyceae (tribophytes or tribophycean), after the common genus *Tribonema* (Graham et al. 2009). They are also informally frequently known as yellow green algae. Xanthophycean are non-motile, unicellular or colonial eukaryotic algae, with a unique pigmentation which gives a yellow or fresh green appearance. Members of this group are photosynthetic organisms which live primarily in freshwater, although a substantial number is found in marine waters, in damp soil, or on tree trunks (Andersen 2004; Maistro et al. 2009). Cells appear yellow-greenish or brownish-green depending on the relative amounts of chlorophyll-*a* and xanthophylls. Many of the freshwater species the Xanthophyta have not been isolated into unialgal culture, so most data come from field studies (Wehr 2010). Yellow green algae incline to be ecologically limited to small water bodies and damp soil. There are no such study which indicate that any member of the group contaminate water source. Molecular data have shown that members of Xanthophyceae are most closely related to the Phaeophyceae (Ariztia et al. 1991; Potter et al. 1997; Lee 2008).

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D. Sahoo, J. Seckbach (eds.), *The Algae World*, Cellular Origin, Life in Extreme Habitats and Astrobiology 26, DOI 10.1007/978-94-017-7321-8_9

1.2 General Characteristics

- Occurrence: Mostly freshwater and a few marine representative
- Pigments: Chlorophyll *a*, *e*, β carotene and xanthophylls
- Pyrenoids: Usually absent
- Reserve food material: Chrysolaminaran, Oil and fat
- Cell wall: Rich in pectic compounds and composed of two equal pieces overlapping at the edges.
- Structure: Eukaryotic unicellular motile to simple filamentous,
- Flagella: Present, two unequal, situated anteriorly. Longer one tinsel and shorter one whiplash
- Reproduction: Vegetative, Asexual and Sexual (Mainly Isogamous, Anisogamy is rare, Oogamous in *Vaucheria*)

1.3 Classification

The Xanthophyceans are much less species-diverse than other major groups such as Chlorophyceae, Rhodophyceae or Phaeophyceae, with about 100 genera and 600 species. Many of the genera contain only a few species (Hoek et al. 2009). The class Xanthophyceae is classified based on thallus structure. Xanthophyceans exhibit morphological diversity and includes motile and coccoid forms, as well as palmelloid, filamentous and siphonaceous forms. However, parenchymatous and heterotrichous forms are conspicuously absent. The orders of Xanthophyceae are distinguished on the basis of their vegetative development. Many of the same vegetative tendencies observation in the Chlorophyceae and Chrysophyceae are paralleled by members of this class (Bold and Wynne 1985).

Fritch (1935) divided this class into four orders

1. **Heterochloridales**: It includes motile forms and its derivatives e.g. *Mischococcus*
2. **Heterococcales**: It includes coccoid forms e.g. *Halosphaera*, *Myxochloris*
3. **Heterotrichales**: It includes filamentous forms e.g. *Tribonema*, *Neonema*, *Heterococcus*, *Heterodendron*
4. **Heterosiphonales**: It includes siphoneous forms e.g. *Botrydium*, *Vaucheria*

On the basis of pigments (Carotene and Xanthophyll) and reserve food material (Leucosin and Oil), Smith (1955) kept Class Xanthophyceae along with Chrysophyceae and Bacillariophyceae in the division Chrysophyta. Prescott (1969) also followed this system of classification but instead of classes he divided the phylum Chrysophyta into three sub-phylums namely Xanthophyceae, Chrysophyceae and Bacillariophyceae. Prescott (1969) divided subphylum Xanthophyceae into five orders.

1. **Rhizochloridales**: Unicellular, Rhizopodial e.g. *Stipitococcus*
2. **Heterocapsales**: Non-motile, colonial, gelatinous e.g. *Gloeochloris*

3. **Hetercoccales**: Unicellular or colonial, autospores and internal cell divisions for reproduction e.g. *Chlorallanthus*, *Chlorobotrys*, *Ophiocytium*
4. **Heterotrichales**: Branched or unbranched, differentiation of basal and distal cells e.g. *Tribonema*
5. **Heterosiphonales**: coenocytic e.g. *Botrydium*, *Vaucheria*

Bold and Wynne (1985) kept class Xanthophyceae along with Chrysophyceae, Prymnesiophyceae, Eustigmatophyceae, Raphidophyceae and Bacillariophyceae in division Crysophyta. They further described following six orders in class Xanthophyceae.

1. **Heterochloridales**: This order consist of naked, unicellular, anteriorly biflagellate monada, some with a capacity to be temporarily amoeboid e.g. *Chloromeson*,
2. **Rhizochloridales**: This order have a dominant amoeboid stage, unicellular or multicellular; zoospores are produced. Broad or delicate pseudopodia characterise the vegetative stage of members of this order. Both solitary types and larger, multinucleate palmodial types are known e. g. *Chlamydomyxa*
3. **Heterogloeales**: This order is comprised of palmelloid forms, solitary or colonial, permanently provided with or without a gelatinous envelope. Reproduction is by zoospores or autospores e.g. *Heterogloea*, *Characidiopsis*
4. **Mischococcales**: Largest order of the class, containing 11 family. These algae have immobile coccoid forms, solitary or colonial. Walls are distinct and are smooth or sculptured in one or two pieces. Reproduction is by zoospores or autospores. e.g. *Botrydiopsis*, *Botryochloris*, *Mischococcus*, *Ophiocytium*
5. **Tribonematales**: Members consist of simple or branched filaments with cross walls; the filaments may be uniseriate or multiseriate. Reproduction is by cell division, zoospores or aplanospores e.g. *Tribonema*
6. **Vaucheriales**: These algae are multinucleate vesicles or filaments, lacking cross walls. Reproduction is by zoospores, aplanospores or by iso-aniso or oogamy e.g. *Botrydium*, *Vaucheria*

On the basis of flagella numbers and position (anterior tinsel and posterior whiplash type); chlorophyll *a* and *c*; fucoxanthin; storage product such as chrysolaminarin occurring in vesicles Lee (2008) kept class Xanthophyceae along with 12 other classes namely Chrysophyceae (golden-brown algae), Synurophyceae, Eustigmatophyceae, Pinguiophyceae, Dictyochophyceae (silicoflagellates), Pelagophyceae, Bolidophyceae, Bacillariophyceae (diatoms), Raphidophyceae (chloromonads), Phaeothamniophyceae, Phaeophyceae (brown algae) and Prymnesiophyta (haptophytes) under phylum Heterokontophyta. Lee (2008) further divided class Xanthophyceae in following four orders.

1. **Mischococcales**: Small coccoid cells.
2. **Tribonematales**: Filamentous organisms, not coenocytic.
3. **Botrydiales**: Globose multinucleate thallus with colourless rhizoids.
4. **Vaucheriales**: Filamentous coenocyte.

Hoek et al. (2009) also kept Class Xanthophyceae under Heterokontophyta but mentioned the following seven orders.

1. **Chloramoebales**: flagellate organisation
2. **Rhizochloridales**: amoeboid organisation
3. **Heterogloeales**: Palmelloid (tetrasporal) organisation
4. **Mischoicoccales**: Coccoid organisation
5. **Tribonematales**: Filamentous organization
6. **Botrydiales**: Siphonous organisation, sexual reproduction isogamous/anisogamous
7. **Vaucheriales**: Siphonous organisation, sexual reproduction oogamous

Wehr (2010) divide division Xanthophyta in three classes (Table 1).

1.4 Occurrence

The diversity of freshwater yellow-green algae is large, but their biology, ecology, and biogeography are known for only a few of the more common taxa. Majority of Xanthophycean forms are fresh water but a few are found growing in marine habitats

Table 1 Diagnostic characteristics of freshwater algae distinguishing the three main classes from the Xanthophyta

	Raphidophyceae	Eustigmatophyceae	Tribophyceae
Common morphologies	Unicell, flagellate	Unicell, coccoid	Unicells, Colonies, Filaments
Flagellar insertion	1 or 2, apical	1 or 2, apical	2, apical
Primary pigments	Chlorophyll *a*, *c*1, *c*2	Chlorophyll *a*	Chlorophyll *a*, *c*1, *c*2
Color of cells or thalli	Yellow-green or yellow-brow	Yellow-green	Yellow-green or green
Major accessory pigments	b-Carotene, Diatoxanthin	b-Carotene, Violaxanthin	b-Carotene, aucheriaxanthin, Heteroxanthin, Diatoxanthin
	Diadinoxanthin, Vaucheriaxanthin	Vaucheriaxanthin	
			Diadinoxanthin
Chloroplasts	Ellipsoid: yellow-green	Parietal or discoid: yellow-green	Discoid: green or yellow-green
Main storage products	Chrysolaminarin	Chrysolaminarin	Chrysolaminarin
Cell wall	None (naked)	Composition uncertain	Cellulose
No. of genera[a]	≈5	≈8	≈100
No. of species[a]	≈10	Unknown	≈600
Common genera	*Gonyostomum*	*Chlorobotrys*	*Botridium, Characiopsis*
	Vacuolaria	*Nannochloropsis*	*Ophiocytium, Tribonema*
		Pseudocharaciopsis	*Vaucheria*

Reproduce from Wehr (2010)
[a]Freshwater taxa only

such as *Halosphaera*. Most members occur as planktons or as epiphytes on other aquatic plants. Several members of Xanthophyceae have colonised alpine and polar environments (Andreoli et al. 1999; Broady 1976, 2005; Darling et al. 1987; Mataloni et al. 2000). Most of the Xanthophyceans are difficult to find because they rarely grow anywhere in abundance. The main exception to this is the species of the filamentous genus *Tribonema* (Fig. 1) which form bright green growth in cold freshwater. *Tribonema* occur in great quantities in bog pools that have been enriched by bird dropping. On the other hand, species of siphonus *Vaucheria* are distributed widely.

1.5 Range of Thallus Structure

Members of Xanthophyceae show well- marked parallelism with Chlorophyceae in their thallus structure which includes motile, palmelloid, dendroids, filamentous and siphoneous form (Kumar and Singh 1971). Members of this class show a wide

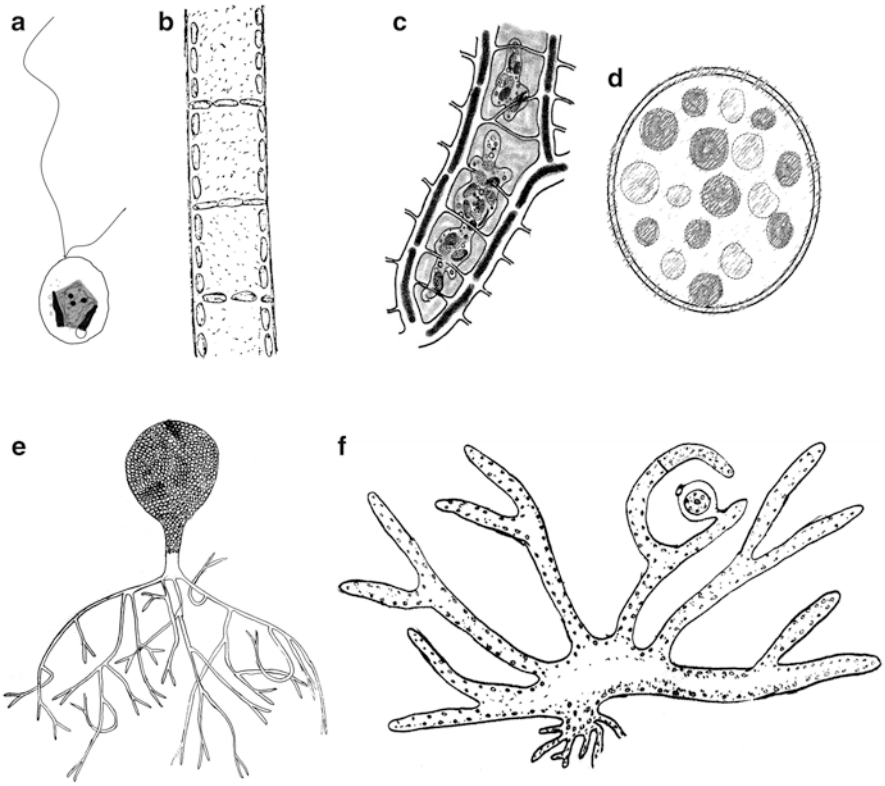


Fig. 1 Range of thallus organisation in class Xanthophyceae (**a**) *Chloromeson* (**b**) *Tribonema* (**c**) *Myxochloris* (**d**) *Botrydiopsis* (**e**) *Botrydium* and (**f**) *Vaucheria*

range of forms. Like green algae, the plant body may be unicellular and motile as in *Chlorochromonas*, palmelloids with a swimming phase as in *Chlorosaccus* or a rhizopodial cell as seen in *Chlorarachnion*. Some members also exhibit a coccoid habit as in *Botrydiopsis*. In another series, the vegetative plant body is palmelloid and nonmotile as in *Mischococcus*. A truly filamentous series is well represented by unbranched filaments of *Tribonema* and siphonus condition is exemplified by the coenocytic thalli of *Botrydium* and branched filaments of *Vaucheria* (Fig. 1). Xanthophyceans lack highly elaborate pseudo-parenchymatous or truly parenchymatous thalli as observed in advanced members of the Chlorophyceae. Hoek et al. (2009) discussed the following levels of organisation which can be found in Xanthophyceae (Fig. 1):

- Unicellular flagellate (monadoid) e.g. *Chloromeson*
- Amoeboid e.g. *Rhizochloris*, *Myxochloris*
- Palmelloid e.g. *Gloechloris*
- Coccoid e.g. *Chloridella*, *Botrydiopsis*, *Characiopsis*, *Ophiocytium*
- Filamentous e.g. *Tribonema*, *Hetrodendron*
- Siphonous e.g. *Botrydium*, *Vaucheria*

1.6 Pigment

The characteristic yellow green colour of Xanthophytes is due to their parietal chromatophores which have chlorophyll *a* and *e*, along with *β*-carotene and several xanthophylls. They differ from the other closely related classes such as Chrysophyceae and Bacillariophyceae by the absence of chlorophyll *b* (Bold and Wynne 1985). In earlier studies chlorophyll *c* were not detected in Xanthophyceae but later chlorophyll *c* were detected in some members in Xanthophyceae (Sullivan et al. 1990).

1.7 Storage Food

For most members of this class the nature of the food reserves has been little investigated, although chrysolaminaran (leucosin a glucose polymers), oils and fats are usually reported. Testing for the presence of starch (with dilute iodine in potassium iodide solution to detect the black staining reaction of starch) results in a negative reaction (Bold and Wynne 1985). The principal storage product is probably a *β*-1,3 linked glucan similar to paramylon, although lipids have been suggested as also being important (Lee 2008).

1.8 Cell Structure

The yellow green algal group is known also as the heterokonate because there are two structurally different flagella (or sometimes but one flagellum), rather than flagella of equal length and morphology which in general characterize the isokonate of the Chlorophyceae (Prescott 1969). The class is characterised by motile cells with a forwardly directed tinsel flagellum and a posteriorly directed whiplash flagellum (Fig. 2) (Lee 2008). The cell wall is composed of pectic substances. Pectic acid and pectose are the chief constituents. The cellulose as a cell constituent of the cell wall is meager but the cell wall in *Botrydium* is wholly of cellulose. The cell wall has tightly overlapping halves (Graham and Wilcox 2000; Graham et al. 2009). In *Tribonema*, the cell wall is composed of 'H' shaped pieces which overlap each other alternately (Fig. 3) (Chapman 1962; Chapman and Chapman 1981; Lee 2008). The number of chromatophores in a cell, depending upon the species, varies from one to

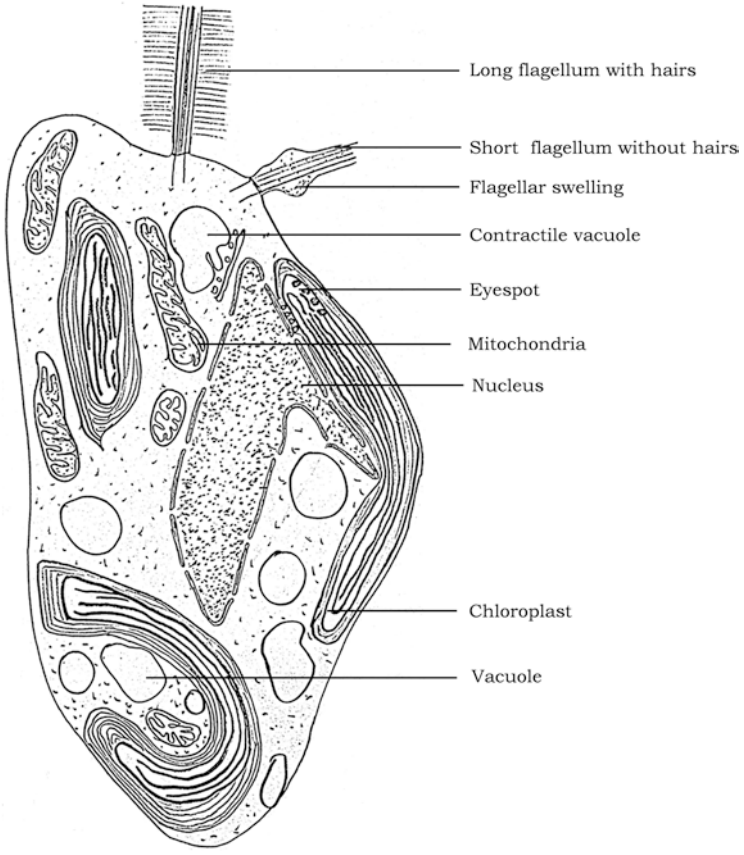


Fig. 2 Ultrastructure drawing of a zoospore of a typical member of the Xanthophyceae (Adapted from Hibberd and Leedale 1971; Lee 2008)

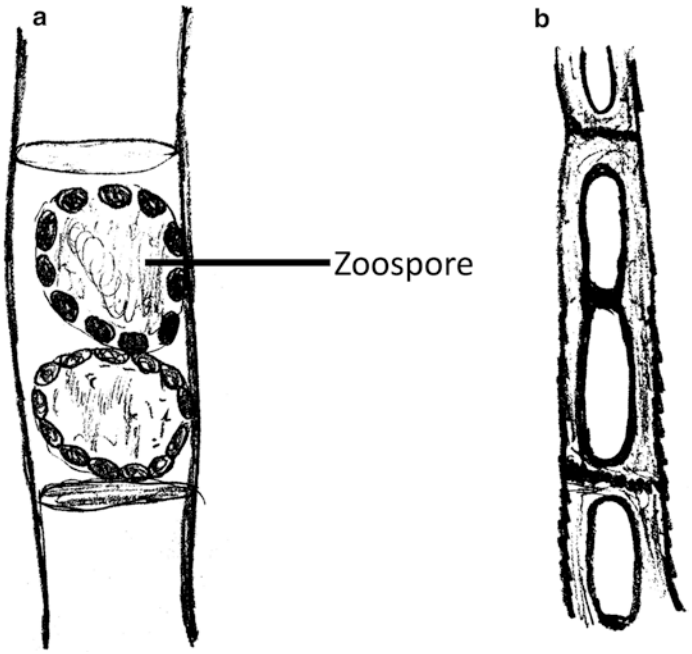


Fig. 3 (**a**) *Tribonema viride*, zoosporangium containing two zoospores and (**b**) H-shaped cell walls of *Tribonema* remaining after liberation of zoospores

several. Chromatophores usually lack pyrenoids but if present, are naked and these do not serve for the storage of food. Main difference of yellow green algae from green algae is the uniformly simple shape of the chloroplast, usually discoid and parietal in position. The members of Xanthophyceae are both uninucleate and multinucleate. The eyespot in motile cells is always in the chloroplast and the chloroplasts are surrounded by two membranes of chloroplast endoplasmic reticulum (Fig. 2). The outer membrane of the chloroplast-endoplasmic reticulum complex is usually continuous with the outer membrane of the nucleus. Mostly walls of non-motile cells are composed of two overlapping halves. The thylakoids are grouped into bands of three (Lee 2008).

1.9 Reproduction

Xanthophyceans exhibit very little regularity in the formation of reproductive bodies (Chapman 1962; Chapman and Chapman 1981). The usual methods of reproduction are vegetative, asexual and sexual (Graham et al. 2009; Kumar and Singh 1971; Lee 2008). In Xanthophyceans, the chief mode of multiplication is by

biflagellate zoospore formation, with the forward tinsel flagellum usually being four to six times longer than the shorter whiplash flagellum (Lee 2008). Zoospore develops during favourable conditions. Aplanospores and even cyst/endogenous spores are formed when condition are not favourable. An aplanospore may germinate directly or it may give rise to zoospore which germinates to produce new plant. Cysts are formed by modification of entire vegetative cells and help species survive conditions unsuitable for growth of the vegetative phase or populations. Silica may occur in cyst walls and cyst may consist of two overlapping parts (Graham et al. 2009). In some forms, under certain conditions vegetative cells function as akinetes due to development of thicker walls and accumulation of abundant food materials. Fragmentation is limited to the tetrasporine and filamentous colonies, and is because of breaking of the colony into parts (Lee 2008). Statospore are produced in some Xanthophyceans algae and they are unlike those occurring in the Chrysophyceae in that they consist of two parts of about equal size, overlap (Bold and Wynne 1985). Sexual reproduction is rare and in most of the known examples is isogamous (Chapman 1962; Lee 2008). Sexual reproduction has only been well established in three genera *Botrydium*, *Tribonema*, and *Vaucheria*.

1.10 Phylogeny

Majority of genera are motile and unicellular which forms comparatively a small group of algae. Pascher (1931), Fritch (1935), Smith (1955), Prescott (1969) and others in comparison to Chlorophyceae, advocate distinct parallel evolution and similarity in the origin of the group from the flagellates. But the sequence of evolution possibly did not go too far since Xanthophyceae lack so highly differentiated and elaborate thalli as observed in green algae. Members of Xanthophyceae resemble those of Chrysophyceae in the habit, reserve food, composition of cell wall and the flagellate stages. Therefore, many authorities agree to place this group with Chrysophyceae and Bacillariophyceae under the division or phylum Chrysophyta. But some members also possess chlorophyll *c* and cell wall is chiefly composed of pectic materials hence, proposes an independent status for Xanthophyceae. Phylogenetic analyses of Xanthophyceae have had limited taxon/gene selection or have focused on particular taxa. Recently, Xanthophyceae has been significantly revised by Adl et al. (2005) who assigned all the taxa to Tribonematales except for *Vaucheria* that was assigned to Vaucheriales. According to Maistro et al. (2009) any of the recent classification is followed, the taxonomic arrangement of Xanthophyceae does not reflect phylogeny. In order to clarify this situation at both micro- and macro-taxonomic levels they have broadened the taxon sampling and have applied a multigenic approach using both plastidial rbcL and psaA and nuclear SSU rDNA genes. Many genera comes under Xanthophyceae after SSU rDNA analysis which were not covered under the Xanthophyceae earlier (Potter et al. 1997).

1.11 Vaucheria*: Life Cycle Study*

1.11.1 Systematic Position

Fritch (1935)	Prescott (1969)	Bold and Wynne (1978)	Lee (2008)
Division-Algae	Phylum-Chrysophyta	Division-Chrysophyta	Group- Eukaryota
Class-Chlorophyceae	Subphylum-Xanthophyceae	Class-Xanthophyceae	Phylum-Heterokontophyta
Order-Siphonales	Order-Heterosiphonales	Order-Vaucheriales	Class- Xanthophyceae
Family-Vaucheriaceae	Family-Vaucheriaceae	Family-Vaucheriaceae	Order- Vaucheriales
Genus-*Vaucheria*	Genus-*Vaucheria*	Genus-*Vaucheria*	Family-Vaucheriaceae
			Genus- *Vaucheria*

1.11.2 Introduction

Genus *Vaucheria* is named after Jean Pierre Etienne Vaucher, a Swiss clergyman and botanist (Graham et al. 2009). It represents the climax of siphoneous habit amongst the Xanthophyceae. *Vaucheria* shows affinities with different groups (Table 2). Earlier *Vaucheria*, with three other genera, *Vaucheriopsis, Dichotomosiphon* and *Pseudodichotomosiphon*, was positioned in the family Vaucheriaceae under order Siphonales of the class Chlorophyceae. The resemblance was so great that even Fritsch (1935) considered them to have arisen from a common ancestor. But the revelation of features such as the absence of chlorophyll b, presence of chlorophyll e, excess of carotenoids over chlorophyll, storage of food reserve in the form of oil and the unequal length of the paired flagella of the compound zoospore, led to its removal from the Chlorophyceae and inclusion in Xanthophyceae (Smith 1955; Kumar and Singh 1971). At present *Vaucheria*, is the lone genus in the family Vaucheriaceae (Guiry and Guiry 2015). The discovery of tinsel and whiplash type of flagella in the motile stage of *Vaucheria* is indicative of the Phycomycetes having been derived from *Vaucheria*- like ancestors.

1.11.3 Occurrence

There are 267 species (and infraspecific) names in the database at present, of which 84 have been flagged as currently accepted taxonomically (Guiry and Guiry 2015). Widespread in freshwater, brakish and marine habitats, *Vaucheria* is often amphibious, living on mud that is periodically immersed in water and then exposed to the

Table 2 Affinities of *Vaucheria*

Xanthophyceans characteristics	Chlorophycean characteristics	Oomycetes characteristics
Siphoneous, coenocytic, acellular organisation of the thallus	Multinucleate, aseptate thallus	Coenocytic nature of the thallus
	Oogamous sexual reproduction	Cell wall composition
Discoid chloroplast		Oogamous sexual reproduction
Cell wall composition		
Flagella morphology of motile cells		
Food reserve		

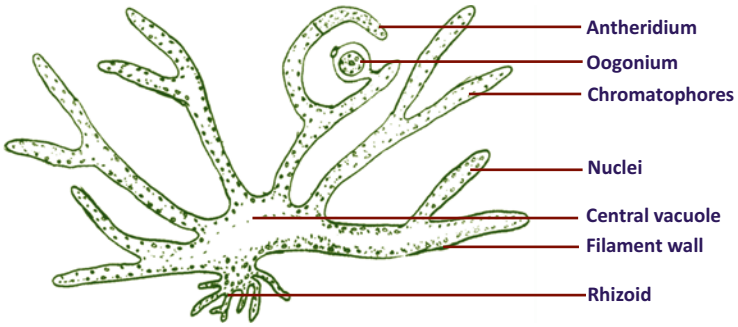


Fig. 4 ***Vaucheria***: Thallus structure

air (Bold and Wynne 1985). Most of the species are more commonly represented in temperate region especially in such well aerated situation as the flowing waters of waterfalls or streams. Many occur in stagnant ponds, ditches and shaded bodies of shallow freshwater . A few species like *V. piloboloids* are marine. The terrestrial species form extensive yellowish or deep-green, dense covering or mat on wet-soil and in flower pots in green houses, thus commonly known as "Waterfelt". Two widely distributed species are *V. sessilis* and *V. geminata.* While *V. sessilis* occurs both on land and in water; *V. uncinata* is aquatic and *V. amphibia* is amphibious.

1.11.4 Thallus Morphology and Ultrastructure

The thallus is composed of yellowish-green, cylindrical, tubular but coarse filaments, branched at irregular intervals (Fig. 4). Thallus consists of a colourless basal rhizoidal portion from which arise green, erect aerial filaments with apical growth and monopodial branching. Growth of the filaments is restricted to the apex which has a large number of vesicles, mitochondria, and dictyosomes. Chloroplasts, nuclei, and the large central vacuole are not found at the growing tip (Fig. 5) (Lee 2008; Ott and Brown 1974). *Vaucheria* has a relatively thin cell wall within which the cytoplasm is restricted to the periphery of the coenocyte, with the centre being

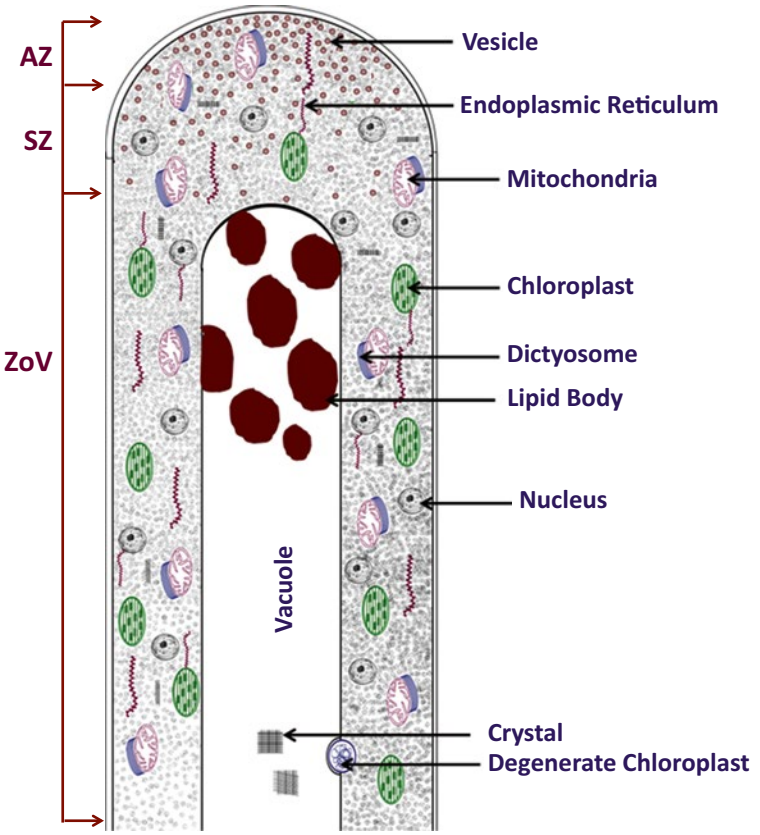


Fig. 5 ***Vaucheria***: Schematic representation of the longitudinal section of the tip of a vegetative filament. *AZ* Apical zone, *SZ* Sub-apical Zone, *ZoV* Zone of vacuolation (After Ott and Brown 1974)

occupied by a large central vacuole (Lee 2008). Cell wall divided into an inner layer of cellulose and an outer layer of pectic substances (Prescott 1969). The cytoplasm is pushed to the cell periphery by a large vacuole and contains many nuclei and discoid plastids. Cytoplasm contains many small chromatophores. The chromatophores contain chlorophyll-*a*, -*e* and carotenoids. It lacks chlorophyll-*b*. Chromatophores are followed by numerous minute nuclei. Pyrenoids not present in the chromatophores. The reserve food is oil or fat (lipid), and gets stored within the cytoplasm in the form of countless droplets. In *Vaucheria*, cytokinesis is not usually followed by mitosis, so the cells retain multiple nuclei. The septa appear only during the formation of reproductive organs. Overall it is suitable to call *Vaucheria*, as an acellular, coencytic organism, rather than a unicellular or multicellular form. Absence of cross-walls makes the thallus vulnerable for infection as the parasitic fungus spreads readily throughout the plant body. Also, a minor injury may cause the discharging of the entire thallus. In case of such an emergency, a septum is formed to localise the injured part and to save the rest of the filament.

1.11.5 Cytoplasmic Streaming

Organellar streaming is a conspicuous attribute of *Vaucheria*, and presumably function to circulate materials within *Vaucheria*'s very large cells (Fig. 6) (Graham et al. 2009). Cytoplasmic streaming takes place in the area of the large central vacuole and directly involves the nuclei, mitochondria, and their associated dictyosomes. The cytoplasmic streaming involves two separate systems, the first based on microtubules that move the nuclei, and the second based on microfilaments that move the mitochondria and their associated dictyosomes. There is no definite streaming in the chloroplasts migration pattern but have a random movement, which is not associated with either microtubules or microfilaments (Lee 2008). Blatt and Briggs (1980) observed longitudinally-oriented cytoplasmic fibrils on the cortical region of the cytoplasm in *V. sessilis.* They found that the fibrils appear to guide the streaming of these organelles. The action spectrum for chloroplast movement in *Vaucheria* is

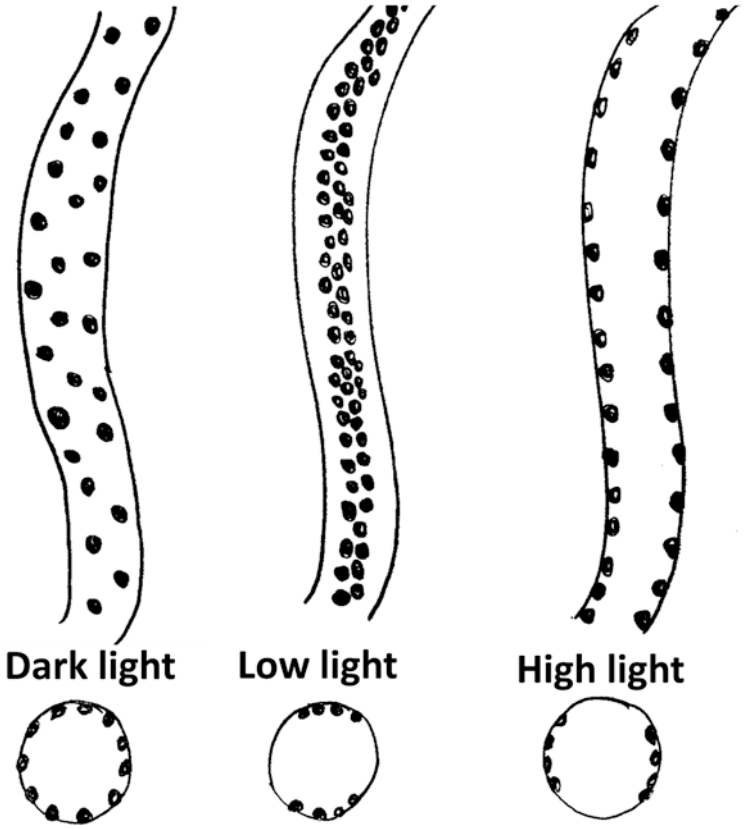


Fig. 6 Position of chloroplasts of *Vaucheria* in the dark/low/high light intensities (Adopted from Lee 2008)

similar to that of higher plants and closely resembles that of the action spectrum of flavins, from which it has been concluded that flavins located in the plasma membrane act as the photoreceptive pigments. There are two possible mechanisms to explain chloroplast movement. In the first or "active movement" the chloroplast moves relative to the rest of the protoplasm, whereas in the second or "passive movement" the protoplasm moves, carrying with it the chloroplast and other organelles. In *Vaucheria*, passive movement occurs. If chloroplast movement is followed at high or low intensity, it can be seen that not only chloroplasts, but also other organelles and inclusions are rearranged by light. Furthermore, if only a small area of the filament is irradiated with a spot of light, a strong accumulation of cytoplasm plus inclusions can be observed at this place. The organelles are actually trapped in a portion of the cytoplasm as they stream through the cell. Illumination of an area of *Vaucheria* results in the formation of an actin fibre network that acts as a trapping mechanism (Blatt 1983; Lee 2008).

1.11.6 Vegetative Reproduction

It multiply in a profile manner vegetatively by fragmentation. In this method, the thallus breaks up accidently into short segments, which ultimately form new individuals.

1.11.7 Asexual Reproduction

Zoospore: This is the commonest method of asexual reproduction in aquatic forms of *Vaucheria* (Morris 1971). Zoospores are normally large, multiflagellate and multinucleate. Zoospore of *Vaucheria* is a compound structure formed as a result of the failure of the protoplast within the zoosporangium to divide into uninucleate, biflagellate zoospores. Thus, it may be more appropriate to term it a compound zoospore or synzoospore. There are some factors which promotes zoospore formation such as:

- Low light intensity or complete darkness
- Change from running to still water
- Weak concentration of nutrients in the culture medium

Zoosporangia develop at the end of side branch. Zoospores are produced alone, within zoosporangia. Zoosporogenesis controlled by an endogenous circadium rhythm (Bold and Wynne 1985). During zoospores production a large number of nuclei and chloroplasts, stream into and accumulate in the swollen tip before it is separated from rest of the filament by a transverse septum. Zoosporangium starts appearing deep green due to diminishing of vacuole. At this stage, the nuclei in the young zoosporangium come to lie external to the chromatophores in the colourless cytoplasm at the periphery within the plasma membrane. A pair of centrioles is associated with each of the numerous nuclei within the zoosporangium (Graham and Wilcox 2000; Graham et al. 2009). The entire protoplast of the zoosporangium contracts to form an oval multinucleate mass – the incipient zoospores. Flagella are

produced through the vesicle membrane, and the vesicles migrate to the plasmalemma. The nuclei with their flagella pairs thus come to lie in the peripheral area of the cell (Lee 2008). The mature zoospores escape through a narrow aperture which is formed by the gelatinisation of the wall at the distal end of the zoosporangium. The zoospore swim, sluggishly for few minutes comes to rest and after being motionless flagella vanish completely. The zoospore rounds off and becomes covered with a thin cellulose wall. At this stage, the chromatophores move outwards and nuclei inwards. The zoospore then elongates in one or two opposite directions in the form of tubular outgrowths. One of these undergoes branching to form the colourless lobed holdfast, while the other continues to grow indefinitely to produce the yellowish-green, tubular filament. There is no eyespot or pyrenoids were reported in the zoospore.

Aplanospores: Instead of producing zoospores, terrestrial individuals may have the entire contents of the sporangium develop into a non-motile aplanospore (Lee 2008). The aquatic species produce aplanospore rarely. The aplanospores develop at the ends of short laterals or terminal branches in aplanosporangia, a club-shaped body which remains separated from the rest of the thallus with the help of a septum. A septum separates the terminal aplanosporangia from the branch. Single rounded thin-walled aplanospore simply droops out from the aplanosporangia by the irregular rupture of the sporangial wall. After liberation, germination of aplanospores in *Vaucheria longicaulis* proceeds through three stages of development. Stage I begins with the initiation of germination which lasts normally 2 h. At this time the rate of germinating filaments is very high (266 ± 12 μmh^{-1}). A sharp decline in the growth rate of germinating filaments (96 ± 4 μmh^{-1}) is a sign of starting stage II which lasts 4 h. During the next 4 h (stage III), filament germinate by a higher growth rate (168 ± 8 μmh^{-1}). Growth rates stabilise and remain unchanged during subsequent development (Oliveira 1992; Oliveira and Fitch 1988).

Akinetes: Extreme desiccation or low temperature can produce akinetes in some aquatic and terrestrial species of *Vaucheria*. In this condition the branched filaments divide into a row of short segments by thick, gelatinous cross-walls. These resting, multinucleate, thick-walled segments (loaded with oil) are known as the cysts, hypnospores or akinetes. These structures are produced in dichotomous branches in rows and also known as **Gongrosira stage** because it resembles a green algae *Gongrosira*. On returning of favourable condition these cysts can germinates either into new filaments or sometimes the protoplast of the akinete divides into small protoplasmic pieces. These pieces liberates through a pore in a cyst wall and move for some time before come to rest. It then secretes cell wall around it and develops into a new filament (Chapman 1962).

1.11.8 Sexual Reproduction

Vaucheria is usually homothallic with oogamous type sexual reproduction. The life cycle in *Vaucheria* is diplontic with predominant diploid phase (Fig. 7). Most of the species are monoecious (homothallic), e.g. all the freshwater or terrestrial

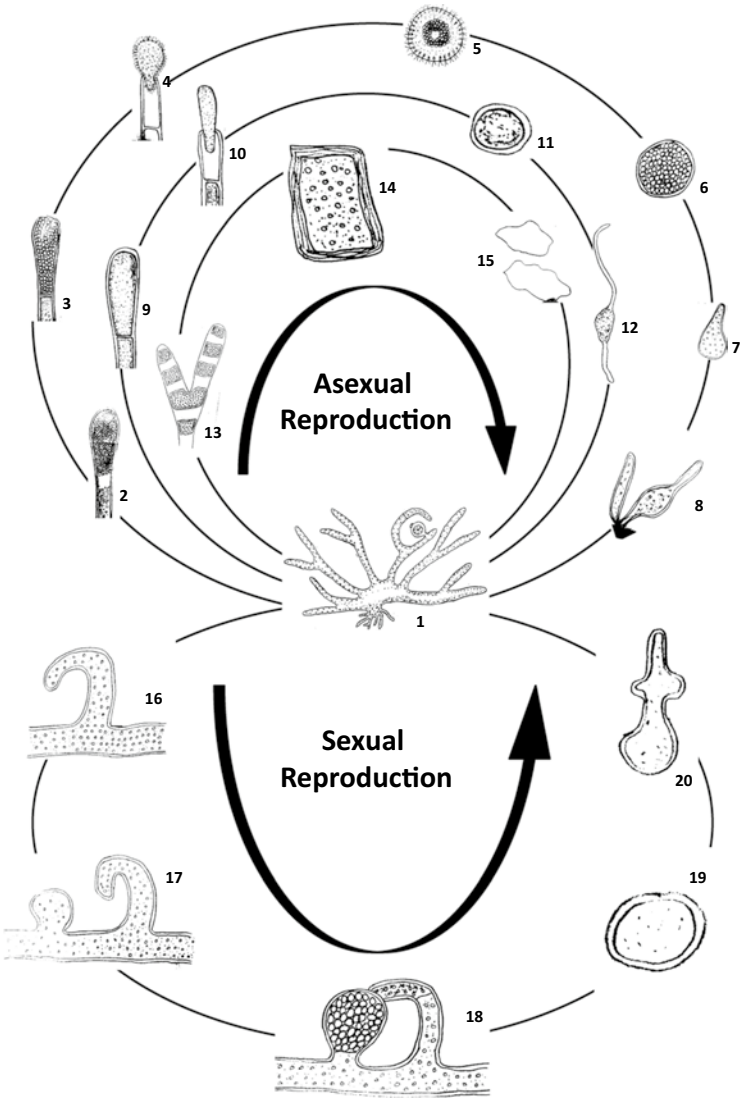


Fig. 7 *Vaucheria*: life cycle: *1 Vaucheria* vegetative thallus, *2–3* Development of zoosporangium, *4* Liberation of zoospore, *5* Flagellated zoospores (synzoospore), *6* A deflagellated zoospore, *7–8* Germination of zoospore, *9* Development of aplanosporangia, *10* liberation of aplanospore, *11* Released aplanospore, *12* Germination of aplanospore, *13* Filament with chain of akinetes (Gongrosira stage), *14* A single akinete, *15* Germination of amoeboid cysts, *16* Development of antheridia, *17* Development of oogonia *18* Antheridia and oogonia comes closer for fertilization, *19* Liberated single zygote and *20* Germination of zygote

forms. *V. dichotoma*, *V. mayganadensis* and *V. litorea*, which are marine, are usually dioecious (heterothallic). The male and female gametes differ greatly in size, form and structure. The male sex organ is called anthridium, and the female sex organ as oogonium.

Oogamous sexual reproduction is easily observed in *Vaucheria* because of the conspicuous nature of the antheridia and oogonia (Bold and Wynne 1985). In the monoecious or homothallic species, the two sex organs usually occur close to one another at intervals along the same filament, arising as a lateral outgrowth (**Sessile type**). Species like *V. geminata and V. terrestris*, the sex organs develop on special side branches (**Geminata type**). The side branch consists of a pedicel, either with a terminal antheridium and a number of lateral oogonia (*V. hamata*), or a terminal oogonium with an anthridium located laterally below it. I dioecious or heterothallic species both sex organs occurs on different plants.

Antheridia of *Vaucheria* show significant variation, and many subsections that have been established within the genus are distinguished on the basis of antheridia (Bold and Wynne 1985). The mature anthridium is a cylindrical, slender and tubular structure. Antheridium usually curved, like a horn, but in some species it may be straight as in *V. aversa*. In some species the mature anthridium (e.g. *V. thureti*, *V. piloboloids*) is reported to be colourless, but in some others species it is green. Usually it is placed high up on a branch and is separated by a septum. The young antheridium contains cytoplasm, nuclei and chloroplast. The mature antheridium has the spermatozoids produced in a specific area between the central and peripheral cytoplasm. Male gametes can liberate through a single aperture or through many apertures. A Small liberated gamete or sperm is an oval, spindle or pear-shaped and colourless structure with two laterally inserted and opposite flagella of unequal length. The short one points forwards, and the long one points backwards. The nucleus is elongated and wormlike, as are the three or four mitochondria. There is neither a chloroplast nor an eyespot, but there is a Golgi body near the basal bodies of the flagella. The proboscis consists of eight or nine microtubules running beneath the plasmalemma with vesicles in between the microtubules (Lee 2008).

The sessile (or sometime sub-sessile) oogonium of *Vaucheria* is a spherical or ovoid structure. It is separated from the supporting filament by a cross wall at its base, and develops a short rounded beak at the tip towards maturity. The oogonium also initially multinucleate (also known as wanderplasm), but all the nuclei except one migrate out from the differentiating oogonium into the subtending filament (Bold and Wynne 1985). The mature oogonium contains a single large nucleus located in the centre, and numerous chromatophores and then protoplasmic contents round off to form a single, ovum or egg. The mature oogonium produces a beak, the tip of which gelatinises, forming an aperture. Reserve food is stored in the form of oil droplets.

Fertilization is accomplished by the spermatozoids fusing with the egg protoplasm through the aperture in the oogonium. The antheridia and oogonia situated close together may dehisce almost same time, or either of them may open from a few minutes to 2 h before the other dehisces. At the tip of the oogonium a pore is formed, where from colourless cytoplasm oozes out through the apical aperture. At this stage, the chromatophores and oil droplets move to the centre of the ovum which is surrounded by a colourless layer of lining cytoplasm. Several sperms emit-

ted from the apical pore in the wall of anthridium gather around it. During fertilization, several spermatozoids enter the oogonium through the aperture formed at the beak region, but only one of them fuses with ovum. The male and female nuclei do not fuse immediately. The small male nucleus lies near the female nucleus and increases in size until it has swollen to nearly the same volume before it fuses with the female nucleus. After fertilization, the aperture of the oogonium is plugged. Zygote is green in colour initially but it gradually turns red due to degeneration of chlorophyll. The zygote develops a zygospore after secreting several layers thick wall around itself. When the original wall gets decayed, the zygospore gets liberated. It then undergoes a period of rest (Prescott 1969). The chloroplast disappears. The resting zygote contains a number of reddish or brownish bodies. Without formation of meiospores, after the dormant period, the zygospore germinates directly into a new filament. The thick zygospore wall ruptures and through the split portion a colourless germ-tube, or a lateral outgrowth of the germ tube, emerges.

1.12 Botrydium*: Life Cycle Study*

1.12.1 Systematic Position

Fritch (1935)	Prescott (1969)	Bold and Wynne (1978)	Lee (2008)
Division – Algae	Phylum-Chrysophyta	Division-Chrysophyta	Group- Eukaryota
Class- Xanthophyceae	Subphylum-Xanthophyceae	Class-Xanthophyceae	Phylum-Heterokontophyta
Order-Heterosiphonales	Order-Heterosiphonales	Order-Vaucheriales	Class- Xanthophyceae
Family-Botrydiaceae	Family-Botrydiaceae	Family-Botrydiaceae	Order- Botrydiales
Genus-*Botrydium*	Genus-*Botrydium*	Genus-*Botrydium*	Family-Botrydiaceae
			Genus-*Botrydium*

1.12.2 Occurrence

Botrydium is a widely distributed terrestrial alga found frequently on muddy or damp soils near the bank of temporary or permanent pools, ponds and stream. The vesicles like part of the *Botrydium* grow above the soil, whereas the underground portion is produced in the form of rhizoidal structure. *B. granulatum* the most common species of *Botrydium*, is often confused with *Protosiphon* as both are associated on areas of drying mud (Chapman 1962). There are 13 species (and infraspecific) names in the database at present, of which 11 have been currently accepted taxonomically (Guiry and Guiry 2015).

1.12.3 Thallus Morphology and Ultrastructure

Botrydium and *Vaucheria* are exception to the general case that tribophyceans are microscopic. Thalli are composed of green vesicles which may be globose, spherical or pear-shaped. Individual vesicle may reach several millimetres in size and thus be visible to the unaided eyes (Graham et al. 2009). However, the shape may vary with the environmental changes e.g. it becomes cylindrical when grows in shade. Sometimes the vesicles which lie on the surface of mud are dichotomously branched. A vesicle is well rooted in mud by colourless and branched rhizoids (Fig. 8). Rhizoidal portion is usually colourless. According to some, the vesicles are incrusted with calcium carbonate. The whole plant body is without septa and there is a large continuous central vacuole with a vacuolar sap which extends throughout the thallus. i.e. the vesicle and rhizoids. Cell wall is mainly composed of cellulose. There is a thin, delicate lining layer of cytoplasm just below the cell wall and it incorporates a large number of small nuclei and chloroplast (Lee 2008). The cytoplasm occupies a thin peripheral layer and contains many plastids and nuclei. Hence the body is described as **coenocytic** and **siphonous** (multinucleate and tubelike, without cross-wall). The cytoplasm also includes numerous discoid, lenticular or fusiform chromatophores in one or more layers in the peripheral region of the above ground vesicle. Chromatophores are often connected to each other by cytoplasmic strands. Oil and leucosin accumulate as product of photosynthesis. Pyrenoids like bodies, occurring in the chromatophores of young plants, have been considered to be central thickened parts of chromatophores by some phycologist.

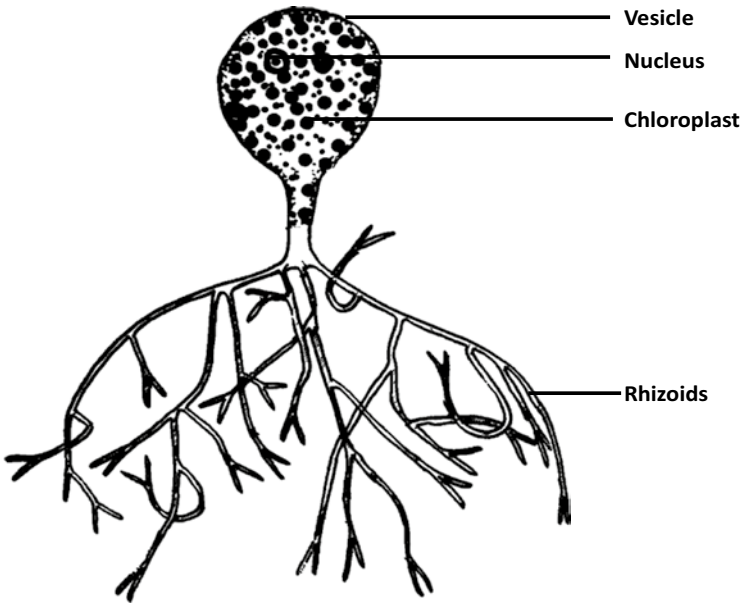


Fig. 8 ***Botrydium***: Vegetative morphology

1.12.4 Reproduction

The life cycle of *Botrydium* involves a haploid multicellular vegetative phase and a diploid unicellular zygote (Fig. 9). *Botrydium* can reproduce by vegetative reproduction or asexual reproduction or sexual reproduction.

Vegetative: It is rare and may be brought about the budding of mature vesicles.

Asexual: Mainly it takes place by means of biflagellate zoospores, or by the formation of aplanospores or hypnospores. The zoospores are produce in very large numbers in the vesicle during favourable condition such as high humidity and free water. During their formation the vesicular coenocytic protoplast fragments into uninucleate parts, each parts develop into a pyriform biflagellate zoospore. Each zoospore has an oval body with a pair of unequal flagella at the anterior end, amongst

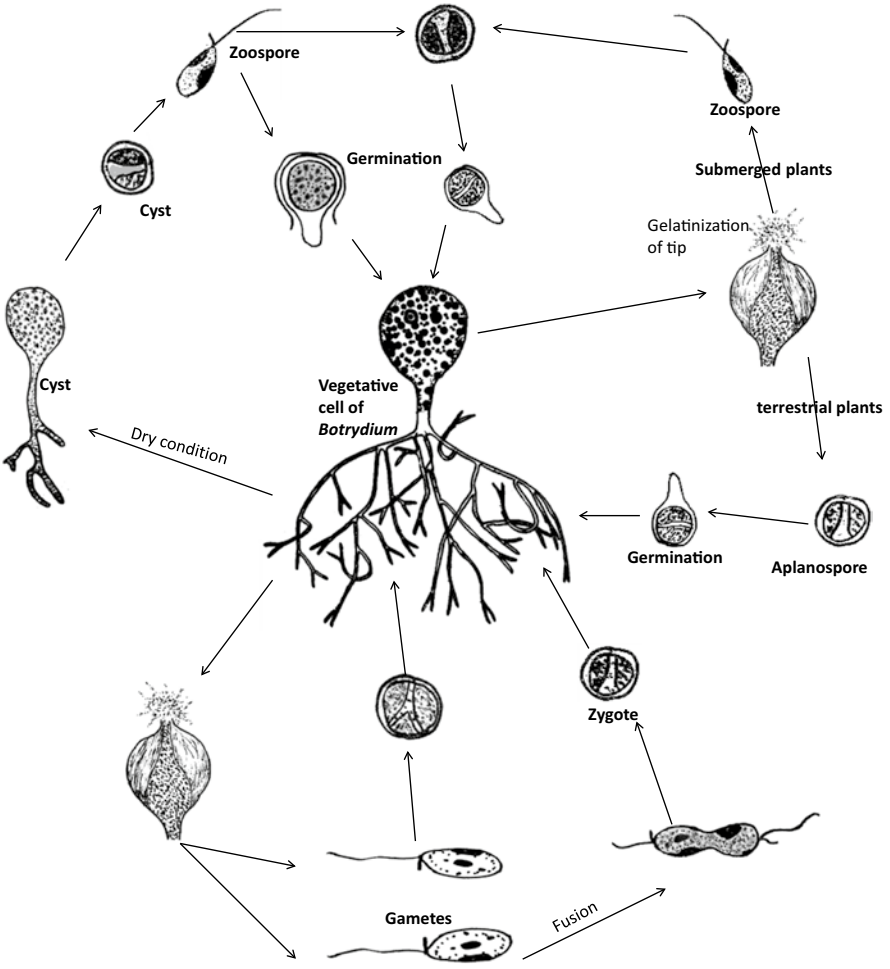


Fig. 9 The life cycle of *Botrydium* (Adapted from Lee et al. 2008)

them longer being pantonematic and the shorter acronematic. Generally the zoospores differentiate directly into vegetative thalli. Sometimes their behaviour is facultative and they may also act as gametes. Under certain conditions, uninucleate or multinucleate aplanospores are formed instead of zoo spores. During adverse environmental condition when water is less abundant, aplanospores transform into uninucleate or multinucleate hypanospores (Graham et al. 2009). In the formation of these spores, three to five chloroplasts become associated with a nucleus in the mother cell, and cleavage occurs with each zoospore containing the above organelles. If the spore is an aplanospore, then a wall is secreted; if it is a zoospore, no wall is formed. Both uninucleate and multinucleate aplanospores germinate directly into new thalli. On the other hand, the uninucleate hypanospores behave like aplanospores in respect of germination but the multinucleate hypanospores produce uninucleate aplanospores or zoospores which then give rise to new thalli. *Botrydium* also produces cysts or resting spores during periods of dry conditions (Lee 2008). The cyst may develop either singly or in large numbers. Singly developed cysts are also called the *macrocysts*. The cysts develop in the aerial parts of the alga. Under certain conditions, the cysts develop even in the rhizoids as in *B. granulatum*. Such cysts are called the *rhizocysts* (Rattan 1977). These rhizocysts are globose or oval in shape and develop serially. During the development of rhizodal cysts, the cytoplasm of the vesicle migrates into the rhoizoids, where cysts formation take place. After tiding over unfavourable condition, the cysts germinate either directly or through zoospores and give rise to new plants (Lee 2008; Rattan 1977).

Sexual: Species of *Botrydium* may be dioecious or monocious. Biflagellate zoogametes, morphologically identical with zoospores, are formed in the same way as the zoospores. The fusing gametes may be similar or dissimilar and accordingly the sexuality is either isogamous or anisogamous with the cells being either homothallic or heterothallic (Lee 2008). Gametes are liberated by gelatinisation of tip of the vesicle. On liberation, they pair together by their anterior ends, unite laterally and form a rounded or globose zygote. At the time of germination, the zygotic nucleus (diploid) undergoes meiosis. Normally four to eight haploid biflagellate zoospores are produced from a germinating zygote and each of these gives rise to a new plant.

2 Euglenophyceae

2.1 Introduction

Euglenoids are among the most ancient lineage of eukaryotic algae (Graham et al. 2009). Euglenoids are green (rarely colourless), solitary unicells (except a pseudocolonial genus *Colacium*). These swimming cells inhabit fresh water almost entirely, though a few are found in brackish or marine habitat (Prescott 1969). The chlorophyllus members of the Euglenophyceae share with the Chlorophyceae the presence

of chlorophyll *a* and *b* in their chloroplast. Other than this, they differ in many respects from green algae in cellular organisation and biochemistry. Euglenophytes store their excess photosynthate as **paramylon**, a β-1, 3 polymer of glucosen that is present in the cytoplasm and not in the chloroplast like the α-1,4- linked polymer of glucose, the starch of the Chlorophytes (Bold and Wynne 1985). Many euglenoids lack plastids and shows variable nutritional types. Non photosynthetic euglenoids feed upon organic particles such as bacteria or unicellular eukaryotes or absorb dissolved organic compounds, and all require vitamins (Graham and Wilcox 2000; Graham et al. 2009). Following are some identifying features for euglenoids

- Presence of chlorophylls *a* and *b*,
- One membrane of chloroplast endoplasmic reticulum,
- A mesokaryotic nucleus,
- Flagella with fibrillar hairs in one row,
- No sexual reproduction,
- Cytoplasm contain Paramylon or chrysolaminarin as the main storage product.

2.2 Classification

There are more than 40 genera and 800–1000 species of euglenoids (Graham et al. 2009; Hoek et al. 2009). Fritsch (1935) divided the class into three families:

1. Euglenaceae,
2. Astasiaceae
3. Peranemaceae

Smith (1955) and Prescott (1969) kept class/subphylum Euglenophyceae under division/phylum Euglenophyta. Prescott (1969) further divided it into two orders

1. **Euglenales**: Majority of the euglenoids included in this order. Most have chloroplast but there are few colourless genera too; almost all genera have pigment spots. Flagella vary in number from 1 to 3 which can be acronematic or pleuronematic. Most of the representatives are free living, some are attached and one genus ***Euglenomorpha*** occurs in the digestive tract of the frog.
2. **Colaciales**

Bold and Wynne (1985), Lee (2008) kept class Euglenophyceae under division Euglenophyta. Bold and Wynne (1985) and Lee (2008) described three orders

1. **Eutreptiales**: Two emergent flagella, one directed anteriorly and the other laterally or posteriorly during swimming; no special ingestion organelle, very active euglenoid movement.
2. **Euglenales**: Two unequal flagella (out of which only one emerge from the reservoir and canal, very active euglenoid movement).

3. **Heteronematales**: colourless, phagotrophic, lack eyespots, two emergent flagella, the longer flagellum directed anteriorly and the shorter one directed posteriorly during swimming; special ingestion organelle present.

Hoek et al. (2009) divided this class into six orders.

1. **Eutreptiales**
2. **Euglenales**
3. **Euglenomorphales**
4. **Rhabdomonadales**
5. **Sphenomonadales**
6. **Heteronematales**

Only the first three orders contain both green and colourless species; the remaining orders consist entirely of heterotrophic organisms.

2.3 Occurrence

In nature members of this class tend to grow in small pools or ditches that have been enriched with organic compounds (Hoek et al. 2009). Euglenophytes are widely distributed, occurring in fresh water, brackish and marine waters and also on moist and mud. They are often abundant and may form water blooms in ponds, marshes, swamps, fens, bogs, tanks and puddles, especially those to which livestock have access, and this may be correlated with the facultatively heterotrophic existence of some of them (Lee 2008; Bold and Wynne 1985). Their nutritional diversity also explains how euglenoids can ecologically link components of aquatic microbial food web. Elsewhere, euglenoids can form floating green scum on water bodies or mud and sandflats estuaries. The green colour disappears in full sunlight as the euglenoids creep away from the surface. Some members of euglenoids are also parasitic e.g. *Khawkinea*, *Euglenamorpha*, and *Hegneria*. Usually rivers, lakes, and reservoirs (larger bodies of purer water) have sparser populations of less common euglenoids as planktonic organisms. Marine euglenoids are comparatively more common. These occur in the open sea, in tidal zones among seaweeds, and as sand inhabitants on beaches. Species of *Eutreptia*, *Eutreptiella*, and *Klebsiella* occur exclusively in marine or brackish water (Lee 2008).

2.4 Pigment

Euglenophyceae is the only other class which has the same chlorophyll as the Chlorophyceae (chlorophyll *a* and *b*). Members of Euglenophyceae also contain *β*-carotene, antheroxanthin, astaxanthin, and neoxanthin (not in all genera). The

pigments are contained mostly in oval plates or discs (which may be star shaped or ribbon shaped). In some representatives of Euglenophyceae chloroplast is diffused and not definitely organised. Pyrenoids may be present either in the cytoplasm or on the face of the chloroplast.

2.5 Cell Structure

The great majority of the euglenoids are unicellular flagellates. They are monads. A few, however, have stages during which the cells are enclosed within a mucilage capsule (Hoek et al. 2009). Euglenoids are cylindrical or fusiform, flattened or round in cross sections. Some genus, especially *Phacus* has flattened, disc like cells which, however, are sometimes somewhat triangular in cross section because of longitudinally flange (Prescott 1969). There is no true cell wall; instead, euglenoids cells are bounded by plasmalemma exteriorly and just within it by a proteinaceous layer of periplast (*pellicle*) which is made up of 80 % protein and rest lipids and carbohydrates. Pellicle has four main components: the plasma membrane, repeating proteinaceous units called strips, subtending microtubules, and tubular cisternae of endoplasmic reticulum. Some euglenoids have a flexible pellicle that allows the cells to undergo a flowing movement known as **euglenoid movement** (Lee 2008). Some euglenoids can change their shape such as *Euglena gracilis* changes its shape two times per day when grown under the synchronising effect of a daily light-dark cycle (Lee 2008). The periplast or pellicle is often spirally striated, punctate, or granulate. Thus euglenoids are naked except in those genera in which a **lorica** (which has an aperture through which the single flagellum extends) is present such as in *Trachelomonas* and *Strombomonas* (Prescott 1969; Bold and Wynne 1985). In addition, the cells are often surrounded by a thin layer of mucilage, which is secreted by muciferous bodies situated beneath the pellicle. These bodies are in communication with the exterior of the cell by canals through which mucilage may be extruded (Bold and Wynne 1985). Mucilage production by the Golgi bodies can result in the formation of palmelloid or encapsulated stages. When euglenoids have two flagella one may be nonemergent from the anterior invagination, which consists of a canal and a reservoir (Hoek et al. 2009). The canal is a rigid structure, whereas the reservoir easily changes shape and is regularly distorted by the discharge of the contractile vacuole. The rigidity of the canal is maintained by microtubules that form a flat helix around the canal, in much the same position as hoops on a barrel. The reservoirs are the only parts of the cell covered solely by the plasmalemma (Lee 2008). Euglenoids flagella are rather coarse as compared with those of Chlorophyceae. They have usual (9 + 2) arrangement of microtubules and in addition a paraflagellar rod. One or many rows of fine hairs may present on the flagella emerge from basal bodies located just beneath the reservoir (Bold and Wynne 1985; Prescott 1969). There are two basic types of flagellar movement in the class. The first group (including the Eutreptiales and Euglenales) has the flagellum continually motile from base

to apex, resulting in cell gyration with the anterior end of the cell tracing a wide circle. The second group (including *Peranema*, *Entosiphon*, and *Sphenom onas*) has the flagellum held out straight in front of the cell with just the tip motile, resulting in smooth swimming or gliding locomotion in contact with the substratum or air–water interface (Lee 2008). The orange-red eyespot or stigma occurs in the colourless cytoplasm in the anterior of green euglenoids cells (Fig. 10). Unlike other algae in which it is located within the chloroplast, the stigma lies about the level of the flagellar swelling. Similar eyespots are found in some Dinophyceae (Hoek et al. 2009). Chloroplasts vary in forms among the different species and genera. They may be small simple discs; large and plate like with entire or dissected margins; or ribbon like and arranged in stellate fashion. Unlike Chlorophycean the euglenoids chloroplasts are surrounded by two membranes of the chloroplast envelope plus one

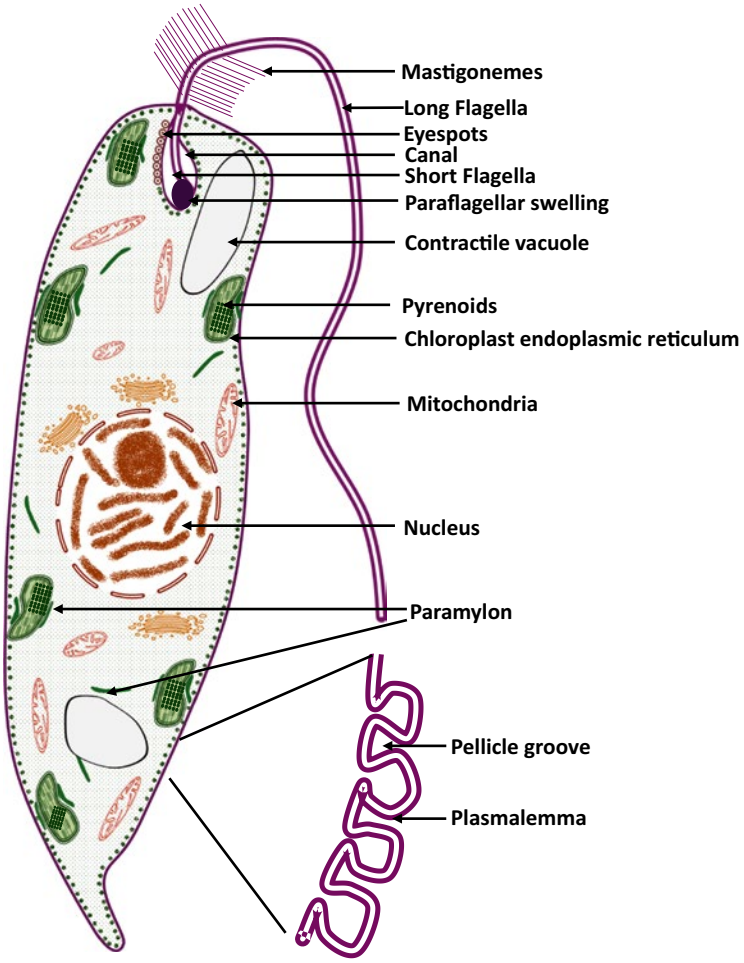


Fig. 10 A diagrammatic drawing of the fine structure of the euglenoid cell

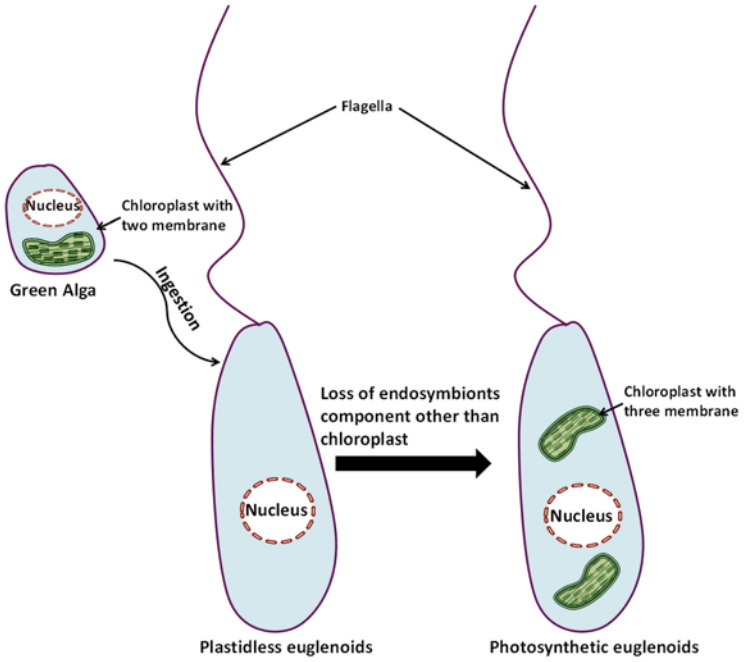


Fig. 11 A diagrammatic drawing secondary endosymbiosis in euglenoids

membrane of chloroplast endoplasmic reticulum; the latter membrane is not continuous with the nuclear membrane (Marin 2004; Lee 2008). It has been suggested that this type of chloroplast are derived from symbiotic green algae (Fig. 11). Phylogenetic analysis indicates that euglenoids plastids did not arise from the same type of green alga that donated its plastid to chlorarachinophytes (Graham and Wilcox 2000; Graham et al. 2009). A contractile vacuole lies at the anterior on the euglenoids. Vacuole has an osmoregulatory function, expelling excessive water taken into the cell (in every 15–20 s). Mitochondria are of typical algal type.

Colourless euglenoids always have more mitochondria than do equivalent-sized green ones (Lee 2008). The nucleus is often readily visible in living individuals in the centre or posterior of the cell. Like Dinoflagellates the euglenoids nucleus is of the mesokaryotic type, having chromosomes that are permanently condensed during the mitotic cycle, a nucleolus (endosome) that does not disperse during nuclear division, no microtubules from chromosomes to pole spindles, and a nuclear envelope that is intact during nuclear division (Lee 2008). The chromosome number is usually high, and polyploidy probably occurs in some genera (Lee 2008). The chloroplast DNA occurs as a fine skein of timy granules, which extends through the whole chloroplast (Hoek et al. 2009).

2.6 Phototaxis and Euglenoids

Euglenoids are positively phototactic to light of low intensity and negatively phototactic with respect to bright light and darkness because of these eyespots. *Euglena* bleached of its chlorophyll but retaining its eyespot and photoreceptor (paraflagellar swelling) is still positively phototactic, eliminating chlorophyll and chloroplasts in the phototaxis directly. A *Euglena* bleached of all pigments but retaining its photoreceptor is negatively phototactic; this rules out the possibility that the carotenoids of the eyespot are directly stimulatory in phototaxis. A *Euglena* lacking a photoreceptor and all pigments is no longer phototactic. There is a circadian rhythm in phototaxis in *Euglena*, with phototaxis operative during the light period and not operative during the dark period. Even if light is introduced during the normal dark period, the *Euglena* cell does not respond phototactically (Lee 2008).

2.7 Evolutionary History and Mode of Nutrition

In 1674 a Dutch cloth merchant Antonie van Leeuwenhoek wrote to a friend about his discovery of a tiny wriggling creature, that was "green in the middle and at either ebd white". Today we know this organism *Euglena virdis*, the first described microscopic protists (Triemer and Farmer 2007). Yet in 300+ years since it discovery, *Euglena* and its relatives remain largely unknown. Euglenoids variously claimed by both protozoologist and phycologist, they have long fascinated biologist with "animal like" fluidity as well as unique ability to carry out photosynthesis. Morphological studies in last century, and nature of plastid which can be acquired through secondary symbiosis, transformed our thinking about their phylogenetic affinities and their evolutionary history (Triemer and Farmer 2007). *Moyeria* is considered as most ancient fossil euglenoids (about 410–460 million years old). *Phacus* and *Trachelomonas* are similar to fossils produced in the Tertiary, beginning about 60 million years ago. Euglenoids share several structural features with two other groups of unicellular flagellates: **Diplonemids and kinetoplastids** (Table 3, Fig. 12). Kinetoplastids are of great medical importance because of parasite such as *Trypanosoma* which cuse sleeping sickness in Africa and Chagas disease in Latin America. Diplonemids are heterotrophic flagellates that primarily consume particles, though some can be parasitic (Graham et al. 2009).

In spite of having chlorophyll *a* and *b* green euglenoids are not absolutely photoautotrophic but are photoauxotrophic because they require one or more vitamins in culture (Bold and Wynne 1985). The Euglenophyceae have a number of modes of nutrition, depending on the species involved. Comparative study of modern euglenoids reveals that cell structure and behaviour have evolved together with changes in mode of nutrition. The earliest euglenoids likely consumed bacteria and give rise both to a lineage of osmotrophs and a lineage that was able to consume eukaryotic prey cells. The latter group considered as ancestral o plastid-bearing euglenoids,

Table 3 Comparison of euglenoids with diplonemids and kinetoplastids (Graham et al. 2009)

Features	Groups		
Flagella emerge from a cellular depression (pocket or ampulla)	Euglenoids	Diplonemids	Kinetoplastids
Reproduction mainly by longitudinal mitotic division	Euglenoids	Diplonemids	Kinetoplastids
Paraxonemal rod or paraflagellar rod stiffens the hair-covered flagella	Euglenoids	and	Kinetoplastids
Disk or paddle shaped mitochondrial cristae	Euglenoids	and	Kinetoplastids
The reserve polysaccharides is **paramylon** (a β-1,3 linked glucan)	Euglenoids only		
Surface structure or pellicle	Euglenoids only		

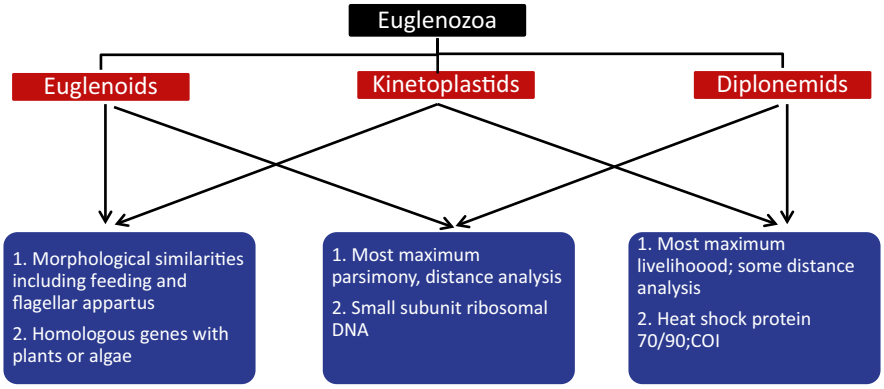


Fig. 12 Three possible phylogenetic relationship among euglenozoan groups showing sister taxa relationship between euglenoids, kinetoplastids and Diplonemids (Courtesy Triemer and Farmer 2007)

some of which subsequently lost photosynthetic pigments but retained their plastids. Graham et al. (2009) divided euglenoids in following groups on the basis of nutrition.

- Early bacteria-consuming euglenoids e.g. *Petalomonas* and *Notosolenus*
- Plastid less, osmotrophic euglenoids e.g. *Distigma*, *Rhabdospira*, *Rhabdomonas*, *Monoidium*, *Gyropaigne* and *Paramidium*
- Euglenoids that consume eukaryotic prey e.g. *Entosiphon*, *Paranema*,
- Plastid-bearing euglenoids e.g. *Euglena*

Most of the phylogenetic molecular analysis revealed that Euglenoid is a distinct group of organisms with uncertain affinities to other protists, but because of their cell morphology, gene composition and ecological diversity, the euglenoids remains

as one of the most interesting and fascinating groups of organism. With most of the major genera now in place on the phylogenetic tree, the future will focus on sub-generic problems.

2.8 Reproduction

Sexual reproduction does not occur in euglenoids. Asexual reproduction occurs by longitudinal division, proceeding from apex to base (Morris 1971). During this process cytoplasm becomes two headed. Prior to mitosis the nucleus migrates to the region just below the flagellar pocket. During mitosis nuclear membrane remain unchanged as in some other protists, animals and land plants (Fig. 13). **Cysts** are formed by euglenoids as a means of surviving unfavourable periods. The cell rounds off and secretes a thick sheath of mucilage that survives for months until the cell emerges by cracking the cyst. Cysts production is triggered by low nutrient levels or by low nitrogen to phosphorous ratio (Graham and Wilcox 2000; Graham et al. 2009). The formation of cysts involves loss of flagella, increase in the number of paramylon granules, swelling and rounding of the cells, and deposition of a layered mucilaginous enclosure that consist primarily of polysaccharides.

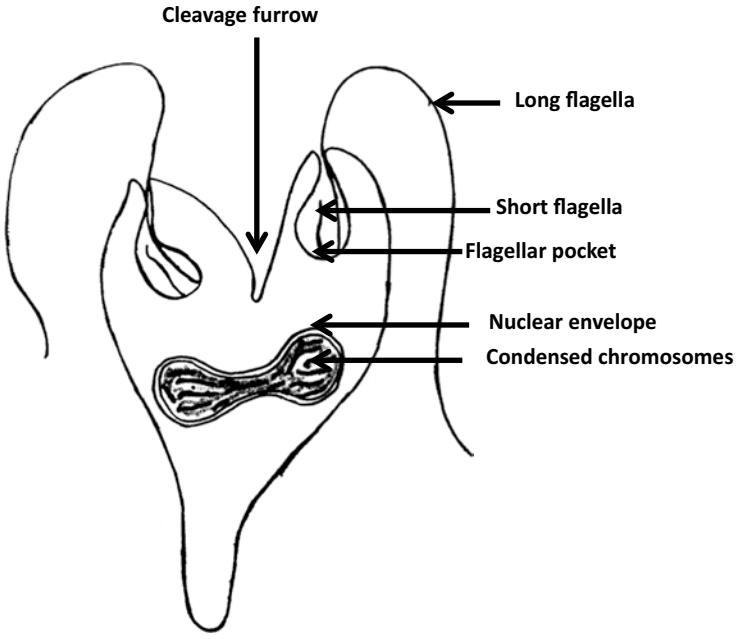


Fig. 13 Closed mitosis in euglenoids-i.e. the nuclear envelope remains intact (Adopted from Graham et al. 2009)

3 Dinophyceae

3.1 Introduction

The term dinoflagellates originate from the Greek word *dineo*, meaning "to whirl" (Graham et al. 2009). Dinoflagellates are a diverse assemblage of biflagellate unicellular organism, which constitute important components of marine, brackish, and freshwater bodies. In general, cells are golden-brown but some are blue, and a few marine genera are chlorophylless but have various pigments in solution (Prescott 1969). Majority of dinoflagellates are motile (Chapman 1962; Morris 1971). Nonmotile forms of dinoflagellates are an example of their diverse adaptation to their habitats. Beside photosynthesis, saprophytic, parasitic, symbiotic and holozoic patterns all are being represented by members of dinoflagellates (Bold and Wynne 1985). Main features of this class are as follow:

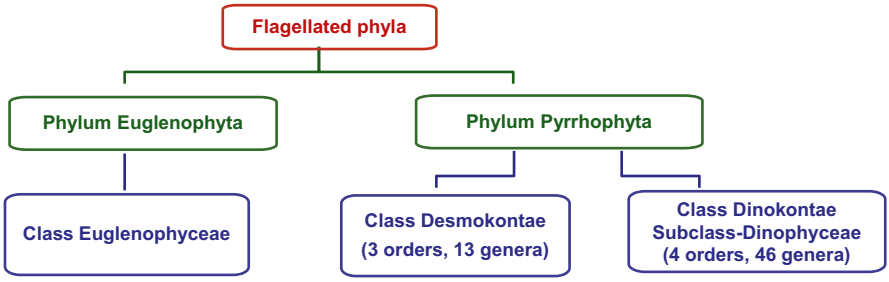
- Presence of chlorophyll *a* and *c*
- Presence of β-carotene and xanthophylls
- Starch as reserve food
- Theca as cell wall which is primarily composed of cellulose
- Distinct type of nucleus (combination of prokaryotic and eukaryotic characteristics)
- Unequal heterodynamic flagella

3.2 Classification

Dinoflagellate research has a 225-years-old history (Moestrup and Daugbjerg 2007), but the classification of dinoflagellates is continually in a state of flux. Some of the complexities are due to the fact that both zoologist and botanist have constructed classification schemes and different criteria have been used to group and to separate the numerous species and genera. The current revolution in dinoflagellate taxonomy, classification and phylogeny is outcome of a combination of molecular studies and high resolution electron microscopy, including reconstruction of the cell ultrastructure based on serial sectioning (Moestrup and Daugbjerg 2007). At present more than 2000 living and 2000 fossil species of dinoflagellates are known, belonging to about 130 genera (Hoek et al. 2009). Initially Fritch (1935) divided the class Dinophyceae into following six orders:

1. **Desmomonadales**
2. **Thecatales**
3. **Dinophysiales**
4. **Dinoflagellata**
5. **Dinococcales**
6. **Dinotrichales**

Altogether a different classification was used for dinoflagellates by Prescott (1969) and Smith (1955) as summarised here.



Bold and Wynne (1985) kept class Dinophyceae along with Ebriophyceae, Ellobiophyceae, Syndiniophyceae and Desmophyceae in division Pyrrhophyta. He further described six orders in class Dinophyceae.

1. **Blastodiniales**: Attached parasitic forms on invertebrates and fishes; amoeboid, coccoid or multicellular colonies; feeding trophocyte with a theca of thin plates; marine
2. **Dinamoebales**: Free living amoeboid cell, solitary, nonthecate; cyst stage with a wall, releasing gynodinioid zoospores upon germination.
3. **Dinophysiales**: Cells motile, solitary, free-living (autotrophic or heterotrophic), with a theca of 18 (rarely 19) plates, including 2 that are much larger than others; reduced epitheca.
4. **Dinocloniales**: Immobiles, filamentous forms, branched, uniseriate with a cell wall; gymnodinioid motile stage.
5. **Gloeodiniales**: Palmelloid vegetative stage of cells aggregated in mucilaginous colonies; motile cells *Hemidinium*-like, with a layer of thin thecal plates and an abbreviated cingulum.
6. **Gymnodiniales**: Cells motile, solitary, free-living; cells covering without thecal plates (but with numerous peripheral vesicles that may have contents homologous to thecal plates).

Lee (2008) and Hoek et al. (2009) divide division Dinophyta in a single class Dinophyceae. Lee (2008) divides this class into following four orders:

1. **Prorocentrales**: Cell wall divided vertically into two halves; no girdle; two flagella borne at cell apex.
2. **Dinophysiales**: Cell wall divided vertically into two halves, cells with elaborate extensions of the theca.
3. **Peridiniales**: Motile cells with an epicone and hypocone separated by a girdle, relatively thick theca.
4. **Gymnodiniales**: Motile cells with an epicone and hypocone separated by a girdle; theca thin or reduced to empty vesicles.

On the other hand Hoek et al. (2009) mentioned 8 more orders and divided this class into following 12 orders.

1. **Gymnodiniales**: e.g. *Gymnodinium micrum*, *G. splendens*, *Symbiodinium microadriaticum*, *Polykrikos schwarzii*
2. **Gloeodiniales**: e.g. *Gloeodinium montanum*
3. **Thoracosphaerales**: e.g. *Thoracosphaera heimii*
4. **Phytodiniales/Dinococcales**: e.g. *Phytodinium globosum*
5. **Dinotrichales**: e.g. *Dinoclonium conradii*
6. **Dinamoebidales**: e.g. *Stylodinium sphaera*, *Dinamoebidium varians*
7. **Noctilucales**: e.g. *Noctiluca scintillans*
8. **Blastodiniales**: e.g. *Blastodinium spinulosum*
9. **Syndiniales**:
10. **Peridiniales**: e.g. *Peridinium synctum*, *P. balticum*, *Protoperidinium*, *Ceratium hirundinella*, *Gonyaulax polyedra*
11. **Dinophysiales**: e.g. *Dinophysis*, *Triposolenia intermedia*, *Amphisolenia globifera*, *ornithocercus splendidus*, *Histioneis josephinae*
12. **Prorocentrales**: e.g. *Prorpcentrum micans*

3.3 Occurrence

Dinoflagellates is a group of unicellular or colony-forming protists among them nearly 80 % are free living, marine, planktonic, or benthic flagellate, while 20 % are from similar habitats in freshwater (Moestrup and Daugbjerg 2007; Hoek et al. 2009). Some dinoflagellates are parasitic (e.g. in copepods) or symbiotic (e.g. in corals). Estimation of total number of species is very difficult as taxonomy needs a complete revision. Approximately 50 % of the total known species are phototrophic whereas the remaining are heterotrophic (phagotrophic), free living, or parasitic (protozoa). Number of mixotrophic species is being found (Moestrup and Daugbjerg 2007). Many planktonic species bear horns, ridges or wings which often have bizarre forms Dinoflagellates are known from polar, temperate and tropical waters, but tend on the whole to be more predominant in warm water communities. Thus dinoflagellates are present throughout the year in tropics and during spring to summer in temperate region. Except some benthic exceptions the great majority of dinoflagellates are planktonic representative (Hoek et al. 2009). Although dinoflagellates are predominantly unicellular, flagellate organism the list below (Hoek et al. 2009) summarise the various types of thallus organisation found in dinoflagellates (Fig. 14).

- **Flagellate unicells**: e.g. *Peridinium*, *Ceratium*, *Gymnodinium*, *Prorocentrum*, *Gonyaulax*, *Polykrikos*, *Dinophysis*, *Triposolenia*, *Amphisolenia*, *Ornithocercus*, *Histioneis*
- **Amoeboid unicell**: e.g. *Dinamoebidium*, *Stylodinium*
- **Palmelloid (tetrasporal) colonies**: e.g. *Gloeodinium*
- **Coccoid unicell**: e.g. *Dinococcus*, *Phytodinium*
- **Filamentous organisation**: e.g. *Dinothrix*, *Dinoclonium*

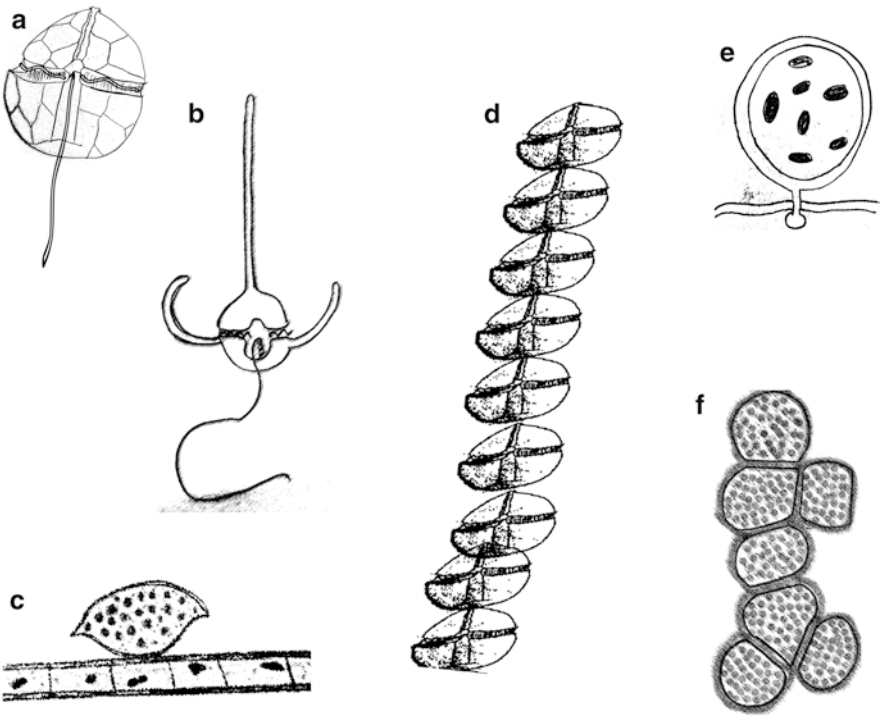


Fig. 14 Range of thallus organisation in class Dinophyceae: (**a**) *Peridinium* (**b**) *Ceratium* (**c**) *Dinococcus* as an epiphyte on *Melosira* (diatom) (**d**) *Gonyaulax* (**e**) *Stylodinium* as an epiphyte on *Oedogonium* (**f**) *Dinothrix*

3.4 Pigment

This group is characterised by presence of chlorophyll *a* and *c* (c_2); β-carotene; and Xanthophylls (peridinin, neoperidinin, dinoxanthin, neodinoxanthin, diatoxanthin etc.). Chlorophyll *b* is absent. Peridinin is the main light harvesting pigment in most photosynthetic dinoflagellates and is closely associated as water soluble peridinin-chlorophyll *a* protein complex. Peridinin capture light energy in the blue-green range of 470–550 nm, which is present in aquatic habitats and inaccessible to chlorophyll alone (Graham et al. 2009; Hoek et al. 2009).

3.5 Storage Food

Like Rhodophyceae the principal reserve food of dinoflagellates is starch which is synthesised outside the chloroplast in the form of grains. While in Chlorophycean member starch gets synthesised inside the chloroplast. Lipid is also found as reserve material in the form of globules and droplets.

3.6 Flagella

This group is considered to be the champion swimmers amongst algae due to their high rates of 200–500 μms^{-1}. However, dinoflagellates are slower than *Mesodinium* the photosynthetic symbiosis between a ciliate and a cryptophyte (Lee 2008; Graham et al. 2009). One of the chief characteristics of Dinophyceae is the presence of two flagella (Prescott 1969) which are inserted into the cell in the area of the intersection of the **girdle/cingulum** and **sulcus** (Lee 2008). An acronematic, posteriorly directed flagellum (or longitudinal flagella) is located in a longitudinally oriented groove, the sulcus, and a flattened or ribbon-like flagellum (or transverse flagella) is located in a transverse groove, the cingulum/girdle. The transverse flagellum is about two to three times as long as the longitudinal flagellum and has a helical shape (Lee 2008). Cingulum encircles the cell in the equatorial region or closer to one or other pole (Bold and Wynne 1985). On the other hand Desmophyceae have flagella originating from the anterior end (which can be divided into right and left halves). Anterior and posterior halves in naked dinoflagellates (e.g. *Gymnodinium*) also known as **epicone** and **hypocone**, whereas in armored dinoflagellates (e.g. *Peridinium*) two hales known as **epitheca** and **hypotheca** (Bold and Wynne 1985).

3.7 Cell Structure

As discussed above most dinoflagellates cells consist of two parts, an anterior or top epicone/epitheca and a posterior hypocone/hypotheca which are separated by cingulum (Fig. 15). Dinoflagellate's beautiful and ornamental covering resemble superficially with diatoms as the two groups differ in both structure and chemical composition. The cell covering in dinoflagellates (also known as **amphiesma** or armour) is a useful means of subdivision this group. In many species each alveolus contains a flat thecal plate composed of cellulose (**armored** or **thecate**), whereas in other species alveoli are devoid such content (**naked** or **unarmored**) (Graham et al. 2009). The distinction of naked and armored dinoflagellates is not absolute due to presence of some transitional types of cell covering. Basic structure of all dinoflagellate's amphiesma remain same, consisting of several layers of membrane: an outermost continuous membrane, flattened vesicles and an innermost continuous membrane sometimes interpreted to be the plasmalemma (Bold and Wynne 1985; Lee 2008). The dinoflagellates armour is divided into an upper (**apical**) and a lower (**antapical**) half, and consist of polygonal plates, which fit tightly against each other (Hoek et al. 2009). The wall of armored dinoflagellates is arranged in **thecal plates** followed by **pellicle** and plasmalemma. The number and arrangement of plates in the theca are one of the most useful criteria in the systematics of armored dinoflagellates. Although relatively rare, scales occur outside the plasma membrane in some dinoflagellates (Lee 2008).

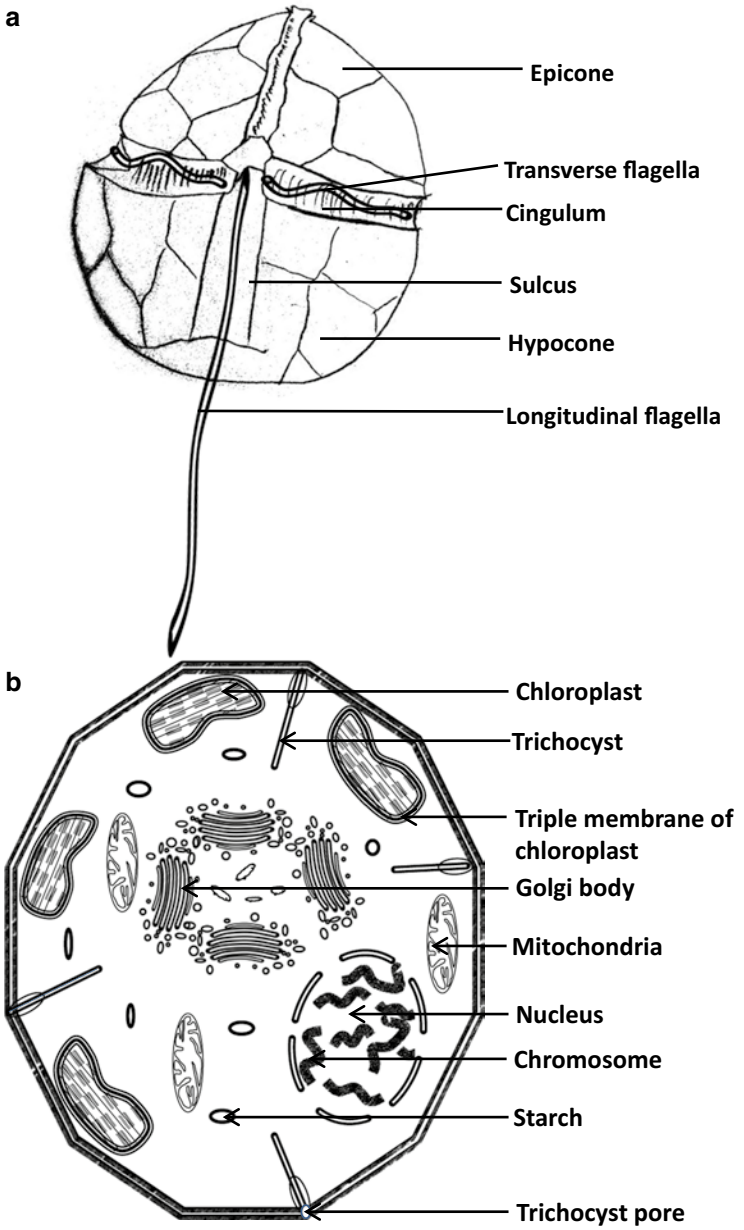


Fig. 15 Light (**a**) and electron microscopic (**b**) drawings of dinoflagellates (Adopted from Lee 2008)

The nucleus of dinoflagellates (also known as **dinokaryon** or **mesokaryon**) has many unusual properties, including the persistence of the chromosome in a condensed condition during interphase and the attachment of the chromosome to the nuclear envelope. Centriole are lacking except in some parasitic species (Bold and Wynne 1985). Similar to bacteria attachment of genetic material with membrane also noted in some members of dinoflagellates. Dinoflagellates nucleus is relatively large (~half volume of cell) which contain relatively less histone protein (with some differences to eukaryote). A dinoflagellate have much more DNA in their nuclei, ranging from 3.8 pg per nucleus in *Cryptothecodinium* cohnii to 200 pg per nucleus in *Lingulodinium polyedrum* corresponding to about 200,000 Mb (in comparison, hexploid *Triticum* wheat is 16,000 Mb and the haploid human genome is 3180 Mb) (Lee 2008). Nucleus may be spherical/U/V/Y- shaped. Number of chromosome may range 12–400. However *Syndinium* contain four chromosome due to which it normally used as a model system to study cell division in dinoflagellates. Unlike eukaryotes nuclear envelope and nucleolus are persistent throughout nuclear division (Bold and Wynne 1985; Hoek et al. 2009).

Many unusual structures in dinoflagellates have been reported such as **pusule** (similar osmoregulatory function as to that of a contractile vacuole). These structures are invagination of the plasmalemma which lies near the flagellar bases. A variety of eyespots or light sensitive organelles ranging from simple collections of carotenoid-containing lipid globules (*Woloszynskia coronate*) to the most elaborate of any light sensitive organelle also known as **ocellus** (*Nematodinium armatum*) were reported in dinoflagellates. Followings are some of the unusual features of dinoflagellates (Bold and Wynne 1985; Hoek et al. 2009):

- **Trichocysts** are rodlike, proteinaceous body and discharged into medium by a rapid hydration process. Trichocysts may be upto 100/cell. These structures are very similar to the Trichocysts of ciliate protozoa, except that the protozoan Trichocysts capped with a spine. Trichocysts develop within the Golgi apparatus and are produce within a sac. According to some experts trichocysts release cause a jet-propulsive response that is useful in escaping from predator because after discharge they become much longer and thinner. Relatively simple sacs that release mucilage to the cell exterior known as **mucocysts** (Graham et al. 2009).
- **Nematocysts or cnidocysts** (comparable structure to Coelenterata) are elaborate ejectile organelles of *Nimatodinium* and *Polykrikos*. They may be upto 8–10/cell.
- **Peduncle** an extendable organelle, composed of compact rows of microtubules and other structure, emerges at the junction of sulcus and cingulum.
- **Muciferous bodies** are present just beneath the cell membrane

3.8 Unusual Chloroplast of Dinoflagellates

Due to yellow and brown accessory pigments chloroplast normally brown in colour. The chloroplast, where present, are surrounded by three membrane, none of which is connected to the endoplasmic reticulum. However some dinoflagellates have

aberrant chloroplast which represents eukaryotic endosymbionts. Many genera are heterotrophic which lack chloroplast (Hoek et al. 2009). Thylakoids united generally in the form of stacks (lamellae) of three. Pyrenoids can be stalked or embedded within the chloroplast or partly penetrated by thylakoids.

The plastids of most photosynthetic dinoflagellates originated from a secondary endosymbiosis with a red alga (Fig. 16). These plastids are surrounded by three membranes. Out of which, two membranes are of chloroplast envelope and one membrane of chloroplast endoplasmic reticulum. There are a small number of dinoflagellates that have plastids derived from a tertiary endosymbiosis. Tertiary endosymbiosis begins with the loss of the plastid (originally derived from a secondary

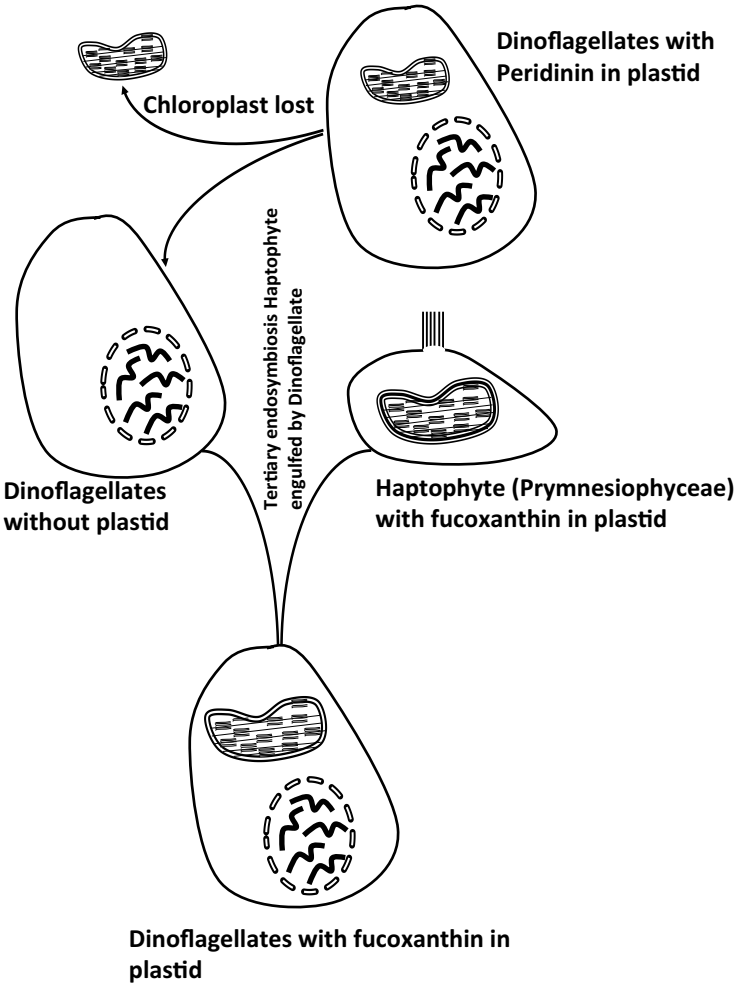


Fig. 16 The sequence of events leading to plastids evolved from a tertiary endosymbiosis (Adopted from Lee 2008)

endosymbiosis) from a dinoflagellate followed by endosymbiosis of an alga from the Prymnesiophyceae (a haptophyte alga). All of the protoplasm of the endosymbionts was lost, except for the chloroplast surrounded by one membrane of chloroplast endoplasmic reticulum, which became the permanent chloroplast of the dinoflagellate (Lee 2008).

Hoek et al. (2009) discussed various endosymbiotic origin of chloroplast in dinoflagellates such as:

- *Peridinium balticum* belong in fact to an endosymbiotic alga, which lives within the *P. balticum* cells. Each chloroplast of the endosymbionts is surrounded by endoplasmic reticulum.
- *P. balticum* also reported containing endosymbionts as vestigial green alga (due to presence of chlorophyll *a* and *b*).
- *Gymnodinium acidotum*, has also been appears to contain an endosymbionts cryptophyte.
- *Dinophysis* includes heterotrophic as well as photosynthetic species.
- *Noctiluca scintillans* were reported containing cells of parsinophycean green alga *Pedinomonas*

On the other hand Moestrup and Daugbjerg (2007) described eight types of chloroplast origin:

- **Type 1**: The peridinin-containing species
- **Type 2**: The Kareniaceae
- **Type 3**: The Dinophysiales group I.
- **Type 4**: The Dinophysiales group II.
- **Type 5**: *Lepidodinium*
- **Type 6**: *Gymnodinium aeruginosum*
- **Type 7**: *Durinskial/Krypthecodinium*
- **Type 8**: *Podolampas*

A number of dinoflagellates contain short-term plastids stolen from their food source (kleptoplastids) and it is sometimes difficult to distinguish between these plastids and permanent plastids. Dinoflagellates have freedom advantage to replace the original plastid with a new one because they have transferred most of the plastid genome to the nucleus of the cell, making them the only eukaryotes that encode the majority of the plastid genes in the nucleus (Lee 2008).

3.9 Reproduction

Dinoflagellates that spend most of their life cycle as unicellular flagellates, cell division are the most common mode of reproduction (Bold and Wynne 1985; Graham and Wilcox 2000; Graham et al. 2009). Cell division in dinoflagellates may be of longitudinal type or transverse type or simply oblique bipartitioning types. Bold and Wynne (1985) discussed three different modes of division:

- In naked dinoflagellates pinch apart by a process of constriction and meanwhile (during separation) a new amphiesma formed around each product
- In Armored dinoflagellates division of the parental theca may be shed in a process of **ecdysis**
- Splitting of the parental theca into two portions, each product of division retaining one half of the parental theca and synthesise the missing portion

Dinothrix also release zoospore which resemble *Gymnodinium*. In some motile members the cells are held together in loose chains e.g. species of *Ceratium* and *Gonyaulax* (Fig. 14d).

Although sexual reproduction has been studied in relatively few dinoflagellates, it is thought to occur much more widely than actually observed (Graham et al. 2009). As homothallic and heterothallic examples have been reported isogamous and anisogamous both types of sexual reproduction occur in dinoflagellates, although isogamous is more common. Different species have different mode of reproduction such as:

- In *Ceratium* (haploid and haplobiontic), meiosis occurring in marine species at variable times after a protected growth phase of nonrusting **planozygote**. Whereas a resting cyst phase occur in freshwater representative (Bold and Wynne 1985)
- Nitrogen deficiency induced sexuality was reported in many dinoflagellates such as *Peridinium* and *Ptychodiscus* (haploid). Gametes fuse in the area of the sulcus where a fertilization tube is formed which help in formation of planozygote. In *Ptychodiscus* low temperature may cause **excystment** a resting phase (Bold and Wynne 1985).
- In *Gymnodinium* and *Woloszynskia* (haploid) planozygote eventually formed **hypnozygote** (which resemble hypnospores) with spiny to warty ornamentation. These structure help in surviving during extreme low temperature (winter) or high temperature (summer) (Bold and Wynne 1985; Hoek et al. 2009).
- In contrast to above haploid condition some members are also represent diploid condition such as *Noctiluca* and *Crypthecodinium*

Encysted stages: Many dinoflagellates such as *Gonyaulax*, *Peridinium* and *Pyrodinium* are known to produce nonmotile resting cysts by sexual or asexual processes (Graham et al. 2009). These are resting cells in response to unfavourable conditions and many fossil forms are thought to represent encysted stages because their thecae are not affected by such harsh condition. These cysts are often known as **hystrichospheres/hystrichospores** (Hoek et al. 2009). These fossilized dinoflagellates first appeared in the Triassic and reached a peak in the Jurassic and Cretaceous, followed by a decrease in the Tertiary (Lee 2008). The oldest dinoflagellate's cysts date from the Silurian, which is about 300 million years old (Hoek et al. 2009). Hystrichospheres are of two types: those that resemble the shape of vegetative cells and those that are very different. The arrangement of the spines on a cyst often bears a definite relation to the structure of the armour in the cell that produced the cyst (Bold and Wynne 1985; Hoek et al. 2009). The process of encystment or resting spore formation is regulated by a complex interaction of day length, temperature, and nutrient concentration (Lee 2008).

3.10 Red Tides and Dinoflagellates Toxin

Red tides owe their name to the reddish or brown hue of surface water (mainly due to growth of planktonic algae) (Hoek et al. 2009). One of the plagues that struck Egypt was described in the Bible: "all the waters that were in the river were turned to blood. And the fish that was in the river died; and the river stank, and the Egyptians could not drink the water of the river …" (Exodus 7: 17). This description is strongly reminiscent of the poisonous red tides (Lee 2008). Red tides are usually caused by dinoflagellates, particularly photoautotrophic members of *Gymnodinium*, *Gonyaulax*, *Glenodinium*, *Dnophysis* etc. (Hoek et al. 2009). Usually cell density remains between 10 and 20 million per litre during red tide. A number of factors have been suggested as the cause of red tides:

- Dinoflagellates favour warm water near the surface. Red tides are generally a phenomenon of tropical and subtropical areas, but can occur in temperate zone in late spring and summer (Hoek et al. 2009)
- A strong, offshore wind aids upwelling, whereas a gentle onshore wind concentrates the bloom near the coast.
- These red tides can scavenge phosphorous from dissolved organic source such as ATP and store relatively large amounts of phosphorous for later use when ambient level are lower (Graham et al. 2009). Therefore these tides occur at times when dissolved nutrient levels would seem too low.
- Dinoflagellates favour a period of bright, sunny, calm weather before outbreaks.
- Red tides usually occur after an upwelling has stopped. It is thought that preceding blooms of diatoms are favourable for the growth of dinoflagellates but not for the diatoms (Lee 2008).

Many dinoflagellates (about 60 species) can produce powerful toxins which can cause the death of fish and shellfish during red tides. Bold and Wynne (1985) categories these red tides in three category: (1) blooms that kills primarily fish, but few invertebrate, (2) blooms that kills primarily invertebrates and (3) blooms that kill few marine organism but the toxin are concentrated within the siphons or digestive glands of filter-feeding bivalve molluscans causing paralytic poisoning. Most of the toxins producing dinoflagellates are photosynthetic in nature, estuarine, or coastal shallow-water forms that are capable of producing benthic resting cysts, and they tend to form monospecific population (Graham et al. 2009). The dinoflagellates become stuck in the gills of the shellfish, and when shellfish are eaten by humans or animals, poisoning results (Lee 2008). The dinoflagellates which have toxins producing chloroplasts, indicating that the ability to produce toxins may have been derived from endosymbiotic cyanobacteria (Lee 2008). Lee (2008) classified dinoflagellates toxin as:

- **Diarrhetic shellfish poisoning**: Mainly in temperate region, by planktonic dinoflagellates (e.g. *Exuviaella*, *Dinophysis*, *Prorocentrum* etc.). It is caused by the polyether carboxylic acids okadaic acid, macrolide toxins, and yessotoxin. The

polyether carboxylic acid okadaic acid is initially formed inside the dinoflagellate cell as dinophysistoxin-4. This weakly sulphated derivative of okadaic acid is not toxic to the dinoflagellate cell. Dinophysistoxin-4 is produced in the dinoflagellate cell and released to the environment, where it is hydrolyzed to okadaic acid diol ester. Okadaic acid diol ester is lipid soluble and passes through the cell membrane of the shellfish where it is hydrolyzed to toxic okadaic acid.

- **Ciguatera fish poisoning**. Mainly in tropical regions with the common causative agent being *Gambierdiscus* (an edible seaweed's epiphyte). The dinoflagellate contains gambieric acids, ciguatoxins, and maitotoxins, putative Ca^{2+} channel activators that result in breakdown of the cell membrane. The typical course of ciguatera fish poisoning is diarrhea for 2 days, followed by general weakness for 1–2 days.
- **Paralytic shellfish poisoning**: Species of *Protogonyaulax*, *Alexandrium*, *Pyrodinium bahamense*, and *Gymnodinium catenatum* produce a group of toxins that are derivatives of saxitoxin. Saxitoxins are potent neurotoxins acting upon voltage-gated Na^+-channels, preventing influx of Na^+, thereby preventing the generation of an action potential.

Dinoflagellates blooms are responsible for some of the oil deposits of the world, such as North Sea oil deposit. The best studied oil deposits The Kimmeridge Clay oil shales were formed from algal blooms in seas that were to some degree landlocked, with salinity a little beneath the average salinity of the open ocean. These blooms deoxygenated and poisoned the water, providing the temporary anaerobic bottom conditions required for the preservation of organic matter (Lee 2008). Petroleum deposits and ancient sediments contain 4α-methylsteroidal hydrocarbons, which probably originated from 4α-methylsterols in dinoflagellates (Robinson et al. 1984).

3.11 Heterotrophic Dinoflagellates

The fascinating discovery of *Pedinomonas* cells inside *Noctiluca scintillans* has led to the hypothesis that dinoflagellates are fundamentally a group of heterotrophic Protozoa, which have acquired photosynthesis ability (Hoek et al. 2009). At present more than 50 % dinoflagellates do not have chloroplast and are exclusively heterotrophic. In addition, when nutrient conditions are low in the environment many chloroplasts bearing dinoflagellates obtained a portion of their nutrients heterotrophically (Lee 2008). The process of feeding on particles such as the cells of other organism is very common among dinoflagellates which occur among marine and freshwater dinoflagellates, photosynthetic and plastidless dinoflagellates, or armored and unarmored dinoflagellates. There are three main feeding mechanisms (Graham et al. 2009).

- **Direct engulfing of prey**: Mainly in unarmored dinoflagellates which are able to stretch as they engulf insect prey cells and then digest them inside an internal food vacuole. *Noctiluca* has a 300-μm-long food-gathering tentacle that is covered with a slimy exudate. *Polykrikos kofoides* feeds by initially spearing its prey with a nematocyst. The prey organism is pulled into the posterior sulcus, with the prey eventually being completely engulfed into a food vacuole (Lee 2008; Graham et al. 2009).
- **Pallium feeding**: This occurs only in thecate species (e.g. *Protoperidinium* and *Diplopsalisand*) utilises a feeding veil, the pallium, which emerges from the flagellar pore and encloses the prey (Lee 2008).
- **Peduncle feeding**: This is an extensible pseudopod, which arise in the region of the sulcus. Peduncle also contain a rod of closely packed microtubules (**microtubular basket**) e.g. *Gymnodinium fungiforme* and *Pfiesteria piscicida* (Hoek et al. 2009; Lee 2008). Peduncle are also produce by armored species such as *Dinophysis* (marine) and *Peridiniopsis berolinensis* (freshwater) (Graham et al. 2009).

Freshwater dinophyte *Stylodinium sphaera*, attaches itself by a small stipe to the *Oedogonium* and release its content in the form of two amoeboid cells. These amoebae cells settle on intact *Oedogonium* and a few second later one amoebae cell puncture the *Oedogonium* cell wall and ingest its content (Fig. 14e) (Hoek et al. 2009). *Haplozoon*, a completely heterotrophic dinoflagellates, includes about a dozen parasites in the intestines of annelids. On the other hand *Blastodinium* are facultative heterotrophs living in the intestines of marine copepods (Bold and Wynne 1985).

3.12 Symbiotic Dinoflagellates

Symbiosis remains one of the most fascinating phenomena in biology. Green algae generally function as endosymbionts in freshwater system (also known as **zoochlorellae**), but in marine system this role is generally taken by symbiotic dinoflagellates (also known as **zooxanthellae**, which broadly defined to include golden, yellowish, brownish and reddish algal cells) (Hoek et al. 2009). In addition to dinoflagellate zooxanthellae, algae classified in other divisions have also been demonstrated to be symbiotic. Zooxanthellae occur in almost all species of tropical and reef-building corals, jellyfish, protozoans and sea anemones (Cnidaria). At first *Gymnodinium*-like dinoflagellates involved in endosymbiosis but later on these coccoid spheres shape endosymbionts have been assigned to the genus *Symbiodinium* (Hoek et al. 2009; Lee 2008). Cells of *Symbiodinium* grow ten times slower than cultured cells. The degree of dependency of the host on the symbiotic algae is variable (Bold and Wynne 1985). Corals with symbiotic dinoflagellates play an important role in carbon fixation and nutrient cycling (Hoek et al. 2009).

The dinoflagellates provides host cells with as much as 90 % of their organic food and oxygen (Graham et al. 2009). Corals with symbiotic dinoflagellates do not incorporate calcium into their skeletons as fast as corals without symbiotic dinoflagellates. In addition to Dinophyceae living symbiotically inside other organisms, there are other organisms that live inside dinoflagellate cells. *Noctiluca scintillans* is a heterotrophic omnivorous feeder that ingests zooplankton, mesozooplankton, and their eggs (Lee 2008).

3.13 Dinoflagellates and Bioluminescence

Phenomenon of bioluminescence occurs in a wide variety of organisms ranging from bacteria to dinoflagellates to jellyfish and brittle stars to worms, fireflies, molluscs, and fish (Hastings 1983). *Noctiluca* was the first dinoflagellates in which this phenomenon of bioluminescence was recorded (Bold and Wynne 1985). Many marine, dinoflagellates (e.g. *Lingulodinium*, *Protogonyaulax*, *Pyrodinium*, *Pyrocystis*, *Ceratium* and some non-photosynthetic forms such as *Noctiluca*, *Protoperidinium*) are the main contributors to marine bioluminescence, emitting a bluish-green (at 474 nm) flash of light of 0.1-s duration (Graham et al. 2009). Interestingly no freshwater dinoflagellates are capable of bioluminescence (Lee 2008). Basic reaction of bioluminescence is as follow in all organism including dinoflagellates.

$$\text{Luciferin} + \text{O2} \xrightarrow{\text{Luciferin}} (\text{P})^{*} \rightarrow \text{P} + \text{h}\nu$$

In dinoflagellates luciferin, luciferase, and luciferase-binding protein occur in particles called **scintillons/microsource** (flashing units) that are approximately 0.5–1.5 μm in diametre. There are three modes to emit light in dinoflagellates: (1) when stimulated mechanically, chemically, or electrically or (2) spontaneously or (3) late at night they can glow dimly (Graham et al. 2009; Hoek et al. 2009; Lee 2008). In *Gonyaulax polyedra* this phenomenon shows different rhythms with different condition. When cells of *G. polyedra* grown under natural light-dark (LD) cycle, the amount of light emitted under agitated condition is observed to be low during the period of illumination and to reach a peak during the middle of the dark period. By transferring such a culture from LD to DD cycle it can be observed that an **endogenous circadian rhythm** is indeed present. The cycle of peaks of bioluminescence continue for several days, the amplitude gradually decreasing. The long persisting periodicity in stimulated luminescence can also be demonstrated in the same species by placing the cells LL conditions of low-intensity light. Although the peak of light emission is not as great as that reached under LD conditions, the circadian rhythm continue s indefinitely (Bold and Wynne 1985).

3.14 Circadian Rhythms in Dinoflagellates

Dinoflagellates show best known of the rhythms in the algae. If dinoflagellates have been growing in natural illumination or on a light-dark cycle, the amount of light emitted will be markedly dependent on the time of day when measurements are made. Oxygen production or carbon dioxide fixation is also a rhythm in *Lingulodinium polyedrum*. A third rhythm with a circadian period in *Lingulodinium polyedrum* is that of cell division (Lee 2008). A fourth type of rhythm involves the vertical migration of dinoflagellate cells in the water column. Marine dinoflagellates (e.g. *Ceratium*, *Peridinium*, *Prorocentrum* etc.) frequently move into deeper, nutrient-rich waters at night and migrate towards surface during the day (Hoek et al. 2009). The duel (over a 24-h period) migrations of dinoflagellates are 5–10 m in relatively quiet waters (Lee 2008). It will take 6 h of the day (24-h) for 5 m of upward migration around dawn, and a further 6 h for downward migration around dusk at a speed of 500 μms^{-1} (Raven and Richardson 1984). It is thought that plasma membrane control all types of rhythms. According to Roenneberg and Deng (1997) two different systems time the circadian rhythm in dinoflagellates, a red-light sensitive system that delays the timing, and a blue-light sensitive system that advances the timing to dawn.

3.15 Phylogeny of Dinoflagellates

Dinoflagellates are perhaps 600 million years old (although fossil cysts form may be more old) groups of algae increased markedly during late Jurassic period (150 million years ago) to reach a maximum in the middle to late Cretaceous (110–80 million years ago) (Hoek et al. 2009). Fossil records of dinoflagellates are biased; containing only a limited amount of information therefore we have to rely on living dinoflagellates. In his much-reproduced phylogenetic tree from 1980, Max Taylor placed most heterotrophic genera of dinoflagellates at the top of the tree, whilst photosynthetic species occupied many of the basal branches (Moestrup and Daugbjerg 2007). The closest relatives of dinoflagellates are apicomplexans and ciliates together with the paraphyletic group that includes their ancestors, the protalveolates, form Alveolates. Some experts have proposed that alveolates and stramenopiles, together with haptophytes and Cryptomonads, share a common ancestor that possessed a photosynthetic plastid obtained from a red alga by a single secondary endosymbiotic event (Graham and Wilcox 2000; Graham et al. 2009). Gymnodiniales appear to have retained more ancestral characters than any other order. Other orders can be derived from this in two way: (1) by phylogenetic gain or step-wise acquisition of more advance characters and (2) by the loss of characters (Hoek et al. 2009). Overall following picture is a summarised way to represent phylogenetic relationship in dinoflagellates (Fig. 17).

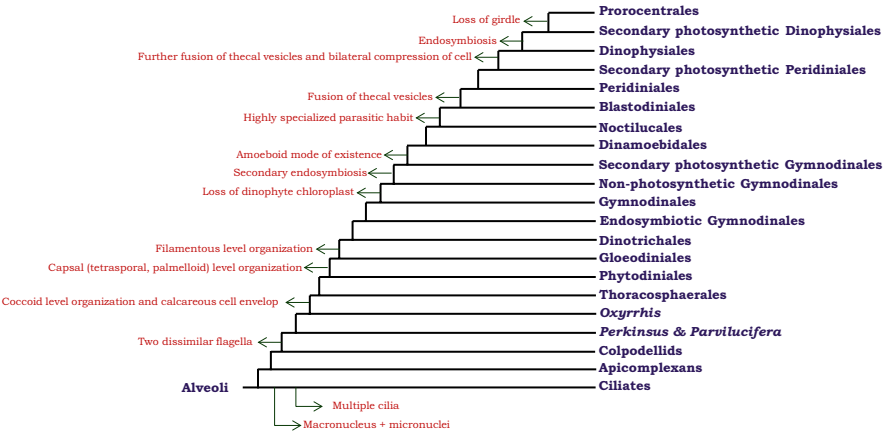


Fig. 17 A hypothetical phylogenetic tree of the Dinoflagellates (Adopted from Hoek et al. 2009; Graham et al. 2009)

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Survey of Algae in Extreme Environments

Joseph Seckbach

1 General Introduction

Prokaryote Archaea, bacteria, cyanobacteria and Eukarya (such as algae, fungi, protistans, and micro-invertebrates) live in almost all of the habitats available on Earth, from the 'normal ambient' environments to the harshest places. These severe habitats are "extreme" from an anthropocentric view (since we cannot live in them without clothing or housing). The 'normal' conditions of life refer to a temperature range of 4–40 °C, a pH level between 6 and 8.5, and a salinity between fresh water and seawater.

The harsh conditions of extreme environments are at the edge of biological growth limits. Among the microorganisms thriving in extreme environments there are those coping with living in: temperature effects, elevated (thermophiles) or lower (psychrophiles) temperature levels, pH effects (acidophiles <4), or alkaliphiles >10), in hyper saline waters (halophiles), anaerobic conditions (some biologists do not usually consider anoxic conditions as extreme since they represent primeval conditions), living in toxic metals, and desiccation. Some oxygenic photosynthetic microorganisms (algae and cyanobacteria) can thrive in most of these areas.

The cyanobacteria (blue-green algae) are among the most ancient oxygenic photosynthetic microorganisms. They inhabited the Earth for almost all its history and caused the oxygenation of the previous anaerobic reduced ancient atmosphere. One can analyze their probable remains in ancient Stromatolites (which were formed >3.5 billion years ago). Some of these cells might "remember" their original harsh environments and have survived through geological times. Others may have adapted to live on the edge of biological existence under marginal physiological situations. After all, the algae (and plants) are the main producers of O_2 and removers of CO_2 from the atmosphere during photosynthesis.

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D. Sahoo, J. Seckbach (eds.), *The Algae World*, Cellular Origin, Life in Extreme Habitats and Astrobiology 26, DOI 10.1007/978-94-017-7321-8_10

Much literature on extremophiles of the three domains of life has been published during the last >40 years. This area of knowledge has been increased with a plethora of articles and books, such those of: Horikoshi and Grant 1998; Seckbach 1999, 2000; Gunde-Cimerman et al. 2005; Rainey and Oren 2006; Gerday and Glansdorf 2007; Stan-Lotter and Fendrihan 2012; Seckbach et al. 2013; Seckbach and Rampelotto 2015). Several conferences have occurred in various places around the world. Roberts (1998) has covered the eukaryotes in extreme environments, and his older data are still valid for today. However volumes dedicated only to algae in harsh conditions are very limited (see e.g., Elster et al. 2001; Seckbach 2007).

The extremophilic organisms may be considered living in conditions and habitats as in oasis or even 'Garden of Eden', and most of them cannot live outside their natural (severe) niches, unless they are merely extremotolerant. Most of the other organisms would not be able to exist under these harsh conditions. We could consider these extremophiles as the pioneers in the evolution of life. Some (extremophilic) unicellular red algae such as the eukaryotic *Galdieria sulphuraria* have genes from prokaryotic microorganisms, which apparently entered via horizontal transfer from bacteria, and Archaea genes (Schönknecht et al. 2013).

In this chapter the algae are our 'stars' since they are thriving in a large variety of habitats from "normal" ambient conditions up to very harsh environments. The cyanobacteria are included with the other algae in this chapter as (blue-green) algae. It is assumed that these extremophile microorganisms have been among the pioneers of early life and also that some of them might serve as analogues for extraterrestrial life.

2 Algal Extreme Environments, Thoughts to Be Not Inhabitable

The algae are phototrophs and need CO_2, H_2O, minerals nutrients, light, and suitable temperature. Via the photosynthetic process, these autotrophs produced simple sugars and more complex compounds while they release oxygen. They live in various habitats of "normal" and extremophilic environments. Some of the extremophiles may have been primeval while others may have adapted later to various harsh condition.

2.1 Algae at High Temperature

Several species of algae thrive at elevated temperatures. Some of the thermophilic cyanobacteria and algae are known from the publications of T.D. Brock a few decades ago (1976, 1978). Among the cyanobacteria is *Chroococcidiopsis* species of which is known for the ability to survive several harsh environments with both high and low temperatures, ionizing radiation, and high salinity. These photosynthetic prokaryotes are usually crypto-endolithic (see more characteristic of this species in Tison et al. 1980). Among the cryptoendolithic are red unicellular algae of

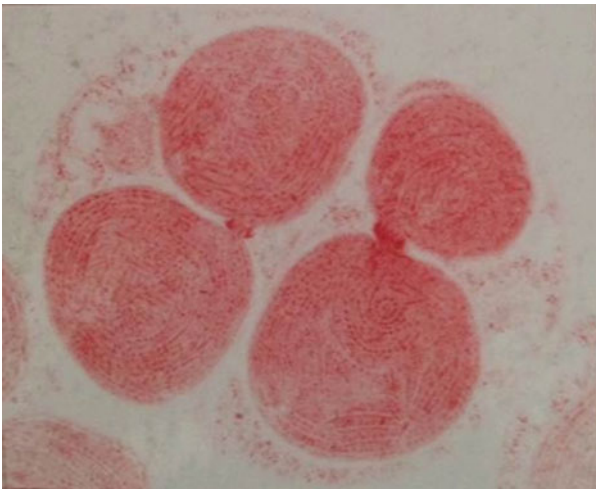

Fig. 1 Cover of **Evolutionary Pathways and Enigmatic Algae**: **Cyanidium caldarium** (**Rhodophyta**) **and Related Cells** (Edited by J. Seckbach 1994). This photo shows dividing Chloroplast of **Cyanidium caldarium** cell (original contributed to the editor by T. Kuroiwa, University of Tokyo)

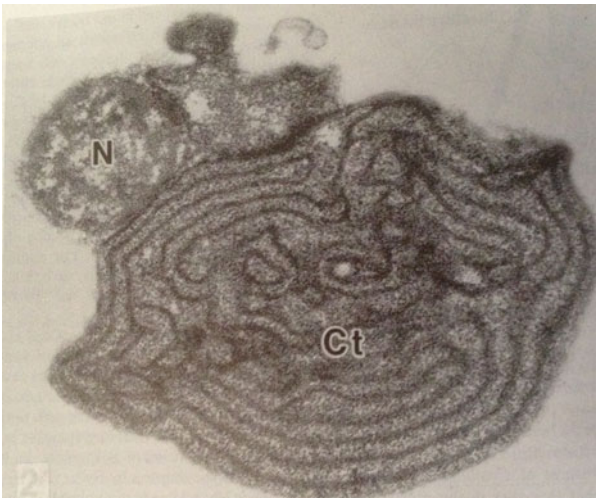

Fig. 2 High magnification of an electron micrograph of ***Cyanidioschyzon merolae*** (member of the Cyanidiaceae group) (Taken form Seckbach J. chapter in ***Evolutionary Pathways and Enigmatic Algae***: ***Cyanidium caldarium*** (***Rhodophyta***) ***and Related Cells***. Kluwer Academic Publishers, Dordrecht, NL) (*N* cell nucleus, *Ct* chloroplast)

Cyanidia group (*Cyanidium caldarium*, *Galdieria sulphuraria* and *Cyanidioschyzon merolae*- see figures) these acido-thermophilic algae survive a max. temperature of 57 °C (Seckbach 1994, 2013). Some green algae (e.g., *Chlorella* species) are known to grow at about 42 °C, while some diatoms grow at 30 °C with a max. of 44 °C, and found in volcanic mud at pH <3 and 70 °C (Quintelo et al. 2008) (Figs. 1 and 2).

2.2 Algae Thrive at Low Temperature

Among the "cold-growing" algae are the psycrophiles. They are observed in the seas and oceans in various algal groups, and in terrestrial areas as ice and snow algae. The snow and ice algae include the *Chlamydomonas nivalis*, *Chloromonas*, the desmid *Mesotaenium*, *Chlorosarcina* and *Chlorella* (Hoham and Ling 2000; Leya 2013). Cold-loving cyanobacteria are species of: *Chroococcidiopsis*, *Oscillatoria*, *Lyngbya*, *Microcoleus*, *Nostoc* and *Phormidium*.

Psychrophilic algae grow in polar regions such as in the high Arctic, Antarctica, and alpine regions. They appear in various colors in melting snow (Hoham and Ling 2000: Leya 2013). Spores of the green alga *Chlamydomonas nivales* appear in the melted snow as bright red, pink, or yellow – green. The snow algae contain astaxanthin as well as other algae with fatty acids. Some spores of snow algae exposed to UV produce flavonoids as antioxidant compounds which serves as a sunscreen in microalgae such as dinoflagellates, diatoms, chlorophytes, and rhodophytes. Microalgae are abundant in the cold seas of the Arctic and Antarctica. Several species of diatoms adapt to cold temperatures as in permafrost and in glaciers.

2.3 Algal Life in Extreme Ranges of pH

2.3.1 The Acidophilic Algae

Among the low pH areas are environments, such as the acido-thermo hot springs of Yellowstone National Park (USA), Iceland hot springs, in Pozzuoli area (near Naples, IT), the non-thermal Tinto River (Spain), and in old abundant mines, which support various algae. Some acidophilic areas may reach a pH range around 3 to O. In general, DNA and ATP are unstable in acid solutions and in most acidophiles the internal pH is close to neutral pH while the external media is acidic (see table of external – internal pH level, in Seckbach 2000, page 112). Among the acidophilic algae are: *Chlamydomonas acidophila*, *Chlorella spp..*, *Chara coralline*, *Scenedesmus quadricauda*, *Dunaliella acidophila*, *Eremosphaera viridis*, *Euglena mutabilis*, *Cyanidium caldarium* with its two cohorts, and the diatoms (e.g., P*innularia*) (Seckbach 2000).

There is a mechanism in the acidophiles that prevents H+ ions from entering the cells. In general, photosynthetic prokaryotes do not grow in acidic media. However at pH 2, the cyanobacteria *Oscillatorial/Limnothrix* and *Spirulina* spp. were found in acid Bavarian lakes (Steinberg et al. 1998). *Chroococcus* sp. was noticed in acid Canadian lakes. It has been reported that the acido-thermophilic algal group of *Cyanidium caldarium* can tolerate 1N H_2SO_4 (Seckbach 1994) and they thrive under a pure CO_2 stream of bubbling gas (Seckbach et al. 1970). The H+ pump is one of the balancing methods against the high acidity in nature. In addition, *Dunaliella acidophila* and *Chlamydomonas acidophila* are known to grow at pH 1–2 in many non-thermal acid waters, as are *Euglena mutabilis* (pH 1–5) and the acidophilic diatoms (pH 0–4) are.

2.3.2 The Alkalophile at Higher pH Ranges

High pH areas are found in carbonate rich soil and in many lakes, such as the Soda lakes in Africa, Mono Lake in California and other areas. In these waters one can find algae at pH 10. Among these is *Arthrospira* [*Spirolina*] which is a major food for flamingoes and a food supplement for humans via naturalist stores. The soda lakes are also high salinity. Among these phototrophs in high pH areas are *Chlamydomonas*, *Dunaliella* and diatoms.

2.4 Salt Environments and Their Oxygenic Photosynthetic Microbial Algae and Cyanobacteria

Halophiles algae grow in saline lakes and other hypersaline waters, such as salterns. One of the best known extreme halophilic environment example is the Dead Sea between Israel and Jordan. Seventy years ago Ben Volcani was the first to publish about life in the Dead Sea which is called in Hebrew the "Salty Sea" (Volcani 1944). The physical and chemical conditions of the Dead Sea are ~430 m under the sea level, with 348 g/l salts. The ions composition in the hyper-salt solution of the Dead Sea is: Mg^{++}, Na^{+}, Ca^{++}, K^{+}, Cl^{-}, and Br^{-}. Another salty environment is the Great Salt Lake (Utah, USA). Under such concentrations of ions there is a serious osmolality problem to be overcome by the halo-microorganisms. Some of them produce special bio-products such as glycerol (as an osmotic-regularity factor). Most halophilic Archaea and some Bacteria have to use an ionic pump for some exchange of ions (Na^{+} moving out while K^{+} is brought into the cell).

The green alga *Dunaliella salina* grows in the Dead Sea (Israel/Jordan) and occurs in the Great Salt lake (Utah, USA) where the salt concentration reaches >30 % and the salinity can be at saturated levels. Numerous species of cyanobacteria are known in hypersaline waters (as for example they occur in Baja California).

The problem of hypertonic external waters with the great osmotic pressure of the salty medium is solved by several physiological mechanisms. Some organisms have an ion^{+} pumping that removes the H^{+} ions, others exchange ions of Na^{+} with K^{+}. Some algae produce special phyco-products such as glycerol and betaines which can balance the external osmotic pressure.

In the Dead Sea, during a heavy precipitation this lake receives lots of running rainwater from its surroundings and its salinity is diluted from the >30 % hypersaline level. When this occurs, some green algae (e.g. *Dunaliella salina*) thrive in the diluted Dead-Sea waters. While in "normal" years of rain, *Dunaliella* is absent from the Dead-Sea. In most hypersaline waters the halobacterial (actually haloarchaea) are nourished by the *Dunaliella* which is the primary (photosynthesizer) producer. This green alga lacks rigid cell wall so that its cells swell or shrink in reaction to the external salinity. In the Dead Sea prokaryotic algae are not important. Diatoms are among the halo algae at higher pH ranges and among the other halophilic algae are *Phomidium*, *Oscillatoria*, *Microcoleus*, and halo-*Spirulina*. Many halophytes spp. produce glucosyl glycerol as an osmotic stabilizer.

2.5 Algae That Tolerate Anoxic Condition

An anaerobic atmosphere occurs at high CO_2 and reduced oxygen levels. In May 2013, the amount of CO_2 in our atmosphere crossed this threshold and has been increased on Earth to reach 400 ppm (the previous level was 300 ppm). But how significant is this milestone? *Cyanidium caldarium* and its cohorts thrive much better when pure CO_2 is bubbled through their medium as compared to aerated cultures (Seckbach et al. 1970). Most algae are not capable of tolerating high levels of carbon dioxide. We have to note here that some phycologists do not consider the anoxic environment as an extreme factor since. See also Anoxia edited by Altenbach, Bernhard and Seckbach (2012).

2.6 Desiccation: Dehydration Tolerance

Algae can survive dry conditions for a long period of time (in dormant stage?) until moisture returns and they "revived". The green alga *Botryococcus braunii* tolerates desiccation and high temperature when dehydrated (Demura et al. 2014). In the *Klebsormidium* (genus of filamentous charophyte alga) several changes were observed during desiccation. This green alga tolerates desiccation as its cell volume is reduced by ~60 % and its cytoplasm appears denser during this desiccation stress. Members of Trebouxiophyceae (class of green algae, in the division Chlorophyta) under water loss (dehydration) and desiccation show various changes in their ultrastructure. There is a rapid decrease in photosynthesis during desiccation. However, since water is essential for all life, the internal cellular organelles and some membranes maintained water molecules (Holzinger et al. 2011). There is a large literature on desiccation tolerance in cyanobacteria.

2.7 Radiation

In areas such as the Antarctica the ozone declines and the UV radiation in the solar radiation of the short wavelengths increases. The oxygenic photosynthetic algae absorb the full solar radiation, including UV radiation. These algae protect themselves by moving away, repairing the damage, or using sunscreen compounds (carotenoids, myxoxanthophyle). *Synechococcus* algal cells were sent into outer space flight for 2 weeks (under high UV radiation) and these cells returned with only a slight reduction in vitality. The alga *Chroococcidiopsis* is also known to resist both heat and UV and gamma radiation. The reader could find further information on the UV tolerance of cyanobacteria in Castenholz and Garcia- Pichel (2000).

3 Astrobiology

The main current candidates for finding traces of extraterrestrial life are Mars, Europa, Enceladus, and Titan. Some rovers on Mars are searching to trace some signals from ancient or current hidden living forms.

Extremophilic environments and their inhabitants may serve as models or analogues for possible extraterrestrial life. Somewhat similar conditions can be postulated for past extraterrestrial surfaces (e.g., in Mars). The recent photos of Mars show that in the past history of this Planet the surface was wetter and warmer than today. Its landscape is engraved with contours of rivers and traces of water falls, which indicate that billions of years ago the red planet was perhaps green and contained life. Are living traces still there buried in the depths of the subsurface? Life forms have also been detected in the subsurface of gold mines in South Africa and in other sub-terrestrial places.

Furthermore, dormant microorganisms have been detected in salt crystal desiccated and after resting for hundreds of millions years, they were revived. The same situation could exist in satellites where once were living activities.

Recently, it was published that in some satellites of the Solar System are saline subsurface oceans buried under the icy sheets, as is the case for Europa, the moon of Jupiter, Enceladus and Titan, satellites of Saturn. To answer the mystery about the origin of life, there is a theory that elements of life was imported from space (Panspermia), perhaps via the bombardment by satellites. So that life forms (like amino acids or even spores or bacteria) were imported from space to Earth by space "messengers" (e.g., asteroids, comets, etc.).

4 Summary

The current chapter deals with algae which grow at the edge of the life. Over the last 40 years, the study of life representatives at extreme conditions have been established and proliferated. Algae are among the extremophiles survive various harsh conditions. Recently, this new area of investigation generated several conferences, published chapters, and printed books on the subject. All of this literature and these meetings have covered the extremophiles of the three domains of life from different angles and aspects. Most of the extremophiles are not able to grow outside their harsh habitats, while for "normal" living organisms these severe environments are lethal.

Algae are growing in various severe extreme conditions. Prokaryotic phototrophs and eukaryotic algae can share some of the severe habitats or, can be separated, thriving in their individual harsh environment. Further away from the "normal" conditions are the habitats of the thermophiles, psychrophiles, halophiles, acidophiles, alkaliphiles and some additional harsh living microbes.

Acknowledgement The author thanks for revising this chapter to Professor David Chapman for his encouragement, he appreciate very much the reviewing of Professors Russell Chapman and Richard Castenholz for their intensive improving the text and its science.

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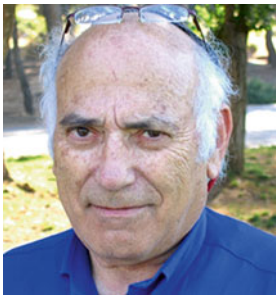

Professor Joseph Seckbach has been the founder and chief editor of Cellular Origins, Life in Extreme Habitats and Astrobiology ("COLE") with ~30 books in that series. He has co-edited other volumes, such as the Proceeding of Endocytobiology VII Conference (Freiburg, Germany) and the Proceedings of Algae and Extreme Environments meeting (Trebon, Czech Republic). See http://www.schweizerbart.de/pubs/books/bo/novahedwig-051012300-desc.ht). His edited the recent volume (with Richard Gordon) entitled Divine Action and Natural Selection: Science, Faith, and Evolution published by World Scientific Publishing Company. He is currently still busy with new books publications and new chapters.

Dr. Seckbach earned his Ph.D. from the University of Chicago (1965) and did a post doctorate in the Division of Biology at Caltech, in Pasadena, CA. He headed a group at UCLA studying the life possibilities on Venus, later he was appointed to the Hebrew University, Jerusalem and spent sabbaticals at UCLA and Harvard University. Dr. Seckbach enjoyed his DAAD-sponsored (The German Academic Exchange) periods in Tübingen, Germany, and at LMU, Munich. He served at Louisiana State University (LSU), Baton Rouge, LA, USA, as the first selected Chair for the Louisiana Sea Grant and Technology transfer.

Beyond editing academic volumes, he has published ~140 scientific articles on plant ferritin – phytoferritin, cellular evolution, acidothermophilic algae, life in extreme environments and on Astrobiology. He also edited and translated several popular books. Dr. Seckbach is the co-author, with R. Ikan, of the Hebrew-language Chemistry Lexicon (two editions by Deveer publisher, Tel-Aviv). His recent interest is in the field of enigmatic microorganisms as the life in extreme environments and Astrobiology. He enjoys visiting many Universities and Institutes and presenting there seminars on his studies.

Part II
Applied Phycology

Algal Biotechnology

Michael Armin Borowitzka

1 Introduction

The term 'biotechnology' was first used in 1917 by Erecky (1917) who defined biotechnology as: '*all lines of work by which products are produced from raw materials with the aid of living organisms*'. Since the 1970s there have been many definitions of 'biotechnology' (Bud 1991), but the most widely accepted is that biotechnology is the use of living systems and organisms to develop or make useful products, or "*any technological application that uses biological systems*, *living organisms or derivatives thereof*, *to make or modify products or processes for specific use*" (definition used in the UN Convention on Biological Diversity). Algal biotechnology can therefore be defined as: "*the technological application of algae* (*both microalgae and macroalgae*) *or their derivatives to make or modify products or processes for specific use*". This definition encompasses the commercial-scale farming of seaweeds and microalgae for biomass as well as the production of specific compounds, the use of algae in wastewater treatment, the use of algal enzymes for specific purposes, the use of algal products in products such as functional foods etc. Although not specifically stated, the above definition also implies a practical and eventual commercial outcome. It does not refer to the technology *per se*, but rather the application of that technology or technologies. The term 'specific use' implies that there is a user – which in business terms means that there must be, ultimately, a customer and a market.

The phylogenetic diversity of the organisms we call 'algae' which include both prokaryotic and eukaryotic organisms (Adl et al. 2005; Borowitzka 2012) is reflected in the extensive range of products which can be produced from algae, as well as the

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D. Sahoo, J. Seckbach (eds.), *The Algae World*, Cellular Origin, Life in Extreme Habitats and Astrobiology 26, DOI 10.1007/978-94-017-7321-8_11

variety of processes in which algae, or products derived from them, can be utilised. For convenience algal biotechnology is generally divided on the basis of the size of the algae under consideration into the biotechnology of the multicellular, large macro-algae or seaweeds, and the biotechnology of the microscopic and predominantly unicellular micro-algae. In this chapter I will be focussing almost exclusively on the microalgae, however the great importance of seaweeds to algal biotechnology must be recognised.

Currently, the quantity and value of wild harvested and farmed seaweeds and their products greatly exceeds that of the microalgae. Statistics available from the United Nations Food and Agriculture Organisation (FAO) show that in 2011 20 million t wet weight (= about 2 million t dry weight) of seaweeds were produced by aquaculture with a value of US$ 5.88×10^9. Seaweeds such as the brown algae *Saccharina japonica* and *Undaria pinnatifida* and the red algae *Kappaphycus* spp, *Gracilaria* spp and *Chondrus crispus* are mainly used to produce hydrocolloids such as alginate, agar, agarose, carrageenan and fucoidan (Bixler and Porse 2011), whereas other species such as the red alga *Pyropia* (*Porphyra*) spp and the green algae *Ulva* and *Caulerpa* are mainly used as food (Zemke-White and Ohno 1999). The cultivation methods used are described by Sahoo and Yarish (2005). Several elite strains have been bred using classical plant breeding methods (Zhang et al. 2011a, b) and tissue culture and molecular techniques are now widely being used in the breeding of elite cultivars and for propagation of the elite seed stock for cultivation (Reddy et al. 2008; Liu et al. 2011; Xu et al. 2012; Robinson et al. 2013).

2 Microalgae

Microalgal biotechnology started with pioneering studies on the large-scale culture of microalgae in Japan, Germany and the USA in the 1950s (Soeder 1986; Borowitzka 2013b). In the 1970s the first commercial production of *Chlorella* commenced in Japan and Taiwan (Soong 1980), and *Spirulina* harvesting started in Mexico in Lake Texcoco (Durand-Chastel 1980). Commercial culture of *Spirulina* commenced in the 1980s in the USA (Belay et al. 1994). The first microalga used for the production of a high-value fine chemical was the halophilic green alga, *Dunaliella salina*, cultured to produce β-carotene with commercial production commencing in the 1980s in Israel, Australia and the USA (Ben-Amotz 2004; Borowitzka 2005a). This was followed by the establishment of production of astaxanthin from *Haematococcus pluvialis* as a source of astaxanthin in the USA and Israel in the 1990s (Cysewski and Lorenz 2004), and the development of a commercial process to produce the long-chain polyunsaturated fatty acid, docosahexaenoic acid, by heterotrophic cultivation of the stramenopiles *Crypthecodinium cohnii* (dinoflagellate) and *Schizochytrium* sp. (thraustochytrid) in the USA (Kyle et al. 1992; Barclay et al. 2005). Table 1 summarises the main microalgae species currently produced commercially and the products derived from them. However, this table does not include the very large amounts of algae grown as feed in the aquaculture of molluscs,

Table 1 Current species of the major microalgae species produced commercially and their products

Species	Product(s)	Main production locations
Chlorella spp	Biomass, extracts for human and animal nutrition	Taiwan, China, Germany
S*pirulina* (*Arthrospira*) spp	Biomass, phycocyanin	China, Taiwan, India, USA, Thailand
Dunaliella salina	β-carotene	Australia, Israel
Haematococcus pluvialis	Astaxanthin	Israel, USA, China
Crypthecodinium cohnii	Docosahexaenoic acid	USA

crustaceans and fish (Borowitzka 1997; Neori 2011), nor the application of microalgae in other processes such as wastewater treatment (Green et al. 1996; Craggs et al. 2013; Sutherland et al. 2014) and use as fertilizer, or for the production of extracts used for a variety of applications such as in cosmetics, functional foods, and in animal husbandry (Gellenbeck 2012; Schwenzfeier et al. 2013; Kotrbáček et al. 2015). Microalgae are also being considered for the bioremediation of carbon dioxide (Moheimani et al. 2012).

3 Production of High Value Chemicals

Currently, the main high value products produced by microalgal biotechnology are the carotenoids β-carotene and astaxanthin, and the long chain polyunsaturated fatty acids docosahexaenoic acid and eicosapentaenoic acid. Smaller quantities of other products such as the phycobilin pigment, phycocyanin, are also produced (Borowitzka 2013d).

3.1 Carotenoids

The carotenoids which are accumulated in high amounts are secondary metabolites, and this means that they are mainly formed once the cells are no longer in the exponential phase of growth; i.e. they algae are either growing slowly or not at all. This presents a challenge in the development of an effective production process as the algae have a low carotenoid content under the conditions where growth is fastest, and accumulate the carotenoids only when growth is slowed or stopped. This means that either a two-step process must be used, with biomass generation in the first step, and product accumulation in the cells in the second step (Ben-Amotz 1995), or a culture regime at which neither growth nor product accumulation is maximal, but the productivity of product formation is highest (Borowitzka et al. 1984).

The halophilic green unicellular alga *Dunaliella salina* (sometimes also referred to as *D. bardawil* – Borowitzka and Siva 2007) is the best natural source of

β-carotene with the alga reported to contain up to 14 % of dry weight as β-carotene (Aasen et al. 1969). The main commercial production of *Dunaliella* β-carotene occurs in Australia at Hutt Lagoon in Western Australia and Whyalla in South Australia, with smaller production at Eilat in Israel (Borowitzka 2010). The two production plants in Australia use very large, simple, unmixed shallow ponds of a total area greater than 740 ha at each plant, whereas in Israel much smaller paddle-wheel driven raceway ponds are used. *Dunaliella salina* grows best at very high salinities between 20 and 35 % (w/v) NaCl. The β-carotene is accumulated in lipid droplets in the chloroplast and, unlike the synthetic β-carotene which is only in the form of the all-*trans* isomer, the *Dunaliella* β-carotene occurs both as the all-*trans* and the 9-*cis* isomers (Ben-Amotz et al. 1988). The maximum cell β-carotene content which can be achieved by *D. salina* is mainly a function of the salinity the algae is grown at, with higher salinities resulting in higher carotenoid contents (Borowitzka et al. 1990). The rate at which the β-carotene is formed is a function of the growth irradiance with higher irradiances leading to faster β-carotene accumulation (Borowitzka 2013a).

In Australia *Dunaliella* β-carotene production is a continuous culture production process. The growth salinity used is a compromise between the optimum salinity for β-carotene productivity (~22 % NaCl) and a salinity at which potential problems with predatory protozoa and/or the brine shrimps *Artemia* and *Parartemia* can be avoided (salinities >25 % NaCl) as well as the ability to supply sufficient seawater to replace water lost by evaporation from the large ponds (Borowitzka and Borowitzka 1989). The very large ponds, which are about 30–40 cm deep on average, are predominantly mixed by wind and convection currents resulting in low biomass productivity. However, the close-to-optimum climatic conditions mean that the biomass has a high β-carotene content and production occurs over the whole year. This, combined with the low land costs and an extremely efficient proprietary harvesting and β-carotene extraction methods, plus the economics of scale, means that this is the lowest cost microalgae production in the world. The products produced include a range of concentrations of β-carotene either in solution or as crystal suspensions in oil for use in the food, pharmaceutical and nutraceutical industries, as well as dried whole-cell β-carotene-rich *D. salina* biomass which can be used in aquaculture as a pigmenter for prawns or as a feed supplement for cattle and other animals.

Astaxanthin is produced using the freshwater green alga *Haematococcus pluvialis*, and high astaxanthin contents of up to 8 % of dry weight are found in the non-motile aplanospore stage of this alga. The astaxanthin is accumulated in lipid droplets in the cytoplasm of the aplanospores both in the form of free and esterified (3*S*,3′*S*) astaxanthin (Grung et al. 1992; Collins et al. 2011). Unlike *D. salina*, *Haematococcus* must be grown in a two-stage batch mode. In the first stage the *Haematococcus* biomass is generated by culturing the flagellated motile cells in nutrient-rich medium. In the second stage the cells are transferred to low-nutrient medium under high light conditions where they develop into the aplanospore stage and accumulate the astaxanthin. At Cyanotech in Hawaii, the first stage culture takes place in closed photobioreactors and this is then followed by the second stage in open raceway ponds (Olaizola 2000; Cysewski and Lorenz 2004). In Israel,

Algaetechnologies grows both stages in glass tubular photobioreactors outdoors. The current production plant has about 300 km of glass tubing on 10 ha of arid land. AstaReal in Sweden grow *Haematococcus* indoors in artificially lit reactors mixotrophically using organic carbon substrates such as acetate. *Haematococcus* can also be grown heterotrophically, but heterotrophically-grown cells do not accumulate large amounts of astaxanthin (Kobayashi et al. 1992; Orosa et al. 2001).

Haematococcus production is significantly more expensive than the production of *D. salina*, and therefore the *Haematococcus* astaxanthin is too expensive to be used as a pigmenter in the culture of salmonids, even though it is a very effective pigmenter (Sommer et al. 1992). The main markets therefore are for use as an antioxidant for human health.

Microalgae are also potential sources of other carotenoids such as zeaxanthin, lutein and canthaxanthin, however none of these has as yet been developed as a commercial product (Orosa et al. 2000; Blanco et al. 2007; Granado-Lorencio et al. 2009).

3.2 Fatty Acids

Over the last 30 or so years microalgae been intensively studied as potential sources of long-chain polyunsaturated fatty acids, especially eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (AA) and several potential candidate species have been identified (Borowitzka 1988; Ratledge 2010). The only current commercial production is by the heterotrophic culture of the dinoflagellate *Crypthecodinium cohnii* to produce DHA for use in infant formula and other applications (Mendes et al. 2009). The fungi-like thraustochytrids, *Thraustochytrium* and *Schizochytrium* which are related to the dinoflagellates, are also used to produce both EPA and DHA heterotrophically (Barclay et al. 2010).

Many phototrophic algae have been proposed as potential sources of long-chain fatty acids. These include *Lobosphaera* (*Parietochloris*) *incisa* which has a high AA content (Bigogno et al. 2002), *Nannochloropsis* spp and *Phaeodactylum tricornutum* with a high EPA content (Chrismadha and Borowitzka 1994; Chini Zitelli et al. 1999), and *Isochrysis* sp. T. Iso (= *Tisochrysis lutea*) with a high DHA content (Tzovenis et al. 1997), however the productivity of these fatty acids is much lower than in the fungi-like organisms grown heterotrophically. It remains to be seen whether these photoautotrophic algae can be developed into commercially viable sources of these long-chain polyunsaturated fatty acids.

3.3 Other Products and Processes

Microalgae are also grown to produce stable isotope-labelled compounds for use in medical diagnostics and for research (Radmer 1996) or for the production of bioplastics (Samantaray and Mallick 2012). Phycobilin pigments are also produced

from cyanobacteria and the red unicell *Porphyridium* for use as fluorescent markers in immunology and also in cosmetics (Arad et al. 1996; Eriksen 2008). There is also the potential to use microalgae to carry out chemical transformations as a potential step in the production of useful compounds (Rao et al. 1999; Tripathi et al. 2002) or for the breakdown of xenobiotics such as pesticides or hormones (Pollio et al. 1996; Ghasemi et al. 2011).

Microalgae also produce many novel bioactive compounds (Borowitzka 1995) and the range of bioactivities reported from extracts of microalgae in the last 50–60 years includes antioxidant (Goiris et al. 2012; Klein et al. 2012), antibiotic (Kellam and Walker 1989; Nagai et al. 1995; Volk and Furkert 2006), antiviral (Hayashi et al. 1996; Boyd et al. 2009), anticancer (Gerwick et al. 1994; Morlière et al. 1998; Schwatsmann et al. 2001), anti-inflammatory (Baker 1984; Raposo et al. 2013), antihypertensive (Yamaguchi et al. 1989; Samarakoon et al. 2013) and other activities. Many microalgae, especially the dinoflagellates and the cyanobacteria, also produce potent toxins which have some applications in research and as lead compounds for the development of drugs. However, despite decades of research there are only a few drugs from marine organisms currently approved or in clinical trials, and all originate from organisms other than the microalgae (Mayer et al. 2010). However, some of these compounds appear to have a microalgal (mainly cyanobacterial) origin (Gerwick and Moore 2012). An example of this is dolastatin 10, originally isolated from the sea hare *Dolabella auricularia* (Pettit et al. 1987), but which was later found to originate from the cyanobacterium *Symploca* sp., a component of the diet of the sea hare (Luesch et al. 2001). Dolastatin 10 is the basis for the FDA approved drug Brentuximab Vendotin (Katz et al. 2011) for use in Hodgkin's lymphoma and anaplastic large cell lymphoma.

4 Biofuels

Interest in the use of microalgae as a source of sustainable renewable fuels and bioenergy has burgeoned again in the last decade and is again the focus of extensive research and development. It presents particular problems for algal biotechnology, mainly because of the very low value of the product (e.g. biodiesel) and the extremely large scale of production required (Borowitzka and Moheimani 2013b). The concept of using algae to produce biofuels such as biodiesel is not new (Harder and von Witsch 1942), and a major research effort occurred in the early 1990s, especially in the USA and in Japan (see Sheehan et al. 1998; Borowitzka 2013b for summaries), and now even greater efforts are under way. Although technically feasible, the major barrier to commercialisation remains in the fact that production of algal biofuels still is far too expensive (Stephens et al. 2010; Sun et al. 2011) and major improvements are needed in all steps of the process from algae culture to harvesting and fuel production (Fon Sing et al. 2013). The perceived advantages of

algae over terrestrial energy crops are that species exist which can be grown on a saline water source. Furthermore, although higher plant energy crops and algae both require fertilizer for maximum productivity, the effective utilisation of the applied fertilizer is much better for algae as they are grown in enclosed containers such as ponds or photobioreactors. However, large scale production of biofuels from algae will require large amounts of nutrients (N & P) and thus will compete for these with the fertilizer requirements of agriculture, even is wastewater is used. The key issues and requirements for environmentally and commercially sustainable microalgal biofuel production have been recently reviewed by Borowitzka and Moheimani (2013b).

Although much of the effort has been on lipid-rich microalgae for the production of biodiesel, alternatives such as continuous hydrocarbon production using *Botryococcus braunii* are being explored (Moheimani et al. 2014; Griehl et al. 2014). An alternative to using the lipids and hydrocarbons to produce liquid fuels exist where the whole biomass is converted to a fuel feedstock by hydrothermal liquefaction (Eboibi et al. 2014). Hydrogen production using microalgae has also been suggested (Benemann 2000). However, most studies have been limited to small indoor laboratory studies with only a few outdoor studies and the process is far from being scalable and commercially viable (Dasgupta et al. 2010; Geier et al. 2012).

5 Food and Fertilizer

Microalgae have also often been considered as an important protein source especially well suited for developing countries (Soeder 1976). However, despite extensive research in the 1980s on cyanobacteria and green algae in India (Becker and Venkataraman 1982), Thailand (Payer et al. 1978), Peru (Castillo et al. 1980) and elsewhere, this has not become a reality except at village-scale in parts of India (Jeeji Bai 1998). As with microalgae for biofuels, it is the high cost of microalgal protein compared to other sources combined with the unconventional nature of the food that has limited its widespread application (Babu and Rajasekaran 1991).

On the other hand, the use of nitrogen-fixing cyanobacteria as a fertilizer, especially in rice cultivation, is practiced widely in Asia, especially in India and Vietnam (Kulasooriya 1998; Vaishampayan et al. 2001). Microalgae such as *Chlorella* spp. have also been used as soil conditioners to improve soil water retention and nutrient availability to plants (Metting 1988). The possibility of producing nitrogen fertilizer by the culture of N_2-fixing cyanobacteria is also of great interest (Benemann 1979; Moreno et al. 2003; Silva and de Jesus Silva 2013).

Aside from their use as biofertilizers, cyanobacteria and microalgae have been shown to produce plant growth-promoting phytohormones (Stirk et al. 2002; Hashtroudi et al. 2013).

6 Genetic Improvement

Genetic improvement of microalgae to produce strains with superior characteristics such as better light utilisation efficiency or enhanced product formation potentially can be carried out by various means such as:

Breeding
Mutagenesis
Genetic engineering

In those species of microalgae which undergo sexual reproduction there is the potential to use basic 'breeding' methods to enhance desirable traits. The best understood system for this approach is the green alga *Chlamydomonas* having strong + and – mating types allowing genetic manipulation through breeding (Lewin 1951; Pröschold et al. 2005). *Dunaliella salina* has a similar mating system, but as yet no strong mating types have been isolated (Ruinen 1938; Huismann and Borowitzka, unpublished results). However, for most microalgae their life-cycles and sexual reproduction (if any) are poorly understood or sexual reproduction has never been observed and no breeding systems exist. Furthermore, breeding, possibly combined with mutagenesis, is a slow process.

Mutagenesis has been used to produce carotenoid-overproducing strains or strains with other desirable characteristics for several microalgae species such as *D. salina* (Shaish et al. 1991; Jin et al. 2003), *H. pluvialis* (Tripathi et al. 2001; Chen et al. 2003), *Chlorella* spp. (Cordero et al. 2011) and others (Suzuki et al. 1999; Doan and Obbard 2012).

6.1 Genetic Engineering, Molecular Biology and the 'Omics'

Advances in molecular biology of the microalgae in the recent decades, accompanied by developments in metabolomics, genomics, transcriptomics and proteomics as well as in bioinformatics, are providing powerful toolkits which can be applied to the development of new and improved algal strains, products and processes. The genomes of a number of microalgae species have been fully sequenced [e.g. the diatoms *Thalassiosira pseudonana* (Armbrust et al. 2004) and *Phaeodactylum tricornutum* (Bowler et al. 2008), the chlorophyte *Ostreococcus tauri* (Derelle et al. 2006) and several species of the eustigmatophyte *Nannochloropsis* (Pan et al. 2011; Radakovits et al. 2012; Vieler et al. 2012; Jinkerson et al. 2013)], and the sequencing of other microalgae such as *Dunaliella* is in progress. Metabolomics and proteomics are assisting the annotation of the sequenced genomes and the assessment and refining of metabolic network models which will aid efforts to modify selected metabolic pathways (May et al. 2008; de Oliveira Dal'Molin et al. 2011). Similarly, studies of the transcriptomics of microalgae are providing insights into metabolic pathways and their regulation which will provide important aids to enhancing the

production of specific compounds in algae (Cadoret et al. 2012; Gao et al. 2012; Ioki et al. 2012; Kristof and Sheshadri 2012; Lv et al. 2013).

Genetic engineering to produce transgenic cyanobacteria is well developed and, thanks to their similarity to other prokaryotes such as *Escherichia coli*, many methods for genetic manipulation of these organisms so that a wide range of transgenic cyanobacteria have been created since the 1970s (Heidorn et al. 2011; Ruffing 2011). The development of molecular genetic techniques for the in vivo analysis of gene function and regulation, the manipulation of endogenous genes, and the introduction and expression of foreign genes in eukaryotic algae is in a much earlier stage of development and so far is limited to a small number of species (Cadoret et al. 2012; Rasala et al. 2013). The best developed 'molecular toolkits' are for the green algae *Chlamydomonas reinhardtii* and *Volvox carterae*, and the diatom *Phaeodactylum tricornutum*. However, DNA transformation has also been achieved for more commercially important algae species such as *Haematococcus* (Steinbrenner and Sandmann 2006), *Dunaliella* (Wang et al. 2007; Li et al. 2008). The potential of these molecular techniques was first demonstrated by the conversion of the obligate photoautotrophic diatom *P. tricornutum* into a heterotroph through the introduction of a human glucose transporter gene (Zaslavskaia et al. 2001).

By far the best studied alga is *Chlamydomonas reinhardtii* with nuclear transformation first being achieved in the late 1980s, soon followed by chloroplast transformation (Kindle and Sodeinde 1994; Purton 2007). There now exist a range of transformation techniques, selectable markers and reporter genes, sophisticated vectors for foreign gene expression and for silencing of target genes by RNA interference (RNAi), and DNA tagging techniques for identifying novel genes, promoters, and enhancer elements for this alga. The use of *C. reinhardtii* as a 'cell factory' for producing proteins, including therapeutic proteins is being studied intensively but only transgene expression in the chloroplast has led to protein accumulation to economically viable levels (Rasala and Mayfield 2010). Although transgene expression from the nuclear genome offers several advantages over chloroplast expression, such as glycosylation and other post-translational modifications and heterologous protein-targeting to sub-cellular locations or for secretion (León-Bañares et al. 2004), the molecular mechanism(s) for the poor transgene expression from the nuclear genome are not yet completely understood. However, some progress is being made, and recently Rasala et al. (2012) a xyalanase-excreting strain of *C. reinhardtii*.

Molecular methods are also being developed to improve the yield of particular products of commercial interest such as carotenoids or lipids.

In order to achieve commercial production and use of transgenic algae and their products much research and development is still required. Furthermore, the potential environmental or other risks of large-scale production still need to be evaluated (Flynn et al. 2013). The current regulations concerning the use of transgenic plants were not designed for organisms such as algae which have markedly different reproductive and dispersion mechanisms compared to higher plants.

7 Algae Production Systems

The first step in the commercial application of algae is the cultivation of the algae to produce the desired biomass at the required scale and cost. For microalgae the available cultivation systems can be broadly classified as 'open' and 'closed' systems (Borowitzka 1999). In open systems the algae cultures are directly exposed to the environment, whereas in closed systems the cultures are contained within a more or less sealed, and usually transparent, container. Currently almost all commercial microalgae production is in open systems, with the paddle wheel mixed raceway pond being the most common. The other main types of open systems are the extremely large, unmixed shallow ponds used for the cultivation of *D. salina* in Australia, the central pivot ponds used for the cultivation of *Chlorella* in Asia, the very shallow sloping cascade ponds in the Czech Republic for the cultivation of *Chlorella*, and the deep tank systems used in the production of microalgae for aquaculture feed. More details of open systems can be found in recent reviews (Borowitzka 2005b; Borowitzka and Moheimani 2013a). The reason for the popularity of these systems is that the cost of producing algal biomass in such systems is considerably lower than cultivation in closed photobioreactors. Indeed, the major challenge to the development of any new commercial products or processes using microalgae is the ability to produce the algal biomass at a low cost. This has recently led to a re-evaluation of the design of raceway ponds to improve the efficiency of water circulation and thus reduce operating costs (Chiaramonti et al. 2013; Liffman et al. 2013). Commercially viable production also requires the culture to be reliable for long periods with a high productivity.

Although a plethora of different designs and configurations of closed photobioreactors has been developed, the cost of production of algal biomass in these reactors is significantly higher than the cost of production in raceway ponds. The most common types of closed photobioreactors are various configurations of tubular photobioreactors and panel photobioreactors (Pulz 2001; Chini Zittelli et al. 2013). Tubular photobioreactors are used for the commercial production of *Haematococcu*s in Israel and *Chlorella* in Germany and the *Haematococcus* plant at Kibbuz Ketura, Israel, has over 300 km of glass tubing in the culture system. As yet there is no significant commercial production of microalgae using panel-type photobioreactors, although some of these designs are showing promise (e.g., Rodolfi et al. 2009). For the production of microalgae for aquaculture large bags of up to 10,000 L or large bubble-column reactors are often used. Other closed photobioreactor types include the dome-shaped photobioreactors (Sato et al. 2006) used in Hawaii and internally-lit vat-type photobioreactors both of which are used by Fuji Chemicals for the production of *H. pluvialis* and astaxanthin.

There is ongoing debate on the relative merits and disadvantages of 'open' *vs*. 'closed' culture systems (Grobbelaar 2009), but as yet closed photobioreactors have proven suitable only for extremely high value products because of the higher capital and operating costs. Open ponds, such as raceway ponds, have been shown to be suitable for the long-term (i.e. greater than 6 months) culture of a range of freshwater, marine and halophilic algae. In most cases these algae grow in a selective envi-

ronment such as high salinity (e.g., *D. salina*), high alkalinity (e.g., *Arthrospira*) or high nutrients (e.g., *Chlorella*, *Scenedesmus*, *Phaeodactylum*), but other species also have the capability of outcompeting any potential contaminating organisms (e.g. *Pleurochrysis carterae* – Moheimani and Borowitzka 2006; *Tetraselmis* sp – Fon Sing and Borowitzka 2015), and have been grown successfully outdoors for long periods. Similarly, not all algae species can be grown reliably and for long periods in closed photobioreactors where sensitivity to shear created by the circulation system and the predilection of many species to 'stick' to the photobioreactor walls limit the choice of species which can be cultured successfully. Closed photobioreactor systems also have a shorter light path which means that the algae can be grown at a higher cell density potentially reducing harvesting costs.

Irrespective of the culture system used, the reliable high-productivity culture of microalgae requires the selection of strains which are well suited to the environmental conditions, especially the temperature range experience over the whole year that they will be exposed to at the place where they are being grown (Borowitzka 2013c). Furthermore, since light is the energy source for all microalgae (except those grown heterotrophically) the efficient use of the available light is all important and is a critical factor in the design of the culture systems and the operational management of the cultures (Richmond 1996). There is a strong interaction between temperature and light and microalgae use the available light most efficiently when the temperature is optimal (Collins and Boylen 1982). As almost all large-scale cultures are also carbon limited the supply of additional C, usually as carbon dioxide but sometimes also as bicarbonate, is important to achieve high productivities (Moheimani 2013). An excellent overview of the main biological principles of the mass cultivation of photoautotrophic microalgae has recently been published by Richmond (2013).

8 Conclusion

Microalgal biotechnology is still in the relatively early stages of development. Although commercial production of microalgae and microalgal products has been carried out for over 40 years, new products and processes using a wider suite of species are being developed, as are new and improved cultivation systems, harvesting methods and in further downstream processing. New markets and applications are also being developed. Furthermore, the potential of molecular biology and the new 'omics' is still being explored and is also expected to become an important part of the future expansion and success of algal biotechnology.

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Harmful Algal Blooms

Peimin He

1 Introduction

As a natural component of the aquatic food chain, algae are photosynthetic organisms varying from small, single-celled forms to complex multi-cellular forms. In an aquatic ecosystem cycle, the aquatic life is maintained in balance by predator–prey relationships. When conditions more favorable for the reproduction and growth of algae, the rapid growth and accumulation of algae population upon aquatic surface which will upset the balance. Such rapid increases in the algae population are called algal blooms, with some algal cells rapidly increase in numbers to the extent that it dominates the local planktonic or benthic community and form high numbers of cells.

An algal bloom is typically not harmful to people and environment, but some species may cause harm, these are known as Harmful Algal Blooms (HABs). HABs are aquatic phenomena caused by the rapid growth and accumulation of certain microalgae, which can lead to marked discoloration of surface waters, and severe impacts on public health, commerce, and the environment. HABs have been used by the scientific community to describe events where the concentration of one or several harmful algae reach levels which can cause harm to other organisms in the sea, e.g. by killing fish and shellfish, or cause accumulation of algal toxins in marine organisms which eventually harm other organisms who eat the toxic species, e.g. accumulation of algal toxins in shellfish that become toxic to human consumers (Andersen 1996). An algal bloom often results in a color change in the water; most common ones are red, green and brown. These blooms are referred to as red, green or brown tides. Once HABs were all referred to as “red tides”, because certain phytoplankton species contain reddish pigments and the water appears to be colored

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D. Sahoo, J. Seckbach (eds.), *The Algae World*, Cellular Origin, Life in Extreme Habitats and Astrobiology 26, DOI 10.1007/978-94-017-7321-8_12

red. But the description has become a misnomer because not all HABs are red. It can be various hues depending on the species and the photosynthetic pigments they contain. Some HABs has no color, but also impart toxicity at very low densities generally less than 1,000 cells per liter. Others may produce deleterious effects even if toxic algae are present in low concentrations. Some are spatially extensive and persistent, while others are patchy and episodic (Smayda and Trainer 2010). And not all HABs are caused by microalgae but also by cyanobacteria, and protozoan agents. In general, the term HABs is widely used to describe any bloom that is harmful or which has potential harmful effects.

Although not all algal blooms in the ocean produce highly visible effects nor are all blooms harmful, they nonetheless affect life in the ocean and on land in both beneficial and harmful ways. Virtually every coastal nation is affected by HABs (Hallegraeff 1993). HABs are considered an environmental hazard. HABs can pose health hazards for humans or animals through the production of toxins or bioactive compounds. They also can cause deterioration of water quality through the buildup of high biomass, which degrades aesthetic, ecological, and recreational values. Humans and animals can be exposed to marine algal toxins through their food, the water in which they swim, or sea spray. HABs can also result in lost revenue for coastal economies dependent on seafood harvest or tourism, disruption of subsistence activities, loss of community identity tied to coastal resource use, and disruption of social and cultural practices (Lopez et al. 2008).

Scientists worked for decades to understand how HABs develop, change and dissipate, but they have not fully revealed what triggers the beginning of HABs – a mix of biology, chemistry and physics that appears to be different for each bloom. In order to reduce the impacts of HABs on public health, the economy, and coastal ecosystems, it has to understand the causes and effects of HABs and to track harmful toxins to prevent health, economic, and marine resource losses. Three well-documented long-term global trends and patterns in progress have attracted attention as the mechanisms potentially underlying the HAB phenomenon: increasing nutrient enrichment of global coastal waters and epicontinental seas; global warming and associated climatic perturbations; global dispersal and redistribution of bloom species in ballast water. Each has been hypothesized to provide the ecological changes or disturbance driving the global HABs phenomenon (Hallegraeff 1993; Feki et al. 2012). The specific causes of HABs are complex, vary between species and locations, and are not all well understood. In general, algal species proliferate when environmental conditions (e.g., nutrient and light availability, temperature, and salinity) are optimal for cell growth. Other biological (e.g., vertical migration, grazing, viral infection, and parasitism) and physical (e.g., transport) processes determine if enhanced cell growth will result in biomass accumulation. The challenge for understanding the causes of HABs stems from the complexity of these biological, chemical, and physical interactions and their variable influence on growth and bloom development among different species. Further, environmental control and genetic variation of toxin production, vertical migration, life cycles, and cell physiology add to the challenge of understanding HABs dynamics. The complexity of interactions between HABs, the environment, and other plankton complicate the predictions of when and where HAB events will occur (Masó and

Garcés 2006; Lopez et al. 2008). This has led to heightened scientific and regulatory attention, and the development of many new technologies and approaches for research and management (Anderson et al. 2012).

On the other hand, some harmless algal blooms have some benefits to aquatic ecosystems and environment. Harmless algal blooms provide large concentrations of algae and increase productivity of the entire food web which they support, so as to produce organic compounds needed by higher organisms, ranging from oysters, clams, and mussels to human beings. For this reason, algae blooms can help feed and grow fisheries and cetacean populations where algal blooms occur. More algae in the water mean that more carbon dioxide is used from the atmosphere and that more oxygen is released into the atmosphere. Oxygen is necessary for many living things, including humans. Some researchers even made an amazing discovery of artificially creating absorb carbon dioxide. Blooms might act the way of cleansing the ecosystem to suck up damaging carbon dioxide, then sinks, locking away the harmful greenhouse gas for hundreds of years, so as to save the world from global warming (Macfarlane 2009).

2 Harmful Algal Blooms

Harmful Algal Blooms (HABs) are seasonal phenomenon of rapid growth and accumulation of microscopic phytoplankton over rather short time under some right environmental conditions, which usually resulting in red coloration of the surface water (Anderson 1994). During algal blooms outbreak, the algal cells divide faster than they are consumed or die and they accumulate near the surface so that the water takes on the color of the algal pigments. It is widely believed that the frequency and geographic distribution of marine HABs have been increasing worldwide. Most HABs are harmful because oxygen is removed from the water at night and during decay of the plankton, and because the blooms are unsightly and/or odorous (Hallegraeff 1993).

2.1 The Cause of Harmful Algal Blooms

There is, as yet, no general theory adequate to explain the global HAB phenomenon. But it is generally believed that natural character of algal species, human activities and climate changes are thought to contribute to Blooms events.

2.1.1 The Harmful Algae Species

HABs species are a small subset of algal species that negatively impact humans or the environment. According to Intergovernmental Oceanographic Commission (IOC) analyses, the number of marine algal species was approx. 5,000, in which

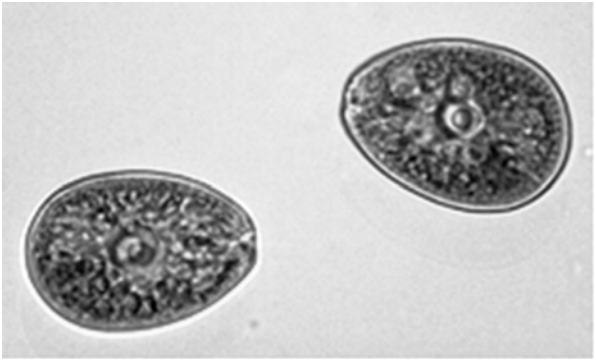

Fig. 1 *Prorocentrum lima* (Ehrenberg) Dodge, 1975

approx. 300 would discolor the water and 75 produce potent toxins at high concentrations (Andersen 1996). The harmful species are distributed among all major taxonomic groups- dinoflagellates, diatoms, haptophytes and cyanobacteria, and some silicoflagellates (Fig. 1).

The algal have been on earth for a long time. Dinoflagellates are ancient, single-celled, eukaryotic organisms that can exist in benthic, parasitic, symbiotic, and free-living forms; ocean currents can transport the latter easily. It has at least one resting form or cyst in their life cycle. The cysts may serve as the seeds for the red tides because they are the renewal of the motile phase of the dinoflagellate when the environmental conditions are appropriate; the motile forms create the blooms and the natural toxins (Kirkpatrick et al. 2004). These cysts facilitate the spread of toxic red tides into new areas and easily transported into new waters by ocean currents, fish, or even humans (via ballast water discharge), and then deposited as a "seed" population that colonizes a new area.

The cells or cysts via anthropogenic activities around the world in ships' ballast water are transported and discharged in areas where they did not previously occur (Hallegraeff and Bolch 1992). Toxic dinoflagellate species that are not endemic to an area can be inadvertently introduced when their cysts are discharged with the ballast tank sediments of bulk container ships these species, which can affect fish- and shellfish-farms, pose a serious threat to public health and aquaculture (Hallegraeff and Bolch 1991). Others are distributed accidentally through the transfer of shellfish for aquaculture. The detection of a harmful species in one area, not previously identified, is a common occurrence. The proposed impacts of this broadcasting on HAB events, therefore, are 'emigration' hypotheses (Smayda 2002).

Brown tide blooms, caused by very small golden brown algae will result in yellow–brown discoloration of the water. Brown tide blooms were caused by the pelagophytes *Aureococcus anophagefferens* Hargraves and *Aureoumbra lagunensis*. The first *Aureococcus anophagefferens* bloom appeared in eastern USA Long Island waters in the late spring of 1985; then blooms have been reported in New Jersey, Delaware, Maryland, has caused destructive brown tide blooms in northeast and

mid-Atlantic U.S. estuaries (Nuzzi and Waters 2004). In 1997, the *Aureococcus anophagefferens* bloom was detected for the first time in Saldanha Bay, South Africa (Probyn et al. 2001). In 2009, the *Aureococcus anophagefferens* bloom was detected along China's east coast on the Bohai Sea (Zhang et al. 2012).

The brown tide blooms have been associated with considerable ecosystem damage in USA and caused considerable environmental damage and economic loss. It have been linked with the starvation and mortality of bay scallops in Long Island, losses of seagrass and reductions in reproductive potential and growth in many shellfish (mussels, hard clams and scallops) decrease of the biomass and diversity of benthic invertebrates Brown tide is a poor food for bivalves and can be potentially toxic (Wazniak and Patricia 2004).

Numerous hypotheses and scenarios have been proposed to explain the initiation of these massive blooms. Harmful algal blooms in coastal waters have been commonly attributed to nutrient loading, some physical and chemical factors were related with these massive accumulations of algae. Bloom initiation and maintenance within Suffolk County appear to be related to *Aureococcus anophagefferens*' ability to use dissolved organic nitrogen (DON) during periods of limited dissolved inorganic nitrogen (DIN) availability. Factors controlling DIN availability include groundwater influx related to meteorological conditions, introduction of septic leachate from on-site wastewater treatment systems, and biological removal (Nuzzi and Waters 2004). *Aureococcus* can achieve maximum growth rates at low light and nutrient levels and are able to utilize a wide variety of organic nutrient (N and P) substrates. Brown tides are promoted by positive feedback mechanisms involving the ability of these algae to grow competitively at low nutrient and light levels, their low rates of grazing mortality, and the associated low grazing-mediated recycling of nutrients. The latter further reduces nutrient concentrations, which further favors competitive population growth of the brown tide species, thereby promoting blooms (Gobler and Sunda 2012).

2.1.2 Nutrients Load to Algal Blooms

Nutrients can come from many natural and manmade sources. Blooms offshore might start with help from nutrients released from sediments or from upwelling. Ocean circulation patterns bring nutrient-rich water up from the bottom. Red tides likely even free up nutrients when they kill fish, which are rich in nitrogen and other nutrients. More manmade nutrients being discharged into the sea – in the form of sewage, animal manure and fertilizers nutrient over-enrichment is considered a prime promoter of HABs and resulted in eutrophication problems (Anderson et al. 2008; Gobler et al. 2012). These inputs not only originate from land but also increase in aquaculture activities can result in increased nutrient loading in certain areas. The increase of nutrient creates a more favorable, nutrient-rich environment in coastal waters in which certain groups of phytoplankton can thrive (Jickells 1998). This over-enrichment leads to diverse impacts including increase turbidity with a subsequent loss of submerged aquatic vegetation, oxygen deficiency, disruption of

ecosystem functioning, loss of habitat, loss of biodiversity, shifts in food webs, and loss of harvestable fisheries and HABs (Rabalais and Nixon 2002). Increasing in nutrient loading have been linked with the development of large biomass blooms, leading to anoxia and even toxic or harmful impacts on fisheries resources, ecosystems, and human health or recreation. Despite many studies pointed nutrients are assimilated differentially by different groups of species, the relationships between nutrient delivery and the development of blooms and their potential toxicity or harmfulness remain poorly understood (Anderson et al. 2002).

2.1.3 Climate Change Impact on HABs

Phytoplankton growth is controlled by factors such as flushing, salinity tolerance, light, nutrients and grazing. Climate change is one of the greatest environmental changes in the world. Both natural climatic variability on a global scale (i.e., the El Niño/Southern Oscillation) and anthropogenic climate variability can provoke changes in the geographical range of the species with increased frequency, duration, and geographic scope (Glibert et al. 2001). Temperature, stratification, upwelling and ocean circulation patterns are climate change factors influenced red tide bloom dynamics.

Temperature and salt concentration determine the density and currents of water. Climate change brings dramatic increases warmer air and ocean temperatures, impacted the ocean include changes to circulation patterns, the frequency of extreme events such as hurricanes, sea-level rise, and ocean acidification resulting from changes to seawater carbon chemistry (Holgate 2007). Global warming has strongly implicates important in frequency of red tides and other HABs. In some areas, sediment disturbance by storms may be important for sustained 'seeding' of blooms. Cold water sinks from the surface. Other water moves across to replace it. Eventually, water at the surface is replaced by water that has risen, or upwelled, from the bottom to the surface somewhere else in the ocean. These upwellings bring nutrient-rich waters to the top. This will have a direct impact on the duration of hypoxic conditions in bottom waters of systems, nutrient cycling within the hypoxic zones (i.e. P, Fe) as well as have a significant impact on competition within the plankton community in surface waters and can trigger algae blooms. Increased warming of surface waters will impact the viscosity and stratification of water columns. Pacific and Atlantic red tide histories show a similar late glacial-early Holocene succession of sustained high production of non-toxic and toxic red tide blooms (Mudie 2002).

2.1.4 Other Factors Affect Harmful Algal Blooms

Manmade physical destruction of habitats, invasions of introduced species, overfishing and aquaculture may foster development of certain HABs. Physical destruction of habitats, such as modifications to water flow has been acknowledged as a likely factor contributing to the increased occurrence of high biomass HABs

(Tilstone et al. 2000). Artificial physical relocation of ports, breakwaters, semi-closed beaches and coastal sheltered areas will change water renovation and flushing rates which are key factors for the algal growth.

Increased aquaculture production and overfishing that alters food webs and may permit harmful species to dominate algal communities. Overfishing of large vertebrates and shellfish, then the decreasing biomass of filter feeding organisms due to over fishing was the first major human disturbance to all coastal ecosystems examined and the alteration of food webs. Historical data show that time lags of decades to centuries occurred between the onset of overfishing and consequent changes in ecological communities (Jackson et al. 2001).

2.2 The Environmental Consequences of Harmful Algal Blooms

Red tide blooms environmental consequences are variable in the scope and severity. There can be classified two types: the high-biomass producers, which are nontoxic to humans and wildlife but degrade ecosystems by forming such large blooms that they alter habitat quality through overgrowth, shading, or oxygen depletion (hypoxia), thus adversely affecting corals, seagrasses, and bottom-dwelling organisms; the toxic events, which cause illness or death in humans and other organisms, including endangered species.

2.2.1 The Aquatic Environment Impact from HABs

High levels of algae may raise the pH of waterbodies. Elevated pH levels are thought to be a by-product of photosynthetic uptake of carbon dioxide. Higher pH levels may be noted late on sunny summer afternoons after photosynthesis as consumed carbon dioxide throughout the day. After sunset, pH levels may fall noticeably since photosynthesis has ended. These extreme fluctuations in pH stress sensitive aquatic life.

The algal overgrowth would low the transparency in the water and cause water discoloration. Sometimes they cover vast areas of the ocean surface and can extend down to the ocean floor; masses of these algae can restrict access for fishing or recreational uses of aquatic system and can block irrigation and navigation channels.

As more algae grow, there are more dead algae to be decomposed. Decomposition by bacteria consumes oxygen and may decrease or even completely deplete dissolved oxygen contents. When the algal die they decompose due to bacterial and fungi activity; in the process oxygen is consumed and the nutrients are released together with carbon dioxide, energy and the emission of unpleasant gases such as methane and hydrogen sulfide due to its decomposition or decay.

2.2.2 The Negative Impacts to Maine Life

Because of nutrients competition, alters the composition of the fish fauna from more to less desirable species and shifts in the abundance and significant reduction in diversity of species (biodiversity) of aquatic organisms. Red tides kills fish and other marine life through suffocation by depriving them of oxygen and producing toxins that can paralyze marine life.

Some harmful algae are non-toxic to humans and wildlife but cause harm to fish and invertebrates by damaging or clogging their gills. The blooms of species which contain barbs that lodge among gill tissues of fish, causing death. Such blooms can cause a great deal of financial damage by killing farmed fish, which are grown in crowded aquaculture pens. Red tide blooms with extremely high levels of algae degrade ecosystems by forming such large blooms that they can adversely affect corals, seagrasses, and organisms living on the sea-bottom. Red tide blooms can generate overgrowth shading to prevent sunlight from reaching beneficial algae and seagrasses limiting their plant growth or even causing them to die. As more algae grow within the aquatic system, there are more dead algae to be decomposed. Decomposition by bacteria consumes oxygen and may decrease or even completely deplete dissolved oxygen contents. Complete lack of oxygen is a condition known as anoxia which can cause fish kills. Nearly all fishes are susceptible, depending on the density of the bloom, the length and mode of exposure, and other factors.

Toxic algae release very powerful toxins which are poisonous at very low concentrations. Marine life is exposed to brevetoxins by eating them, breathing them or touching them. The toxins can also pass through cell membranes, including the blood-brain barrier and skin tissue (Kemppainen et al. 1991). Different forms of marine life vary in their reaction to the toxins. The toxins produce negative effects on the liver of life stock at minimal concentrations but they can lead to the death of other animals. *Karlodinium micrum* (Dinophyceae) produces at least one compound which is hemolytic, ichthyotoxic and cytotoxic to kill fishes that paralyze the fish's gills and cause its death from respiratory failure that suffocation due to gill inflammation (Deeds et al. 2002). Fish kills are both an early warning sign for humans and a sad hallmark of red tide blooms. Fish are exposed to brevetoxins by swimming through blooms and ingesting forms of marine life that have become contaminated with toxins. They are thought to be killed through lack of muscle coordination and paralysis, convulsions and respiratory failure (Kirkpatrick et al. 2004). PSP-causing dinoflagellates and other toxic species may disrupt food webs by harming zooplankton, larval fish and their predators such as fish, marine birds and mammals (Mudie et al. 2002). Red tide toxins are thought to affect invertebrate animals, such as crabs, shrimp, and lobsters. Dolphins, manatees and marine birds can suffer the effects of red tide.

2.2.3 The Human Health Impacts from Harmful Algal Blooms

Toxic algal species can cause a variety of human ailments, contracted either through inhaling airborne toxins, skin contact or, more commonly, eating contaminated shellfish. Only a few red tide species actually produce toxins that are poisonous to people and marine animals. The most well-known red tide toxins are generically referred to as: ciguatera fish poisoning (CFP), neurotoxic shellfish poisoning (NSP), paralytic shellfish poisoning (PSP), diarrheic shellfish poisoning (DSP), and amnesic shellfish poisoning (ASP). These toxins may cause amnesia, stomach cramps, nausea, memory loss, paralysis and even death. The risk of poisoning from consumption of fish or shellfish is serious and of concern to public health authorities in all coastal environments.

Eating shellfish tainted by a red tide can cause food poisoning, vomiting and other nastiness and produce airborne toxins that cause throat and eye irritation, coughing, bloody noses and asthma fits. When red tides hit some coastal areas all events are cancelled and residents are encouraged to wear surgical masks.

Brevetoxins are tasteless, odorless and heat and acid stable. They cannot be easily detected nor removed by food preparation procedures (Baden et al. 2005; Kirkpatrick et al. 2004). To date, shellfish are the primary vector, or pathway, for human brevetoxin exposure. Shellfish reported to be associated with neurotoxic shellfish poisoning (NSP) when contaminated with brevetoxins include oysters, clams, scallops and other filter feeders. Typical NSP symptoms include gastrointestinal symptoms (nausea, diarrhea, and abdominal pain) accompanied by occasional neurological symptoms (headache, vertigo, incoordination). In severe cases respiratory failure has been reported (Kirkpatrick et al. 2004). Humans can also be exposed to brevetoxins through inhalation. The cells of *Karenia brevis*, a kind of dinoflagellates, are fragile organisms that are easily broken open by wave action along the beach. When this happens, the brevetoxins are released and can become aerosolized. When a red tide bloom is near the shoreline, the aerosol of contaminated sea spray will contain toxins that can be carried inland with the prevailing winds. Studies to date show the toxins can travel at least a mile (1.6 km) inland from the shore, and the distance is highly variable and dependent upon environmental conditions, such as wind speed and direction (Fleming et al. 2007). Inhalation of aerosolized brevetoxins causes respiratory irritation, bronchial constriction, coughing and a burning sensation in the eyes, nose and throat.

The detrimental effects of a harmful algal bloom can range from cell and tissue damage to organism mortality, and can be caused by a number of mechanisms, including toxin production, predation, and particle irritation, induced starvation, and localized anoxic conditions. As a result, a bloom may affect many living organisms of the coastal ecosystem, from zooplankton to fish larvae to people. Toxins produced by HA species are generally tasteless and odorless, and heat- and acid-stable.

There is no known cure for most of biotoxin and consumption of large amounts in shellfish or lobster is fatal to humans. Toxins produced by red tide species are generally tasteless and odorless, and heat- and acid-stable. Normal food preparation methods do not prevent intoxication.

2.3 How to Control Harmful Algal Blooms

Although many researches has been done for decades to understand how algal blooms develop, change and dissipate, but it is a mixture of biology, chemistry and physics that appears to be different for each bloom, so it has not fully revealed what triggers the beginning of a bloom. Effort and large budgets were devoted to the elucidation of the biology and ecology of causative organisms and their blooming mechanisms, modeling and mitigation methods.

In order to mitigate the impacts of HABs, it is very essential to detect, monitor and forecast their development and movement. The counteraction techniques are roughly divided into two categories, indirect and direct methods (Shirota 1989). Indirect methods focus on prevention (e.g., reduce nutrient inputs to the ocean), and/or the minimization of bloom impacts (e.g., early warning, cell and toxin monitoring, harvesting bans), are basically important as prevention of red tide occurrences on a long-term scale (Shirota 1989).

2.3.1 Monitoring of HABs

One way to mitigate the potential impact of these blooms is to improve their detection. Historically, detection of these organisms has relied on microscopic methods for distinguishing morphological characters, but microscopic determination of the phytoplankton species composition and cell density is time-consuming and requires a high degree of taxonomic expertise. Current advances in the development of molecular probes are enabling detection of lower concentrations of cells. These have the potential to allow for discrimination of unique strains and even for assessment of the metabolic activity of target species. Remote sensing techniques, including satellite and airplane as well as in situ devices, hold great promise for detecting selected taxa.

Traditional ship-based field sampling and analysis are very limited in both space and temporal frequency; remote sensing technology can be ideal instruments for estimating global phytoplankton biomass, especially in episodic blooms, because they provide relatively high frequency synoptic information over large areas. Chlorophyll a measurements provide biomass estimates which are taxonomically non-specific (Jeffrey et al. 1999). Using optical properties, spectral ratio discrimination, satellite chlorophyll anomaly, fluorescence line height (FLH) measurements,

and climatological data analysis have provided reliable detection, but many cases were not able to fully support accurate identification, monitoring and forecasting of future locations of the red tide blooms, Inaccurate information derived from these methods prevents managers from better anticipating red tides or allowing them to better focus their sampling efforts on threatened aquaculture fish-harvesting areas, particularly in coastal oceanic ecosystems. In recent years, the use of chromatographic method based on high performance liquid chromatography (HPLC) have allowed rapid separation and accurate quantification of over 50 chlorophyll and carotenoid pigments in a single operation (Wong and Wong 2003).

Toxin identification and characterization are the major impediment to effective management of HABs. Some inexpensive, rapid testing toxins kits (Fig. 2) show great promise for use in screening samples to detect and quantify toxins (Hu et al. 2012).

They are also useful for looking at past events to determine the most important factors driving bloom initiation, maintenance, transport, and decline. To investigate the location of cyst beds and abundance of cysts within the beds play a critical role in bloom development. Then to explore optimal growth conditions for various species as well as what factors influence toxin production. Such models may incorporate information on cell life cycles, physiology, grazer impacts, physical processes, and important environmental parameters. This information will ultimately evolve to yield models of population growth, mortality, potentially, predictive models of bloom development, maintenance, dissipation, and impact. Predictive models, along with cell and toxin detection methods, are the ultimate tools for exploring impacts of blooms and developing mitigation strategies.

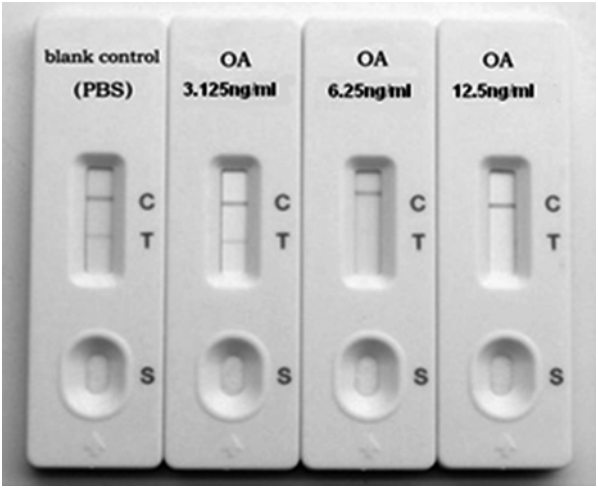

Fig. 2 Immunochromatographic detection of okadaic acid (OA) using test strip

2.3.2 Control of HABs

HABs control is the most challenging and controversial aspect of management. Develop environmentally acceptable strategies for direct intervention in ongoing bloom events for the purpose of eliminating toxic or harmful cells or inhibiting their growth. Direct strategies target the causative organisms directly, and attempt to eliminate them from the water column, or minimize their proliferation. It can be grouped five categories: mechanical, biological, chemical, genetic, physical control.

Mechanical control involves the use of filters, pumps, and barriers (e.g., curtains, floating booms) to remove or exclude algal cells, dead fish, or other bloom-related materials from impacted waters. To date, the most promising strategy for controlling algal blooms in the field is the application of suspended clay particles over the bloom to flocculate and settle the algal cells. The clay particles aggregate with each other and with red tide cells, removing those cells through sedimentation.

Biological control involves the use of organisms or pathogens (e.g., viruses, bacteria, parasites, zooplankton, and shellfish) that can kill, lyse, or remove algal cells. The biological control of HABs by using copepods and bivalves such as oysters has been examined, but the results were minimal because of the huge scale of red tides (Shirota 1989).

Chemical control involves the use of chemical or mineral compounds to kill, inhibit, or remove algal cells. It was attempted in 1957 against the Florida red tide organism using copper sulfate delivered with crop dusting airplanes (Rounsefell and Evans 1958). Chemical control has not been actively pursued, because it is difficult to find an environmentally acceptable chemical that would target a particular red tide species but not cause widespread mortality of other organisms.

Genetic control is similar in many ways to those associated with biological control, the genetic engineering of species that are purposely introduced to alter the environmental tolerances, reproduction or other processes in the undesirable species. It also concerns about the possible negative impacts of introducing a non-indigenous organism to an area.

Physical control is isolation by transferring protected target or set barrier, destroy by ultrasonic or electromagnetic wave, reclaim by pump on board etc. In one study conducted in the 1970s in Japan, techniques to skim the surface water to remove algae were assessed (Secher 2009).

Some examples of direct control include the use of chemicals that kill HABs species during blooms, the addition of clays and chemical flocculants to aggregate cells into larger, more-rapidly settling particles, the use of large scale filters or screens to remove cells, and the addition of biological agents such as viruses, bacteria, or parasites that are lethal pathogens to HABs species.

3 Macroalgae Blooms

Green tide or macroalgae blooms are vast accumulations of unattached green macroalgae associated with eutrophic marine environments. The majority of blooms were reported to consist of species of six genera: *Ulva* (Fig. 3), *Chaetomorpha*,

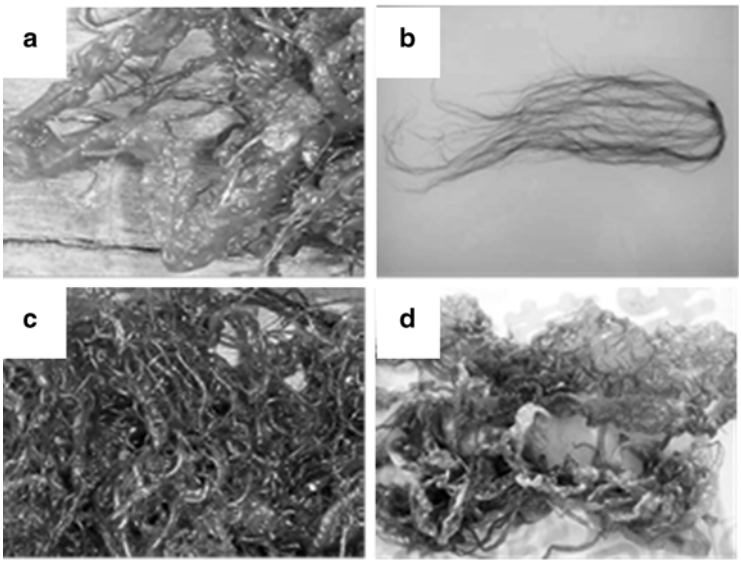

Fig. 3 Morphological changes of *Ulva prolifera* during cultivation in enclosures. (**a**) New filamentous branches from old thallus; (**b**) Longer filamentous thallus; (**c**) Tubular and cystic blade; (**d**) Folded blade

Cladophora, *Rhizoclonium*, *Percursaria* and *Ulvaria*. These seaweeds are ubiquitous throughout the world in marine and estuarine habitats, where they are highly tolerant of variable salinity, temperature and water quality, and grew rapidly in nutrient-rich habitats (Fletcher 1996; Zhang et al. 2013). Green tide blooms are now the main evidence of coastal eutrophication, and are widespread throughout the world and recognized as a common and detrimental phenomenon since thalli of the seaweed entirely cover the surface of the bottom sediment and cause deterioration of quality of the bottom sediment.

In many coastal areas, macroalgal blooms are often dominated by only one out of several co-occurring green algal species. In Washington State, USA, blooms are often dominated by *Ulva* spp. intertidally and Uivaria obscura subtidally (Nelson et al. 2008). Green tides at Tokyo Bay, Japan, were first reported in 1995 and formed by a bloom only of free-floating *Ulva* species (Yabe et al. 2009). In France, several regions are affected including the coasts of Brittany, the Arcachon basin and the Mediterranean lagoons (Merceron et al. 2007). In China large-scale Enteromorpha sp. green tides have broken out for six consecutive years from 2007 to 2012 in the Yellow Sea (Fig. 4).

Although public health and food safety problems weren't discovered, green tide had seriously affected tourism scenery, ecological environment, safety navigation, fishery, marine aquaculture etc., and had become a sustained marine environmental disaster. Blooms of green macroalgae had negative effects on marine intertidal ecosystems, including changing ecosystem structure and decreasing biodiversity. Meanwhile, due to the competition for light and space, green tide could lead to

Fig. 4 The *Ulva prolifera* green tide bloom in the Yellow Sea, China (Aug. 2008)

declining populations or reduced reestablishment capability of seagrass beds. Besides, when the algae died and sunk to the bottom, the consumption of dissolved oxygen may cause a local "dead zone" with hypoxic conditions, resulting in a shift from a high-diversity mixture to low-diversity assemblages of fast-growing annuals and invertebrate, fish, and even marine mammal kills. At last, ecosystem structure and function were affected severely in different ways. So it is urgent to control and manage the tide.

Due to the extremely detrimental effects on the coastal ecology, green tides have been focused by many studies, and the original source of the green tide bloom and the outbreak potential were two main prerequisites before any management decision was made. Indeed, accurate localization of the origin was the first step in understanding this large-scale green tide, so many studies were conducted to track the original species of the green bloom in Yellow Sea by a combination of molecular, morphological, physiological and satellite remote sensing analyses. However, little has been known about the potential of proliferation and growth, which plays an important role in interpreting the occurrence mechanism of the blooms. It is considered that eutrophication, irradiance and temperature may be the driving forces for the blooms of green tide. *U. prolifera* was tubular monolayer cells structure, which was beneficial to absorb nutrients effectively and accelerated its growth. Due to the fact that it can grow without any attachment to the substratum, and capable of proliferating when supplied with nutrient-enriched seawater such as estuaries that associated with sewage effluent, therefore huge mass of the alga may formed in coastal areas where nutrient levels were higher than normal. Seawater quality in the coastal waters of China experienced high level eutrophication and unbalance ratio of nitrogen to phosphate, which provided numerous material base for the bloom of *U. prolifera*.

Green tide blooms are the result of excessive proliferations of some green algae. It is generally supposed that they are caused by the increased nutrient (nitrogen and phosphorus) loads such as river runoff, sewage sources, upwelling events, intertidal tides and so on. Especially when water temperature conditions are favorable to their growth, the formation of macroalgal blooms and green-tide events will happen in many parts of the world.

At present there is nothing we can do but remove by hand the mass of green tides to reduce the damage against intertidal flat or salt marsh ecosystems (Yamochi 2013).

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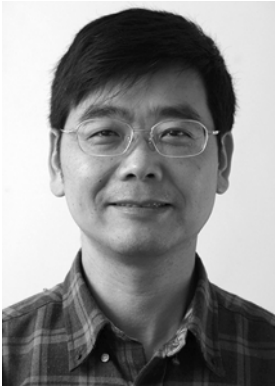

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Phycoremediation

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1 Introduction

Phycoremediation refers to the combination of the words "phyco" meaning "algae" and "remediation" which means "to treat or bring back to the original state"; phycoremediation may therefore be defined as "the use of algae to bioremediate" wastes or wastewaters. The use of algae to treat wastes or wastewaters dates back to the early civilisations where waterways such as rivers and the sea were found useful as toilets where human wastes and later animal wastes were discharged directly as natural disposal systems. Nutrients from the degrading wastes supported the proliferation of microalgae which then provided both photo-oxygenation of the waters and food for the rest of the food web in the aquatic ecosystem.

The algae comprise a large assemblage of photosynthetic organisms ranging in size from the microscopic microalgae or phytoplankton to the macroalgae, more commonly referred to as seaweeds. Algae have high photosynthetic efficiency, grow fast and are considered important primary producers in the aquatic ecosystems. In addition to their ecological importance, algae have been extensively used as food, feed, medicine and as sources of industrial materials, biomedical materials, cosmeceuticals and biopharmaceuticals. In recent years, algae have received great interest in their use as feedstock for biofuel production.

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D. Sahoo, J. Seckbach (eds.), *The Algae World*, Cellular Origin, Life in Extreme Habitats and Astrobiology 26, DOI 10.1007/978-94-017-7321-8_13

1.1 History of Bioremediation

As early as 3500BC in Mesopotamia, disposal of human wastes had been organised in the form of drainage systems and latrines where the wastes were decomposed through natural cycles (Lofrano and Brown 2010). The eighteenth century brought the Industrial Age and by the nineteenth century, the importance of water and wastewater disposal was realised although dilution was still the main principle applied. The revolution in wastewater management was brought about by the establishment of water quality parameters like biochemical oxygen demand (BOD) and the establishment of water quality standards. Further development in analytical tools led to the development of waste treatment technologies.

Waste treatment can be divided into primary treatment where non-degradable solids are separated from the organic, inorganic and dissolved components which are then treated using various technologies. Treatment using physical-chemical methods may be fast and efficient but is costly, with great increments in cost as the waste becomes more dilute. Capital investments in sophisticated equipment and expensive chemical reagents, some of which may be environmentally toxic, are additional limitations to their widespread applications. As a cue from the traditional natural disposal systems, the use of biological systems for treating wastes, especially non-toxic organic wastes, was adopted as more environmentally friendly approaches and transformed to economical and technology-friendly bioremediation processes that have been successfully implemented in many countries worldwide. These bioremediation technologies are based on the use of bacteria (anaerobic, aerobic fermentation; activated sludge) and other microbes (fungi in composting), plants (phytoremediation) and algae (phycoremediation).

Algae are primarily autotrophic, carrying out photosynthesis and requiring mainly sunlight, carbon dioxide, nitrogen and phosphorus, and some minor elements. However, many algae are also capable of metabolising organic carbon compounds in the absence (heterotrophy) or presence (mixotrophy) of light. It has been found that 50 % of algal metabolism in wastewaters is heterotrophic. These heterotrophic algae are responsible for decomposition and removal of the organic components in wastes, and converting them and other inorganic compounds into biomass which may be harvested for use in various applications. The removal of the algal biomass results in purification of the wastewater. If the algae remain in the system, they would contribute to the biochemical oxygen demand (BOD) especially in receiving waters and upon cell death, release the nutrients back into the system. Microalgae have been used for treatment of sewage, agricultural and agro-industrial wastes (effluents from processing of sugar, tapioca starch, rubber, palm oil, fish, seafood, livestock), industrial wastes (textile, paper, metal finishing) and even landfill leachate (Phang and Ong 1988; Phang et al. 2001). The algal biomass may be harvested for use as aquaculture and livestock feed, extraction of pigments and more recently for the lipids and fatty acids which are the precursors of biodiesel. The seaweed industry is well established supporting mainly phycocolloid (agar, carrageen, alginic acid) production, food products and increasingly the cosmeceutical

and biopharmaceutical industries. The culture of seaweeds in wastewaters especially effluents from aquaculture, serves the dual purpose of waste treatment and the supply of a cheaply produced useful seaweed biomass. Phycoremediation has evolved from simple systems involving earth ponds to the high rate algae ponds using microalgae, and co-culture of seaweeds with fish, shrimps in ponds to controlled complex integrated systems like the integrated multi-trophic aquaculture (IMTA) systems involving more than two species. An added advantage in using algae is that the systems can contribute to carbon dioxide removal thereby contributing to management of global warming and climate change.

2 Microalgae and Bioremediation

The term "microalgae" used in this paper refers to both prokaryotic cyanobacteria and eukaryotic algae, which are microscopic in size compared to the macroalgae or seaweeds. They consist of mainly unicellular or filamentous photosynthetic microorganisms. Microalgae contribute to almost half of global primary production and form the basis of the aquatic food chain, and they are also one of the most efficient converters of solar energy to biomass (Masojıdek and Prasil 2010). Some microalgae such as *Arthrospira* (*Spirulina*) have been collected by the natives in Mexico, Africa and Myanmar for food from natural populations in alkaline lakes (Masojıdek and Prasil 2010). While thousands of microalgal strains have been isolated from natural habitats and are maintained in numerous culture collections around the world, only a few strains have been successfully cultivated in large-scale commercial production systems. The microalgae that have been mass cultured for various biotechnological applications include *Arthrospira*, *Chlorella*, *Dunaliella*, *Haematococcus*, *Nannochloropsis* and *Tetraselmis*. The potential of microalgae as feedstock for biofuels, especially biodiesel has attracted much interest in recent years (Chisti 2007). There is a rich diversity of chemical constituents amongst the microalgae, which exhibit bioactivities with applications in the food, cosmetic and agricultural sectors, and in human health (Stengel et al. 2011). Algal compounds of particular commercial interest include pigments, lipids and fatty acids, proteins, polysaccharides and phenolics. Chemical composition of algae may vary with taxonomic, ecological and ambient environmental factors (Stengel et al. 2011).

Phycoprospecting which involves the screening of potential indigenous microalgae with intrinsic characteristics for bioresource production and waste mitigation is considered to be the most sustainable path forward for the development in algal biotechnology (Wilkie et al. 2011). In the case of phycoremediation (waste mitigation using algae), its success depends on several factors including the algal productivity that can be attained, efficiency of nutrient and pollutant removal, and harvesting costs (Olguin 2003). In addition, the use of algal strains with special attributes such as tolerance to extreme temperature, chemical composition with high content of high added value products, a quick sedimentation behaviour, and a capacity for growing mixotrophically, are other factors that should be considered. Strain

improvement using genetic modification technology to enhance the applicability of microalgae for bioremediation is another approach that has been receiving much attention due to the advent of molecular and functional genomic tools (see Sect. 4). Use of filamentous microalgae with high autoflocculation capacity and immobilised cells has also been investigated for phycoremediation. In addition, immobilisation of both cyanobacteria and eukaryotic algae in a wide range of materials including alginate, glass beads, loofa sponge, carrageenan and silica gel has been shown to be useful for the removal of nutrients such as nitrogen and phosphorus, and heavy metals (Hameed and Ebrahim 2007).

The constituents of wastewater vary with the type of wastewater and prior treatment may be required before they can be used to grow microalgae. The total nitrogen and phosphorus concentrations may range from 10 to 100 mg L^{-1} in municipal wastewater and >1000 mg L^{-1} in agricultural effluent (de la Noue et al. 1992). Effluents originating from domestic, agricultural or industrial sources may contain BOD reaching 85,000 mg L^{-1}, as found in palm oil mill effluent (POME) (Phang and Ong 1988). The BOD is mainly attributed to organic compounds including refractory organics such as humic and tannic acids. Such constituents in wastewater serve as nutrients for algal growth, which supplies oxygen for bacterial oxidation of organic matter, and even removal of toxic and recalcitrant compounds.

Examples of the use of microalgae for wastewater treatment are given in Table 1. The major open system used for growing microalgae to treat wastewater is the high rate algae ponds (HRAP). The HRAP, which was first introduced by Oswald and Gotaas (1957), are shallow ponds with typical raceway designs and mixed by paddlewheels. The HRAP system is suitable for mass culture of microalgae in the tropics, where there is warm weather and high abundance of sun light throughout the year. Gross biomass productivities (P_{GB}) ranging from 2.7 to 44.2 g m^{-2} day^{-1} have been reported for HRAP treating various types of wastewater including municipal waste, piggery waste, tapioca wastewater and palm oil mill effluent (Phang et al. 2001). Algal biomass productivity ranging from 2.3 to 12.0 g m^{-2} day^{-1} with significant removal of COD (99 %), NH_4-N (97 %), NO_3 (93 %) and PO_4 (95 %) were attained by *Chlorella vulgaris* grown in HRAP treating rubber effluent (Phang et al. 2001). As rubber effluent is carbon limited (C:N:P = 6:2:1–14:5:1) for algal growth, carbon supplementation in the form of molasses was found to further enhance biomass productivity (61 g m^{-2} day^{-1}). Enhancement of biomass productivity was only effective when molasses was supplemented at noon but not in the evening, which could be attributed to the mixotrophic growth of *Chlorella vulgaris*. In another study, *Arthrospira platensis* grown in HRAP was able to remove almost all the phosphate from anaerobically digested starch factory wastewater (Phang et al. 2000). The HRAP system is also useful for tertiary treatment of the wastewater after undergoing the conventional treatment before discharge. For instance, *Chlorella vulgaris* grown in HRAP has been shown to be useful for final polishing of textile wastewater before discharge, especially for colour removal (Lim et al. 2010).

The efficiency of pollutant removal from the wastewater by microalgae depends on the stage of treatment that the wastewater has undergone. For instance, *Chlorella* sp. grown in municipal wastewater after activated sludge treatment removed very

Table 1 Use of microalgae in bioremediation of various types of wastewaters and waste gases

Microalgae	Waste	Culture system	Biomass productivity	Reduction of pollutants	References
Chlorella sp.	Flue gas from boiler plant containing 6–8 % CO_2	Photobioreactor with inclined lanes	19.4–22.8 g dry weight/ m^2	Reduction of CO_2 by 10–50 %	Doucha et al. (2005)
Chlorella vulgaris	Flue gas from municipal waste incinerator	Photobioreactor	2.5 g dry weight L^{-1} 24 h^{-1}	CO_2 fixation rate $^{-}$4.4 g L^{-1} 24 h^{-1}	Douskova et al. (2009)
Chlorella vulgaris (mixed with bacteria)	Municipal wastewater	Tubular photobioreactors (PBRs) consisting of vertical columns	Peaked at 2.12 g L^{-1}	Removal of 97 % NH_4^+, 98 % phosphorus and 26 % dissolved organic carbon (DOC)	He et al. (2013a)
Chlorella vulgaris (mixed with the bacterium *Azospirillum brasilense*)	Municipal wastewater	Immobilised in alginate beads	–	Removal of 100 % NH_4^+, 15 % NO_3^-, 36 % phosphorus	de Bashan et al. (2004)
Desmodesmus communis versus natural microalgal consortium	Primary effluent from a wastewater treatment pilot plant	Laboratory cultures	Biomass productivity higher for *Desmodesmus communis* than natural microalgal consortium.	Pollutant removal higher by *Desmodesmus communis* than natural microalgal consortium.	Samori et al. (2013)
			Biomass:	*Desmodesmus*-ammonia and phosphorus – 100 % removal	
			Desmodesmus- 0.138 0.227 g $L^{-}1$ s^{-1}		
			Algal consortium- 0.078 g $L^{-}1$ s^{-1}		
Chlorella zofingiensis	Piggery wastewater	Tubular bubble column photobioreactors	Highest biomass productivity in diluted piggery wastewater with 1900 mg L^{-1} COD	Efficient removal of pollutants	Zhu et al. (2013)

(continued)

Table 1 (continued)

Microalgae	Waste	Culture system	Biomass productivity	Reduction of pollutants	References
Chlorella vulgaris	Textile wastewater	Immobilised cultures in carrageenan and alginate	$\mu = 0.18–0.23\ day^{-1}$	Colour removal – 48.9 %	Chu et al. (2009)
Consortium of microalgae (e.g. *Scendesmus* and *Chlorella*)	Anaerobic sludge centrate from wastewater treatment plant	Tubular plastic photobioreactors	Productivity = 3.3 ± 1.5 g dry wt $m^{-2}\ day^{-1}$	Ammonia removal =72 %	Dalrymple et al. (2013)
				Phosphorus removal = 85 %	
Consortium of microalgae (*Chlorella vulgaris*, *Scenedesmus quadricauda*, *Euglena gracilis*, *Ankistrodesmus convolutus* and *Chlorococcum oviforme*)	Landfill leachate	High rate algae ponds	Biomass = 2.00–5.54 g dry weight L^{-1}	COD removal = 91.0 %	Mustafa et al. (2012)
				Ammoniacal nitrogen = 91.0 %	
				Orthophosphate = 86.0 %	
Isochrysis	Modified f/2 medium with Palm Oil Mill Effluent (POME) and inorganic fertiliser	Photobioreactor (1 L) versus outdoor cultures (glass aquarium, 9 L)	Biomass- PBR	NO_3^-	Vairappan and Ang (2007)
			69 mg $m^{-2}\ day^{-1}$	PBR – 38.1 %	
			Outdoor	Outdoor- 46.4 %	
			92 mg $m^{-2}\ day^{-1}$	PO_4	
				PBR – 86.6 %	
				Outdoor- 83.3 %	

low percentage of phosphorus compared to that grown in wastewater before and after primary settling (Wang et al. 2010). In addition, the recycling of the algae as inoculum for the next batch of culture was found to improve the biomass productivity by 58 % in HRAP treating primary settled sewage (Park et al. 2013). It has also been shown that the biomass yield of *Arthrospira platensis* could be further increased if the colour of piggery wastewater was first removed by chitosan (Depraetere et al. 2013). The green alga *Chlorella pyrenoidosa* was found to be able to grow in diluted primary piggery wastewater with biomass productivity ranging from 250 to 1000 mg L^{-1} and ammonium removal of over 90 % (Wang et al. 2012a). In another study, *Scenedesmus obliquus* was found to be able to degrade cyanide in gold mill effluents from an initial concentration of 77.9–6 mg L^{-1} in 77 h in batch culture (Gurbuza et al. 2009).

The use of a consortium of natural microalgal assemblage has been compared with unialgal culture system in terms of biomass productivity and efficiency of removing pollutants from wastewater. For instance, Samori et al. (2013) found that *Desmodesmus communis* showed higher biomass productivity and better removal of ammonia and phosphorus from primary effluent compared to a natural consortium of microalgae. In comparison, a consortium of five microalgae consisting of *Chlorella vulgaris*, *Scenedesmus quadricauda*, *Euglena gracilis*, *Ankistrodesmus convolutus* and *Chlorococcum oviforme* were found to produce higher biomass than a mixed population of pond algae when grown in HRAP treating landfill leachate although there was no significant difference in the reduction of pollutants (Mustafa et al. 2012).

Mixed cultures of algae and bacteria are important in the treatment of wastewater. For instance, in the treatment of municipal wastewater using a consortium of microalgae and bacteria, *Chlorella vulgaris* was found to play a dominant role in removing nitrogen and phosphorus while the bacteria removed most of the organic matter (He et al. 2013a). In addition, oxygen produced by algal photosynthesis can be used by the acclimatised bacteria to biodegrade hazardous pollutants such as polycyclic aromatic hydrocarbons, phenolics, and organic solvents (Muñoz and Guieysse 2006). Addition of bicarbonate may be required to enhance photosynthetic oxygenation to support bacterial degradation of organic pollutants, as demonstrated by Bahr et al. (2011) using a consortium of enriched wild-type methanotrophic bacteria and microalgae for the breakdown of methane, methanol and glucose (model of organic pollutants) in an enclosed algal-bacterial photobioreactor.

In bioremediation, besides suspension culture, immobilised algae have been shown to be another efficient system for removing nutrients and other pollutants from wastewater. For instance, Liu et al. (2012) assessed the capability of *Chlorella sorokiniana* GXNN 01 immobilised in calcium alginate in removing nitrogen and phosphate under autotrophic, heterotrophic, mixotrophic and micro-aerobic conditions. The immobilised cells were found to remove higher percentage of ammonia and phosphate than free-living cells under some of the conditions. In another study, Chu et al. (2009) demonstrated that immobilised *Chlorella vulgaris* was useful in removing colour from textile dyes and textile wastewater. The percentage of colour removal by *Chlorella vulgris* immobilised in alginate was higher compared to

suspension cultures. Immobilised algae have also been used for bioremediation of heavy metals and other xenobiotics. For instance, immobilised *Spirogyra condensata* and *Rhizoclonium hieroglyphicum* were more efficient in removing chromium from tannery wastewater compared to suspension cultures (Onyancha et al. 2008). In addition, immobilised *Chlorella vulgaris* in alginate beads was found to be useful in removing tributylin by biosorption and biodegradation (Luan et al. 2006).

One of the most attractive approaches in phycoremediation is the integration of wastewater treatment and CO_2 biofixation with the production of algae biomass as feedstock for biofuel. This biorefinery strategy will contribute to an overall enhancement of the economic viability of the system for biodiesel production (Olguin 2012; Pittman et al. 2011; Sivakumar et al. 2012). It will not compete for freshwater resources and fertilisers, and thus, reduce the environmental footprint (Park et al. 2011). An example of such integrated system would be the use of seawater supplemented with anaerobically digested piggery wastewater for cultivating *Arthrospira* and producing biogas, biodiesel, hydrogen and other high-value products (Olguin 2012). An integration of piggery wastewater treatment and biodiesel production on a semi-continuous mode using *Chlorella zofingiensis* under outdoor conditions produced a stable net biomass productivity of 1.314 g L^{-1} day^{-1} (Yuan et al. 2013). In another study, *Chlorella zofingiensis* grown in bubble column photobioreactors was found to efficiently remove pollutants in piggery wastewater and produce algal biomass rich in lipids for biodiesel feedstock (Zhu et al. 2013).

Lipids, especially triacylglycerol from the algae biomass can be converted to biodiesel by transesterification (Chisti 2007). Cultivation of *Chlorella vulgaris* in wastewater was found to produce algae biomass suitable for biodiesel production but the lipid productivity and fatty acid profile were dependent on the ammonia concentration of the medium (He et al. 2013b). Microalgae treating wastewater can also be used for biofixation of CO_2 from waste gas streams generating biomass that can be subjected to anaerobic digestion for generation of biogas (Prajapati et al. 2013). The anaerobic digestion of algal biomass can be carried out by different groups of bacteria including hydrolytic, acidogenic, acetogenic and methanogenic bacteria. Production of biogas is advantageous as the process does not require dewatering or further chemical extraction steps as needed in case of other liquid biofuels such as biodiesel. In addition, Batten et al. (2013) worked out using a spreadsheet model to show that combining anaerobic digestion with oil extraction coupled with recycling of water and nutrients on-site could allow algal oil to be produced at a competitive cost of less than US$ 1 per litre.

Integration of microalgal culture with wastewater treatment and aquaculture is another approach that has attracted great interest. Using the Integrated Multi-Trophic Aquaculture (IMTA) approach, Gilles et al. (2013) described a prototype system how nutrients could be recycled in ponds rearing the tilapia *Sarotherodon methanotheron* feeding on *Chlorella*. The wastewater was channelled to a separate pond with rotifers which fed on *Chlorella*, and the zooplankton was then consumed by the fry and juveniles of tilapia. Algae system may be incorporated into one of the treatment stages of aquaculture wastewater. For instance, wastewater from land-based aquaculture farms can be channelled to an algal system for the removal of

dissolved nutrients after the initial treatment which removes solids through anaerobic ponds and sand bed filter (Castine et al. 2013).

Microalgae which are commonly used as aquaculture feeds include *Skeletonema*, *Thalassiosira*, *Tertraselmis*, *Pavlova*, *Isochrysis* and *Nannochloropsi*s due to their high nutritional value, especially their high contents of polyunsaturated fatty acids (Muller-Feuga et al. 2003). Wastewater could serve as a low-cost substrate to generate such microalgae for aquaculture feed. For instance, a modified medium containing 5 % palm oil mill effluent (POME) with 0.075 % inorganic fertiliser was found to improve the production and quality of *Isochrysis* as live feed for aquaculture (Vairappan and Ang 2007). *Isochrysis* grown on POME-modified medium was found to be a suitable supplement (mixed with *Nannochloropsis*) in enriching and improving rotifer cultures. In addition, significant reduction of pollutants from POME such as nitrate and orthophosphate by the microalga was attained (Table 1).

2.1 Mechanisms Used by Microalgae in Bioremediation

In phycoremediation, microalgae not only remove nitrogen and phosphorus which are nutrients for their growth, but are also capable of removing and/or degrading other environmental toxicants such as heavy metals and organic pollutants. Microalgae have developed mechanisms that enhance their tolerance to the pollutants, and enable them to detoxify or degrade the toxicants. Heavy metals such as cadmium and mercury may be transformed by microalgae to form sulphides, which have low solubilities and thus, low toxicities because they are biologically unavailable. For instance, when the green alga *Chlamydomonas reinhardtii*, the red alga *Cyanidioschyzon merolae*, and the cyanobacterium *Synechoccocus leopoliensis* were exposed to cadmium, the heavy metal was detoxified by converting it to cadmium sulfide (Edwards et al. 2013). Another study showed that exposure of 200 ppb of Hg (II) to the red alga *Galdieria sulphuria* resulted in the formation of 90 % of the Hg (II) into metacinnabar (βHgS) within 20 min (Kelly et al. 2007). In addition, the microalgae may produce metallothioneins or phytochelatins for the detoxification of the heavy metals taken in (Perales-Vela et al. 2006). Living cells of *Chlorella vulgaris* have been shown to have the ability to accelerate the aggregation of nickel oxide nanoparticles as well as reduce it to zero valence nickel (Gong et al. 2011).

Biosorption and bioconcentration are the major mechanisms of microalgae in removing toxicants such as heavy metals. Bioconcentration is the accumulation of the toxicants from the environment by the living algal biomass while biosorption refers to the adsorption of the toxicants (e.g. metal ions) on mainly dead biomass (Mehta and Gaur 2005). The ability to adsorb and metabolise heavy metals by microalgae is associated with their large surface: volume ratios, the presence of high affinity metal-binding groups on their cell surfaces, and efficient metal uptake and storage systems (Rajamani et al. 2007). For instance, three green algae, namely *Hydrodictyon* sp., *Oedogonium* sp., and *Rhizoclonium* sp. were found to accumulate heavy metals such

as vanadium and arsenic in wastewater derived from coal-fired power generation (Saunders et al. 2012). The bioconcentration of most of the heavy metals tested was found to be positively correlated with the growth rate of the algae although elements such as cadmium and zinc were concentrated more when growth rates were lower.

Microalgae have been shown to have the capability to adsorb heavy metals, which involves binding of the metals on the cell surface and to intracellular ligands (Mehta and Gaur 2005). The functional sites that bind to metals include carboxyl, imidazole, sulphydryl, amino, phosphate, sulfate, thioether, phenol, carbonyl, amide and hydroxyl moieties (Wang and Chen 2009). The mechanisms of heavy metal biosorption include ion exchange, chelation/coordination, reduction and complexation (Ahemad and Kibret 2013). Factors that affect the capacity of metal biosorption include metal and biomass concentration in solution, pH, temperature, cations, anions and metabolic stage of the organism (Mehta and Gaur 2005). Pretreatment of $CaCl_2$ has been shown to be the most suitable and economic method for activation of the algal biomass for metal biosorption. In addition, the capacity of dead cells in removing heavy metals by biosorption is higher than live cells (Mehta and Gaur 2005). A column filled with immobilised algae or granulated algal biomass can be used for several sorption/desorption cycles for the removal of heavy metals (Mehta and Gaur 2005). There is a great potential of using periphytic algae for removing heavy metals from wastewater. For instance, the dried biomass of *Spirogyra hyalina* has been shown to be useful for removal of cadmium, mercury, lead, arsenic and cobalt (Kumar and Oommen 2012). Of 30 strains of microalgae examined for their biosorption abilities in the uptake of cadmium, lead, nickel, and zinc from aqueous solution, Klimmek et al. (2001) found that *Lyngbya taylorii* showed highest uptake capacities for the four metals. In addition, dead biomass of *Arthrospira* has been shown to be a useful biomaterial for the detoxification of Cr (VI) by reducing it to Cr (III) (Gagrai et al. 2013a). The concentration of functional groups of *Arthrospira* biomass involved in the biosorption of Cr(III) is in the order of: phosphatic >> carboxyl > amine (Gagrai et al. 2013b)

Microalgae have also been shown to be useful in bioremediation of acidic wastewater, especially Acid Mine Drainage (AMD) (Das et al. 2009). The mechanism involves generation of alkalinity by microalgae through the assimilation of nitrate, and this results in the precipitation of metal hydroxides. For instance, in one study, *Arthrospira* was grown in a separate vessel to generate alkalinity and the alkaline water was mixed with AMD in another vessel (Payne 2000). This resulted in the increase of pH of the raw effluent from 1.8 to 9.0 and metals in AMD such as Zn, Pb, Fe and Cu were precipitated as metal hydroxides. In another study, a cyanobacteria (predominantly *Oscillatoria*)-microbial consortium was found to be useful in removing SO_4 and precipitating metals such as Fe, Pb, Zn, Cu and Co from mine wastewater, with pH increased from 2.93 to 6.78 (Sheoran and Bhandari 2005). Extracellular polysaccharides from microalgae may also serve as a carbon source for the growth of the anaerobic sulfate reducing bacteria (SRB), which in turn produce alkalinity and leads to sulfide precipitation from AMD (Molwantwa et al. 2000; Bhattacharya et al. 2008). The metals may also be removed through volatisation by bacteria and microalgae (Wang et al. 2014). For instance,

Ostreococcus tauri has been found to be able to biomethylate arsenic to a volatile form (Zhang et al. 2013). In addition, three cyanobacteria, namely *Microcystis* sp., *Synechocystis* sp. and *Nostoc* sp. were found to accumulate large amounts of arsenic and convert it to a volatile methylated form (Yin et al. 2011).

Studies have shown that a wide range of organic chemicals, including pesticides (Barton et al. 2004; Singh and Walker 2006), phenolics (Semple et al. 1999), tributyltin (TBT) (Tsang et al. 1999), naphthalene (Todd et al. 2002), bisphenol (Hirooka et al. 2005), and oil, petroleum and hydrocarbons (Joseph and Joseph 2001) can be degraded by cyanobacteria and microalgae. Thus, there is great potential of using such organisms for bioremediation of organic pollutants. Addition of nutrients in the form of organic carbon such as glucose has been shown to enhance the growth and ability of microalgae to degrade the organic pollutants (Megharaj et al. 1988; Tikoo et al. 1997; Papazi and Kotzabasis 2007). For instance, the growth and catabolic activity of *Scenedesmus obliquus* in degrading halogenated phenols increased in the presence of 5 g glucose L^{-1} when irradiated at 120 µmol m^{-2} s^{-1} (Papazi and Kotzabasis 2007). In some cases, the degradative products from the organic pollutants may actually enhance the growth of microalgae. For instance, Papazi et al. (2012) reported that *Scenedesmus obliquus* was able to degrade the toxic p-cresol (4-mthylphenol) and use it as an alternative carbon/energy source.

Although some of the organic pollutants may not be completely mineralised, they may be converted to less toxic or nontoxic compounds, which is an important bioremediation strategy (Subashchandrabose et al. 2013). For instance, the freshwater microalgae *Pseudokirchneriella subcapitata*, *Scenedesmus acutus*, *Coelastrum reticulatum* and *Scenedesmus quadricauda* were found to be able to metabolise biophenol, an endocrine disruptor to bisphenol glycosides, which are non-estrogenic (Nakajima et al. 2007). A consortium of pure cultures of six microalgae (*Anabaena cylindrica*, *Chlorococcus*, *Arthrospira platensis*, *Chlorella*, *Scenedesmus quadricauda* and *Anabaena*) inoculated into a pond containing synthetic wastewater was found to be able to remove endocrine disrupting compounds such as estrone and 17ß-estradiol (Shi et al. 2010). It was suggested that the removal mechanisms for the compounds may involve both biosorption and biodegradation. Recently, Dotto et al. (2013) have also demonstrated that *Arthropsira* nanoparticles could be used to remove phenol by biosorption.

Pollutant degradation by microalgae may be linked to photosynthesis as toxicant removal may be inhibited when they are grown in the dark or in the presence of the photosynthetic inhibitor 3-(3,4-dichloro phenol)-1,1-dimethyl urea (DCMU). For instance, the removal of 2,4-dichlorophenol (DCP) by *Chlorella fusca* was inhibited when it was grown in the dark or in the presence of DCMU (Tsuji et al. 2003). In another study, Lima et al. (2004) demonstrated that the removal of pentachlorophenol (PCP) by the consortium of microalgae comprising *Chlorella vulgaris* and *Coenochloris pyrenoidosa* decreased significantly when they were grown in the dark. In contrast, there was better removal of low molecular phenols from olive mill wastewater by *Ankistrodesmus braunii* and *Scenedesmus quadricauda* when they were grown in the dark (Pinto et al. 2003). Consortium of aerobic heterotrophic bacteria with *Synechocystis* PV6803 has been shown to be useful for bioremediation of oil-

polluted sites, as they have the potential of degrading of oil hydrocarbons (Abed 2010). The bacteria were capable of metabolising the photosynthetic and fermentative exudates from the cyanobacterium, and this enhanced their growth. On the other hand, the cyanobacterium could feed directly on metabolites (e.g. organic acids) produced upon the degradation of oil hydrocarbons by the aerobic heterotrophic bacteria.

With regard to pesticide degradation by microalgae, factors such as algal size, density, morphology and metabolic activities are important in affecting the uptake and removal of the toxicants (Subashchandrabose et al. 2013). For instance, high surface area to biovolume ratio of algae provides greater potential for sorption and subsequent interaction with the pesticides. Some microalgae and cyanobacteria are capable of degrading the organophosphorus insecticide methyl parathion and utilise it as a source of phosphorus (Megaraj et al. 1994). Barton et al. (2004) demonstrated that methyl parathion was transformed in *Anabaena* sp. through a reductive process, where it was converted to O,O-dimethyl O-p-nitroephenulthiophosphate and subsequently reduced. Both *Scenedesmus* sp. and *Chlorococcum* sp. were found to convert α-endosulfan, an endocrine disrupting insecticide, to endosulfan sulphate, endsulfandiol, ß-endosulfan, endosulfan aldehyde and endosulfan ether (Sethunathan et al. 2004). In another study, *Chlorella vulgaris*, *Chlorella pyrenoidosa* and *Scenedesmus obliquus* were found to degrade the herbicide, diclofopmethyl by absorbing and subsequently hydrolysing it to yield diclofop inside the algal cells (Cai et al. 2007). However, in some cases, the transformed compounds from organophosphorus pesticides (e.g. fenamiphos) may be more toxic than the parent compound (Cáceres et al. 2008). As a bioremediation strategy, a consortium of microalgae and bacteria may be useful as the bacterial partners can degrade the algal metabolised compounds (Subashchandrabose et al. 2011).

3 Macroalgae and Bioremediation

The macroalgae or seaweeds, together with the marine angiosperms referred to as seaweeds are the main primary producers of the coastal and shallow waters of the world. Together with the less visible phytoplankton, they serve important roles in the biogeochemical cycling of nutrients, carbon and oxygen. Seaweeds unlike the seagrasses, lack the xylem and phloem transport system of higher plants (Lobban and Harrison 1994). Seaweeds are classified based on their pigments into three broad groups; green (Chlorophyta), red (Rhodophyta) and brown (Phaeophyta) seaweeds.

The Chlorophyta are characterised by the presence of chloroplasts with two envelope membranes, stacked thylakoids and chlorophyll *a*, *b* and *c*. All green algae produce starch as the main reserve polysaccharide, which is deposited inside the plastids. The green algae are one of the most diverse groups of eukaryotes, showing morphological forms ranging from flagellated unicells, coccoids, branched or unbranched filaments, to multinucleated macrophytes and taxa with parenchymatic tissues. They are distributed worldwide and can be found in a wide range of habitats

from the Arctic and Antarctic regions to oceans and freshwater lakes as well as in the soil of temperate and arid areas. In the marine ecosystems, the green seaweeds often inhabit the shallow coastal waters and dominate during eutrophication episodes, due to their tolerance of high nutrient levels. As such, the green seaweeds have commonly been investigated as nutrient "scrubbers" in waste effluents. The green seaweeds are rich in starch which are amenable to saccharification and fermentation to bioethanol, thus providing a useful solution to disposal of nuisance seaweed biomass that result from the eutrophication periods.

The red seaweeds (Rhodophyta) are an ancient group of eukaryotic algae, many of which have adapted to living in brackish and seawater. Members of red seaweeds are found in the tropical, temperate, and cold-water phytogeographical regions, but are more abundant in the temperate and tropical regions. The Rhodophyta contain phycoerythrin, phycocyanin, and allophycocyanins as accessory pigments to chlorophyll *a*, in their chloroplasts. The phycobiliproteins allow the red seaweeds to live in deep waters. Storage polysaccharides in the red seaweeds include floridean starch and the commercially important agar and carrageenan.

The Phaeophyta or brown algae are mostly marine algae. Phaeophyta are characterised by the pigment fucoxanthin that gives them the brown colour. The cell wall in Phaeophyta is two layered; inner layer consists of cellulose and outer layer mainly of algin and fucoidan. The brown seaweeds serve as important source of the industrial hydrocolloid alginate as well as food in countries like Japan, Korea and China.

3.1 Advantages of Using Macroalgae for Bioremediation

The main objective in wastewater remediation is removal of nutrients like compounds of nitrogen and phosphorus, and organic contents, that will result in eutrophication and increased oxygen demand, in the receiving waters. Toxic and hazardous compounds like metals, pesticides and recalcitrant compounds will also have to be removed to prevent contamination of water resources and the living organisms within the aquatic ecosystems. Physical-chemical remediation technologies are very expensive; ion-exchange, precipitation and membrane-filtration systems have high capital and operation costs and often require skilled operators. Bioremediation processes are more economical and are based on anaerobic-aerobic fermentation, microbial denitrification and biological removal of nitrogen and phosphorus through primary producers like microalgae, seaweeds, seagrasses and constructed wetlands.

The macroalgae or seaweeds have properties that render them very suitable for bioremediation of wastewaters (Schramm 1991):

(i) Seaweeds can use specific nutrients and combinations of nutrients as well as tolerate the high nutrient levels in wastewaters; e.g. seaweeds can take up ammonium; seaweeds can take up and accumulate phosphorus even when external phosphorus levels are high.

(ii) Seaweeds have high tolerance for environmental conditions like extremes of temperature, light, salinity; eg. *Enteromorpha* is very tolerant of salinity which can range from >30 to <3 ppt.
(iii) Seaweeds can accumulate heavy metals.
(iv) Multispecies systems based on seaweed species with different growth and reproduction periods to treat wastes which may vary with the seasons.
(v) Seaweeds have high productivity potential.
(vi) Seaweed biomass can be used as manure, fodder, production of biochemicals or bioenergy

The high growth rates of seaweeds are accompanied by efficient uptake of the nutrients and carbon dioxide from the system. Plant biomass serves as good carbon sinks. While estimates of carbon stocks in various forest types are available, no equivalent figures for marine biomass are available. Tropical forests can store around 212 Gt C. The tropical biosphere sink contributes to −1.6 GtC per year. In Malaysia, a study at the tropical forest of Pasoh gave CO_2 flux ranging from −2.1 to −2.6 $gC.m^{-2}.day^{-1}$. In the oceans, carbon can be stored in the seawater and marine organisms including phytoplankton, seaweeds and corals. However an increase in the carbon stored in living marine organisms may only be achieved through fertilization of the nutrient-poor ocean waters. In seaweed cultivation, the use of the seaweed biomass as a source of energy will best serve its role in carbon reduction. Net primary productivity in seaweeds range from 20 $gC.m^{-2}.day^{-1}$ (*Laminaria solidungula* in the Arctic) to 1900 $gC.m^{-2}.day^{-1}$ (*Laminaria* in the temperate) and 2500 $gC.m^{-2}.day^{-1}$ (*Sargassum* in the tropics). In Sabah, Malaysia, farms of *Eucheuma* and *Kappaphycus* are maricultured to provide biomass for the extraction of carrageenan (Phang et al. 2010). The floating monoline method is popularly used on the shallow reef areas. The annual production is around 2800 mt generating a revenue of US$5 million. In line with Malaysia's National Agriculture Policy, aquaculture including seaweed cultivation, has been identified as a priority development activity. Todate only 800 ha is under seaweed cultivation, mainly around Semporna, Sabah. More than 20,000 ha of coastal areas throughout Sabah has been identified as potential sites for farming seaweeds. The agarophyte *Gracilaria* has also been identified as a potential seaweed for commercialisation. With an extensive coastline of 4675 km, numerous islands and extensive continental shelves, the potential for developing seaweed 'forests' is immense and the mitigating effect substantial. However, whether seaweed cultivation can contribute to management of carbon dioxide, will reside to a large extent on the productivities achieved with or without fertilization, and the ultimate fate of the biomass. The carbon budget has to be drawn for a seaweed farm and with respect to each potential use of the seaweed biomass, eg. as a source of ethanol, food or industrial material. For biofuel production, the algal biomass needs to be produced at a cost of around one dollar (US) or less per kg. Integration of seaweed biomass production with waste bioremediation is one way of reducing the production cost while contributing to pollution abatement.

Seaweeds are also good accumulators of toxic compounds especially metals. Seaweeds are able to accumulate metals up to very high levels without adverse effects, due to the evolution of defence mechanisms resulting from exposure to increasingly contaminated aquatic ecosystems. The red seaweed *Gracilaria verrucosa* was shown to suppress the survival and growth of the harmful alga *Karenia mikimotoi* by out-competing the microalga for nutrients in an enclosed sea of Hanzhou Bay, China (Huo et al. 2011a, b).

3.2 Mechanisms Used by Macroalgae in Bioremediation

The macroalgae have evolved a diversity of defence mechanisms against environmental stresses especially toxic compounds.

3.2.1 Metal Uptake and Accumulation

The uptake of metals may be through passive adsorption or active uptake, even against a concentration gradient. Uptake and accumulation of heavy metals may be affected by pH, age of algal cells, aeration of culture, presence of other metals. Increase in temperature cause increase in toxicity as well as the reverse, while nitrate, phosphate and organic compounds may reduce metal toxicity. Organic substances like glycollic acid, fulvic acid, humic acid, amino-acids have metal-binding properties and form complex metal compounds making them unavailable for absorption. Mechanisms involved in metal uptake include:

(i) Binding of metals on cell surfaces; functional groups like lipids and polysaccharides, on algal cell surfaces can bind metal ions. The cell wall may contain amino, phosphate, carbonyl, cysteinyl ligands that act as diffusion barriers; ligands are sites for metal ion binding. Some species secrete extracellular polypeptides and organic acids which also form complexes with and thus ameliorate the toxic effect of the metals.

(ii) Phytochelation complexes: production of phytochelatins are induced, via enzymic polymerization of peptide precursors. The phytochelatins similarly complex with and render metals non-toxic to the seaweed.

(iii) Polyphosphate bodies: Pb, Cd, Zn can be incorporated in polyphosphate bodies in algal cells, making them unavailable and hence harmless to the seaweed. However, metals incorporated this way can be transferred up the food chain, and mobilisation of the polyphosphates during P-deficiency, would lead to death of the organism and subsequent release back to the environment.

(iv) Chelation to phenols located in physodes: physodes are located in the axils of brown seaweed thalli, and are small light-refracting bodies in the algal cells that contain fats, proteins, tannins, terpenes, nitrogenous compounds, glycosides and phloroglucinol-like polyphenols. High amounts of phenol (2–3 %

DW) are contained in the physodes; the phenol content increases with age of tissue, increasing salinity and under nitrogen deficiency. Phenols have different affinities for different metals, with high affinity for copper.

3.2.2 Nutrient Uptake

Nitrogen

Seaweeds and plants require essential elements for their growth. Nutrients are important for regulating the growth, biochemistry and reproduction of seaweed. They need from 14 to 21 special elements as nutrients. Essential elements including C, H, O, N, P, Mg, CU, Zn, and Mo are required for all seaweeds.

Ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) are the most common ionic forms of dissolved inorganic nitrogen in aquatic ecosystems. Inorganic nitrogen pollution can result in acidification of water, algal blooms and impact human health. Seaweeds are able to obtain nitrogen from the inorganic sources such as ammonium (1–5 μM) nitrate (1–500 μM) and nitrite (0.1–50 μM) which are concentrated in seawater (Jones et al. 2002). The uptake of inorganic nitrogen and other nutrients is influenced by temperature, water motion, light availability, level of desiccation and ionic form of the nutrient (Lobban and Harrison 1994). Although ammonium is less abundant in seawater than nitrate, which is stored in a lower capacity in internal tissue pools, it is capable of supplying 33–100 % of the total in situ nutrient requirement in several species of seaweeds (Fujita et al. 1988).

Increased aquaculture activities globally, has led to increase of the inorganic nitrogen concentration (eutrophication or hypertrophication) in the aquatic ecosystems. Environmental impacts include hypoxia (low oxygen level) which may result in reduced populations of fish and other aquatic animals. Untreated sewage effluent and agricultural run-off carrying fertilizers are other sources of human-caused eutrophication. Several red seaweeds of the genus *Gracilaria* such as *G. edulis* (Jones et al. 2002), *G. tikvahiae* (Kinne et al. 2001), *G. parvispora* (Nelson et al. 2001), *G. lemaneiformis* (Zhou et al. 2006), *G. birdiae* (Marino-Soriano et al. 2009), *Porphyra* sp. (Pederson et al. 2004) and green seaweeds such as *Ulva* spp. (Neori et al. 2000; Troell et al. 2003) are able to rapidly assimilate ammonium. Qian et al. (1996) reported that *Kappaphycus alvarezii* is able to assimilate waste nitrogen from pearl oyster farms (*Pinctada martensi*). Besides concentration of nutrients, the ionic or molecular form of the nutrients will also affect their uptake rates. For example, nitrogen in the form of ammonium often is taken up more rapidly than nitrate, urea, or amino acids. In addition, uptake rates of nutrients can also be influenced by the concentration of other ions in the medium. For instance ammonium may inhibit nitrate uptake by as much as 50 %. In contrast, *Gelidium*, *Macrocystis* and *Laminaria* can consume nitrate and ammonium at equal rates when they are supplied at the same time (Harrison et al. 1986).

Phosphorus

Phosphorus present in the form of orthophosphate ions (PO_4^{3-}) is required for seaweed growth. Inorganic polyphosphate, phosphate (HPO_4^{-2}) and organic phosphorus compounds are other sources of phosphorus in seawater. Orthophosphate is an essential macronutrient with a central role in all main metabolic activities in plants, especially in photosynthesis and respiration. Seaweeds can take up phosphorus as orthophosphate ions or obtain phosphate from organic compounds through the extracellular enzyme alkaline phosphatase. This enzyme plays a crucial role in energy transfer through ATP and other compounds involved in photosynthesis and respiration (Lobban and Harrison 1994). Phosphorus is usually a limiting factor in seaweed growth (Chopin et al. 1995). Many seaweeds showed high C: N: P ratio (Mizuta et al. 2003). Seaweeds store phosphorus in the form of polyphosphate where it can be utilised especially under conditions of phosphate exhaustion (Lobban and Wynne 1981). *Enteromorpha*, *Acetabularia*, *Ceramium* and *Ulothrix* are able to deposit polyphosphates as source of phosphorus (Kuhl 1962). Before the seaweeds uptake the phosphorus triphosphate and pyrophosphate, they are broken down to orthophosphate. A study on *Laminaria japonica* showed uptake rate of inorganic phosphorus, organic phosphorus and extracellular alkaline phosphatase were increased when the concentration of phosphate decreased to the critical level, and also nucleic acid content showed positive correlation with the critical level of phosphorus content (Mizuta et al. 2003); this result indicated the importance of critical phosphorus content in the seaweeds. Phosphate plays a role in subtropical and tropical water environments, affecting nitrogen metabolism and growth of seaweeds (Copertino et al. 2009; Troell et al. 2003). Seaweeds can produce the alkaline phosphatase enzyme at levels of 32–320 μM to help in phosphorus uptake (Aberg and Fries 1976). Environmental conditions such as light intensity, water temperature and phosphorus concentration and internal factors such as seaweed age, phosphorus content and region of the thalli can affect the uptake rate of phosphorus compounds (Mizuta et al. 2003).

Carbon

Carbon sources in the form of carbon dioxide (CO_2), bicarbonate (HCO_3^-) and carbonate (CO_3^{-2}) are required for algal growth. In the aquaculture system, the main source of acid comes from the metabolic CO_2 produced by fish, shrimp and plant respiration. CO_2 plays a major role in determining the alkalinity and pH of the water. Water with an alkalinity of 40 mg L^{-1} is considered the best concentration to stabilise the water pH for healthy fish growth. The primary photosynthesis pathway is variously referred to as the photosynthetic carbon reduction cycle (PCRC), the reductive pentose phosphate pathway, C_3 photosynthesis and the Calvin cycle (Raven et al. 2012; Lobban and Harrison 1994). Most seaweeds have C_3 pathways, except for a few species and many species suppress photorespiration by CO_2 concentration via HCO_3 (Raven et al. 1990).

3.3 Seaweeds and Remediation of Aquaculture Effluents

Modern shrimp farming, the production of marine shrimp in ponds, started in the early 1970s, and today, over 50 countries worldwide have shrimp farms. The global shrimp farming industry was valued at an estimated USD 16.7 billion in 2010 (FAO 2013), increasing by more than 10 % annually and will reach 50 % of the world's seafood supply in 2030. However, aquaculture has caused serious ecological problems, such as coastal eutrophication due to the release of excess nutrients, which may negatively influence the aquaculture activity itself by increasing the ammonium toxicity and water turbidity (Troell et al. 1999).

The use of high quality feed has been a strategy to increase shrimp productivity (Brzeski and Newkirk 1997) which accounts for more than 95 % of the nutrient input (Krom and Neori 1989). Less than one third of the nutrients are assimilated into the shrimp biomass (Briggs and Funge-Smith 1994) and the remaining is discharged to the environment. In addition, 70–80 % of the ingested protein is excreted to the water, the majority of which (80 %) comprises dissolved nitrogen in the form of ammonium (Porter et al. 1987).

The overload of organic nutrients influences the quality of shrimp farm effluent which then impacts on the marine ecosystem. Eutrophication is a general phenomenon in coastal waters, commonly attributed to the increase in aquaculture of shrimp, fish and shellfish (Liu et al. 2010), resulting in harmful algal blooms and deterioration of water quality (Marinho-Soriano et al. 2009). Aquaculture farms discharge large amounts of nutrients into the marine ecosystem, in the form of excretory products and excess feed (Zhou et al. 2006; Rodrigueza and Montaño 2007; Marinho-Soriano et al. 2009). Of the chemical contaminants entering the marine ecosystem, the main pollutant is nitrogen.

Quantitative comparisons of shrimp farm influent and effluent water have demonstrated that the effluent can contain elevated concentrations of dissolved nutrients, phytoplankton, bacteria, and other suspended organic and inorganic solids. Effluents from fish-cages and intensive fishponds release nutrients to the environment and contribute to water pollution. This is of particular concern in oligotrophic warm-water coastal seas because only 25–30 % of feed nutrients convert to energy and growth of animal (Losordo and Dan-Hobbs 2007). In general, ammonia and nitrite are the most toxic metabolites resulting from the excess nutrients.

The major challenge of the aquaculture industry has been to reduce the negative effects of nutrients in aquaculture effluent. Biofiltration is the most effective method to treat aquaculture effluents for minimising nutrient release from aquaculture ponds into the environment. Biofiltration of aquaculture effluent can be accomplished by microalgae, macroalgae, bacteria and suspension feeders (Shpigel and Neori 2007). Biofiltration in aquaculture pond can effectively reduce water pollution and water exchange costs.

3.3.1 Integrated Multi-Trophic Aquaculture (IMTA)

Integrated Multi-Trophic Aquaculture (IMTA) refers to the farming of different aquaculture species together in a way that allows one species' wastes to be recycled as feed for another (Chopin et al. 2004). IMTA can synergistically increase total output and this system is a practical solution to eliminating the negative environmental impacts of aquaculture waste (Wang et al. 2012b).

Hughes-Games (1977) described the first practical marine fish/shellfish/phytoplankton culture. IMTA has been proposed as an environment friendly aquaculture and fishery resources management system (Troell et al. 2003; Neori et al. 2004). IMTA is more sustainable than monoculture, because in this system animal waste is reutilized by microalgae or seaweeds (Neori et al. 2007). In most studies seaweeds are integrated with fish culture (Buschmann et al. 1994; Troell et al. 2003; Chopin et al. 2001; Neori et al. 2004; Zhou et al. 2006). True IMTA can be land-based, using ponds or tanks, or even open-water marine or freshwater systems. Implementations have included species combinations (Neori et al. 2004) such as shellfish/shrimp, fish/seaweed/shellfish, fish/seaweed, fish/shrimp and seaweed/shrimp (Troell et al. 2003). IMTA in open water (offshore cultivation) with seaweeds attached to lines, have been grown next to cages or nets in which fishes are reared (Chopin et al. 2004; Mao et al. 2009). Therefore IMTA promotes economic and environmental sustainability by converting byproducts and uneaten feed from fed organisms into harvestable crops, thereby reducing eutrophication, and increasing economic diversification (Neori et al. 2004; Troell et al. 2003; Tournay 2006; Mao et al. 2009).

Properly managed multi-trophic aquaculture increases growth without undesired side-effects (Chopin et al. 2004; Tournay 2006). By increasing nutrient assimilation, IMTA can reduce negative environmental impacts. Ecological engineering assists to increase the environmental sustainability, economic and social aims in IMTA (Mao et al. 2009). Typically, carnivorous fish or shrimp occupy IMTA's higher trophic levels. They excrete soluble ammonia and phosphorus in the form of orthophosphate. Seaweeds and similar species can extract these nutrients directly from their environment (Chopin et al. 2001; Neori et al. 2004; Troell et al. 2003). Fish and shrimp also release organic nutrients which feed shellfish and deposit feeders (Lander et al. 2004).

IMTA enables farmers to increase their output by using the byproducts produced by lower trophic levels. Initial economics research suggests that IMTA can increase profits and can reduce financial risks due to weather, disease and market fluctuations (Ridler et al. 2007). Japan, China, South Korea, Thailand, Vietnam, Indonesia, Bangladesh, etc. have co-cultured aquatic species for centuries in marine, brackish and fresh water environments (Chopin et al. 2001; Troell et al. 2003). Fish, shellfish and seaweeds have been cultured together in bays, lagoons and ponds. The red seaweed *Gracilaria verrucosa* was cultivated in an IMTA system with the fish *Pseudosciana crocea* in the open waters of the East China Sea (Huo et al. 2012). It was shown that this IMTA system reduced the eutrophication in the sea by removing PO_4-P, NO_2-N, NH_4-N and NO_3-N by 58 %, 48 %, 61 % and 47 % respectively.

3.3.2 Recirculating Aquaculture Systems (RAS)

Recirculating Aquaculture Systems (RAS) was started in 1960 for developing fish culture. Advantages of this system are (i) reduction in land and water requirement, (ii) effective control of culture system with optimum growth rate of fish biomass. This system provides suitable water supply, through mechanical and biological filtration, continuous water flow by pumping, aeration and oxygenation system (Hutchinson et al. 2004). The biological filtration is fundamental for water treatment to increase the growth of the cultured animals. The extra dissolved nutrients are used by heterotrophic bacteria such as *Nitrosomonas* sp., *Nitrospira* sp. and *Nitrobacter* sp. that digest and oxidise ammonium-ions via the nitrification processes. These microorganisms are accumulated on the solid substrates such as sand, coral gravel in conventional methods or on various plastic structures in modern methods such as grids, corrugated sheets, balls, honeycomb-shaped or wide-open blocks. In a large number of investigations, examples of anaerobic denitrification were successfully used to reduce the concentration of nitrate nitrogen in closed or semi-closed aquaculture systems (Barak und van Rijn 2000). The use of the RAS allows the siting of the farm in water limited locations that are near the market, making the system cost-effective by reducing the transportation time and costs. Higher degree of pollution control in the RAS system provides greater environmental sustainability compared to the traditional aquaculture systems. The effluent of this system can be used for hydroponic agriculture (Summerfelt et al. 2004). Specific-species selection based on market preference is another advantage of the RAS (Timmons et al. 2002).

3.3.3 Advantages of Using Seaweeds for Remediation of Aquaculture Effluent

Similar to other plants, seaweeds require inorganic nutrients for growth. The integration of seaweeds to marine animal cultures has been recognised as the most promising approach to reduce excess nutrients release by aquaculture activity, due to the high nutrient uptake efficiency of macroalgae, their fast growth rates, and economical and practical aspects of their cultivation (Chopin et al. 2001; Neori et al. 2004). Several studies have demonstrated the efficiency of seaweeds as biofilters in aquaculture (Hayashi et al. 2008; Neori et al. 2000; Carmona et al. 2006; Msuya et al. 2006). These studies have shown seaweeds are a suitable candidate to use in mariculture for bioremediation and economic diversification. The integration can benefit economy and environment in a sustainable manner in coastal waters at warm seasons (Zhou et al. 2006). Several studies have been carried out to evaluate the effect of seaweeds on aquaculture treatment and reducing their negative effects on the environment (Table 2).

The majority of the researches on integrated mariculture are based on the integration of biofiltering seaweed and fish aquaculture. A few studies have been focused on the integration of seaweed with shrimp culture (Kinne et al. 2001;

Table 2 Use of seaweeds in remediation of aquaculture wastewaters

Seaweed	Type of wastewater	Culture system	% Nutrient removal	Growth rate	References
Ulva pertusa	Black rock fish (*Sebastes schlegeli*) effluent	Outdoor recirculating batch tank system; 10 g per L seaweed density; culture period of 2 weeks	83 % ammoniacal nitrogen; 54 % dissolved inorganic nitrogen; 22 % nitrite and nitrate; 31 % phosphate	N/A	Kang et al. (2011)
Ulva lactuca	Synthetic media: NH_4-N, NO_3-N, NH_4-N	Batch culture system in lab	92 % ammonium; 32 % nitrate	16.4 % day^{-1} in NH_4Cl 9.4 % day^{-1} in $NaNO_3$	Ale et al. (2011)
Enteromorpha (*Ulva*) *intestinalis*	Sea bass effluent	Lab. culture; water flow of 0.5, 1.0 and 2.0 volume per day; 2 g per L seaweed density; culture period of 7 days	85.3–99.6 % phosphate	0.08–0.17 % per day	Martinez-Aragon et al. (2002)
Ulva rotundata	Sea bass effluent	As above	80.1–88.2 % ammonium	0.06–0.09 % per day	Hernandez et al. (2002)
Ulva clathrata	Shrimp effluent	Outdoor tank; 1 volume change per day; 7 g per L seaweed density	70–82 % ammoniacal nitrogen 50 % phosphate	N/A	Copertino et al. (2009)
Kappaphycus alvarezii	Shrimp effluent	Indoor batch tank culture; culture period of 5 days	94.5 % phosphate; 54.5 % nitrate; 92.3 % nitrite; 42.0 % ammonia	3.6 % per day	Mitra et al. (2012)
Gracilaria birdiae	Shrimp effluent	Lab. aquaria; 2 g per L seaweed density; culture period of 28 days	34 % ammonium; 93.5 % phosphate; 100 % nitrate	2.6 % per day	Marinho-Soriano et al. (2009)
Gracilaria lemaneiformis	IMTA with scallop	Outdoor batch tank system; 0.069, 0.14, 0.26, 0.35 g per L seaweed density; culture period 3 weeks	83.7 % ammonium; 70.4 % phosphorus	0.55–2.52 % per day	Mao et al. (2009)
Kappaphycus alvarezii and *K. striatum*	Milk fish effluent	Aquarium tank of 71 L; culture period of 28 days	41–61 % ammonium	2.7–4.4 % per day	Rodrigueza and Montano (2007)
Gracilaria lemaneiformis	Fish (*Sebastes fuscescens*) effluent	Outdoor tank batch culture; 350, 550, 700, 850 g m^3 seaweed density; period of 30 days	80 % ammonium; 60 % phosphate	1–3.76 per day	Zhou et al. (2006)

Lombardi et al. 2006). Studies on the environmental impact of shrimp aquaculture are limited and have mostly focused on in-pond water quality (Pillay 1992).

In order to mitigate the environmental impacts due to effluent discharge and maintain sustainable prawn farming, various methods have been proposed to address the issue of nutrients discharged from prawn aquaculture, one possible method is integrating prawns and seaweed (Neori et al. 2004). Ryther et al. (1975) suggested a biofilter system based on the seaweed nutrient uptake rate for reducing nutrients in effluents. Several studies have demonstrated that seaweed-based integrated aquaculture systems are able to improve water quality and environmental performance by removing nutrients (Buschmann et al. 1996, Phang et al. 1996). Development of integrated mariculture may facilitate the aquaculture industry to avoid non-compliance and gain both direct and indirect benefits from improving water quality and coastal ecosystem health. The general benefits from integrated practices are additional income from co-cultured crops and reduction of nutrient release to the environment.

Integrating suitable seaweed species into an aquaculture system is very important to provide sustainable eco-friendly aquaculture. The success of macroalgae as a biofilter depends on the characteristics of the selected species (Neori et al. 2004), which must also be ecophysiologically adapted to the local environment and culture conditions. Selection of seaweed species for use in an integrated aquaculture system must involve consideration of both economic value and biofiltration capacity (Neori et al. 2004). Several seaweed species have high biofiltering capacities and thus can be used for efficient removal of dissolved phosphorus (P) and nitrogen (N) from wastes of intensive fishpond system (Haglund and Pedersen 1993; Krom et al. 1995; Neori et al. 1996). In addition, seaweeds have high primary productivity of ≥3000 g C m^{-2} $year^{-1}$ (Gao and McKinley 1994) and the harvest of the seaweed biomass may result in carbon drawdown, indicating high potential for CO_2 remediation (Chung et al. 2011).

Many species of seaweeds are used in aquaculture including Rhodophyta such as *Gracilaria*, *Gelidium*, *Porphyra*, *Eucheuma*; Phaeophyta such as *Sargassum*, *Laminaria*, *Undaria* and Chlorophyta like *Ulva* and *Enteromorpha* (Briggs and Funge-Smith 1994; Neori et al. 2004). Species of *Gracilaria* (Buschmann et al. 1994; Troell et al. 1997, 1999; Nelson et al. 2001; Jones et al. 2002) and *Ulva* (Krom et al. 1995; Neori et al. 1996, 1998, 2000) were considered in the integrated biofilter system and showed high efficiency in the removal of waste inorganic nutrients. The integrated farming of *Gracilaria chilensis* and salmon cages in Chile had the potential to remove at least 27 % of released phosphate from the fish farm (Troell et al. 1997; Martínez-Aragón et al. 2002). Most of the work on the use of seaweeds as biofilter is based on ammonium biofiltration, and seaweeds such as *Gracilaria birdiae*, *Gracilaria edulis* and *Gracilaria lemaneiformis* can efficiently remove dissolved nutrients from fish and shrimp effluents and produce a biomass with useful products (Marinho-Soriano et al. 2009). Numerous studies also have been carried out on co-culture of seaweeds in fish tanks or cages (Buschmann et al. 2001; Troell et al. 1999).

Several studies were focused on bioremediation potential of the green algae (Chlorophyta) like *U. clathrata* (Copertino et al. 2009) and *U. pertusa* (Kang et al. 2011). Fish-pond biofilters with the green seaweed *Ulva lactuca* L. remained clean and functional for years with minimal maintenance while producing high yields (200 g wet weight, 30 g dry weight $m^{-2}day^{-1}$) using only fish-pond effluents for water and nutrition (Vandermeulen and Gordin 1990; Cohen and Neori 1991; Neori et al. 1991). Seaweed utilise ammonia and phosphate released by fish and bacteria. Various strategies for integrating seaweed cultivation with fish culture have been successful. During the day time seaweeds produce dissolve oxygen (D.O.), increase pH and simultaneously take up nutrients (Brzeski and Newkirk 1997). In culture of shrimp and *Sargassum* sp., the concentrations of nitrate, nitrite, total ammonium nitrogen (TAN), dissolved inorganic nitrogen (DIN), total nitrogen (TN), total phosphorus (TP) and phosphate (PO_4^{3-}) were significantly ($p<0.05$) lower than during monoculture (Mai and Futedar 2010). In integrating seaweeds with fish, production was more than twice that of a monoculture (Troell et al. 1999). *Gracilaria* could remove as much as 90–95 % of the ammonium in the effluent waters released from salmon tanks (Troell et al. 1997; Buschmann et al. 1994). *Gracilaria* cultivated by fish cages had higher growth rate up to 40 % compared to controls.

4 New Approaches and Technologies for Phycoremediation

Strain improvement through genetic engineering is one of the new biotechnological approaches used to further enhance the applications of algae in bioremediation. Expressed sequence tag databases of several microalgae have been established, and the nuclear, mitochondrial, and chloroplast genomes of some species, especially *Chlamydomonas reinhardtii* have been sequenced (Zeng et al. 2011). For instance, RNAi-based strategy has been successfully used in *Chlamydomonas reinhardtii* to reduce the antenna size to reduce photoinhibition and thus, enhance photosynthetic efficiency under high light conditions (Mussgnug et al. 2007). Improved photosynthetic efficiency and growth of the algae may further increase biomass production and removal of pollutants from the wastewater. In addition, molecular cloning and expression of heavy metal accumulator genes and xenobiotic degrading enzyme coding genes may enhance the bioremediation efficiency of microalgae.

The advent of functional genomic tools, such as high-throughput screening of differential gene expression has contributed to better understanding of the mechanisms of adaptation of microalgae to environmental pollutants at the molecular level. Such information is essential if transgenic approaches are to be used to enhance the applicability of microalgae in bioremediation. For instance, mRNA differential analysis showed that 13 cDNA sequences were expressed in *Chlamydomonas reinhardtii* under cadmium stress (Rubinelli et al. 2002). Some of the genes expressed were related to photosystem I and II maintenance, cysteine biosynthesis and iron deficiency. In another study, a specific gene was found to be much more pronouncedly transcribed in a tolerant strain of *Scenedesmus acutus* than the wild

type strain when subjected to chromium stress (Torelli et al. 2008). The product for the gene translation was postulated to be a surface or secreted protein involved in external chromium detoxification. In addition, a transgenic strain of *Chlamydomonas reinhardtii* overexpressing the gene coding for an isomerase enzyme showed high tolerance to excess nickel, and increased accumulation of nickel and other metals such as zinc, iron, copper, manganese and magnesium (Zheng et al. 2013). The genes involved in metal detoxification could be the possible targets for manipulation to enhance heavy metal tolerance in microalgae.

Detoxification of heavy metals in microalgae involves the peptides metallothioneins, especially the post transcriptionally synthesised class III methallothioneins or phytochelatins (Perales-Vela et al. 2006). The occurrence of phycochelatins has been reported in ten divisions and 24 genera of algae (Gaur and Rai 2001). Phycochelatins not only play a role in detoxification of heavy metals, but also in mitigation of oxidative stress, as demonstrated in *Phaeodactylum tricornutum* exposed to copper (Morelli and Scarano 2004). The genes encoding such proteins have not been well studied in algae, and could be another target for manipulation. Proteomic analysis could be another approach to identify signature proteins that are up- or down-regulated in microalgae in response to the presence of specific pollutants including heavy metals (Singh and Nagaraj 2006). For instance, exposure of *Chlamydomonas reinhardtii* to cadmium resulted in the decreased abundance of both large and small subunits of the ribulose-1.5-bisphosphate carboxylase/oxygenase, and several enzymes involved in photosynthesis, Calvin cycle and chlorophyll biosynthesis (Gillet et al. 2006). In contrast, proteins involved in glutathione synthesis, ATP metabolism, response to oxidative stress and protein folding were up-regulated in the presence of cadmium. A proteomic approach could provide further insight into the physiological changes in microalgae in response to the stress due to the toxicants. It may also provide further information on bioremediation-related genes and their regulation.

There should be appropriate measures to prevent the release of live transgenic microalgae into the environment as they may have an adverse impact on the ecosystem. For instance, transgenic microalgae with enhanced heavy metal binding capacities could accelerate the biogeochemical cycling of heavy metals and their accumulation in the food chain (Rajamani et al. 2007). Besides physical containment, multiple mutations may be introduced into the host strain to preclude the growth of the transgenic microalgae in the wild. An alternative strategy would be to use non-viable transgenic algae with enhanced metal binding capacity and selectivity.

5 Concluding Remarks

There has been extensive research on the use of both microalgae and seaweeds for phycoremediation. The advantage of algal systems is that they are based on biopurification processes that exist naturally in the ecosystem. The achievements of phycoremediation processes are manifold; recyclable water, useful algal biomass as

feedstock of valuable compounds like food, feed, fertiliser, biofuel and the potential for carbon reduction from the environment. Of the diverse uses for algal biomass, it is likely that the discovery of useful strains of both microalgae and macroalgae with high potential for use as feedstocks for biofuel production, will provide the push for expansion of phycoremediation systems. The benefits here would include cost reduction due to supply of nutrients from wastes such as aquaculture and agricultural effluents, while the environment would benefit from the resulting cleaner water, carbon reduction and a carbon neutral biofuel.

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Bioactivity of Secondary Metabolites from Macroalgae

Gilles Bedoux and Nathalie Bourgougnon

1 Introduction

Intertidal marine ecosystem constitutes so a constantly fluctuating and stressful environment where algal growth, development, reproduction and productivity are drastically affected by changes in temperature, humidity, salinity, irradiance, nutrient availability, predator-prey and biotic competitive interactions. As an extremely harsh environment, organisms inhabiting intertidal systems must be very well-adapted to the severe conditions, and natural selection has ensured survival of intertidal macroalgae. In general, seaweeds can survive in constantly changing environments due to complex metabolic networks capable of detecting stress signals that allow them to respond appropriately and adapt to new conditions. This sensing of stress signals and their transduction into appropriate responses is crucial to the acclimation and survival of intertidal macroalgae (Potin et al. 2002; Parages et al. 2014). To survive in such a marine dangerous environment, plants obviously have to develop some various self-defences such as the production of chemical deterrents base of chemical ecology (Harlin 1987; Hay and Fenical 1988; Hay 1996; Potin et al. 2002) and they can actively alter their susceptibility to various attackers. Allelopathy in marine aquatic environments may generally provide a competitive advantage to algae in their biotic interaction with other primary producers or animals for competition between different photoautotrophs for resources, space, light, or nutrients. Hence epiphytism, which reduces the availability of light and increases hydrodynamic drag, provides a permanent threat. The release of allelopathically active compounds interfering with settlement and/or growth of competitors in their vicinity is an adaptive trait developed by primary producers against competitors.

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D. Sahoo, J. Seckbach (eds.), *The Algae World*, Cellular Origin, Life in Extreme Habitats and Astrobiology 26, DOI 10.1007/978-94-017-7321-8_14

Seaweeds produce a wide range of secondary metabolites, many of which exhibit a broad spectrum of activity (Da Gama et al. 2002; Ioannou and Roussis 2009). The ecological roles of secondary metabolites produced by seaweeds have recently been investigated in studies that chiefly emphasize the chemical mediation as a defense against herbivores (Macaya et al. 2005; Gagnon et al. 2006). These compounds can also inhibit settlement or development of fouling organisms (including viruses, bacteria, fungi and other algae) (Hay 1996; Hellio et al. 2001, 2002; de Nys and Steinberg 2002; Bazes et al. 2006, 2008; Silkina et al. 2012). Although a large body of evidence to show that algae are endowed with chemical defenses has been available for some time, the idea that many of these defenses are induced following challenge by bioaggressors has emerged only recently. Finally, given the importance of the primary biomass in the euphotic zone, it is likely that the defense strategies developed by benthic and planktonic algae play a major role in the structuring of marine communities (Potin et al. 2002). The exploration of this chemical diversity for pharmaceutical and cosmetic purposes has revealed important chemical prototypes for the discovery of new agents (Kornprobst 2005; Mayer 2002; Mayer and Hamann 2004; Mayer et al. 2007; Ioannou and Roussis 2009; El Gamal 2010). Since 1960, till today more than 15,000 novel compounds have been isolated from marine organisms (Cardozo et al. 2007).

2 Examples of Environmental Constraints

2.1 Grazing/Herbivory

In coastal marine systems, the grazing of seaweeds by fishes or sea urchins can be intense and may cause wounds and diseases. This effect is often variable in time and space (Hay 1996). In response to herbivory, macroalgae have evolved a variety of chemical mechanisms including spatial and temporal escapes, species association, tolerating or deterring herbivores (Hay and Fenical 1988; Kurata et al. 1998; Macaya et al. 2005; Gagnon et al. 2006). Some species also increase concentrations of defensive compounds in response to grazing on their tissues (Sotka et al. 2002). Induction of reduced palatability (Renaud et al. 1990) or of increased levels of secondary metabolites (Hammerstrom et al. 1998) can occur in as little as 1 day. Such a rapid response to attack should allow seaweeds to reduce their susceptibility on a time scale that is ecologically meaningful for numerous types of marine herbivores. Induced defenses could be effective against these consumers because they remove smaller amounts of tissue over extended periods of time (giving the plant time to respond) and because mesograzers are more intimately associated with their host plants, allow changes in the chemistry of an individual plant to have a greater impact on both plant and herbivore fitness. Other characteristics of the ecology and physiology of seaweeds suggest that they could induce resistance due to cues received from the grazing of neighboring plants. Seaweeds often form dense monospecific stands

where individuals are in close physical contact (Dayton 1985; Schiel and Foster 1986) and water movement is reduced (Eckman et al. 1989; Komatsu and Murakami 1994), potentially facilitating the transmission of chemical signals between neighboring plants.

For example of grazing, A classic example of a putative chemical defense is found in the brown algal order Desmarestiales, where certain species produce and store high concentrations of sulfuric acid (H_2SO_4) within cell vacuoles, resulting in extremely low internal pH (down to ~0.5 pH). Sulfuric acid is probably not the only chemical compound player in chemical defense mechanisms and is associated in conjunction with other factors (e.g. aspects of the abiotic environment, availability of alternative prey) (Gagnon et al. 2006).

2.2 Antifouling

Seaweeds live in the photic zone of the world's oceans and are subjected to high fouling pressure, but frequently show low epibiont coverage (Thabard et al. 2011). According to Molisch (1937), allelopathy covers biochemical interactions, both stimulatory and inhibitory, among different primary producers or between primary producers and microorganisms. Seaweeds are continuously being challenged by micro-organisms (including viruses, bacteria, fungi and other algae). Nutrients, and allocation of resources to secondary metabolites are factors modifying seaweed susceptibility to herbivores and fouling (Hay 1996), but these alterations could be due to some physical changes as desiccation and light quality (Amade and Lemee 1998). Some marine organisms have developed antifouling defense mechanisms. The production and secretion of allelochemicals by aquatic macrophytes could be an effective defense strategy against other photosynthetic organisms competing for light and nutrients (van Donk and van de Bund 2002; Bazes et al. 2006, 2008). Although bacteria do not compete with macroalgae for light or most nutrients, they may enhance attachment of primary producing microalgae (Gross 2003). Prevention of epiphyte growth or other epibionts competing on macroalgal tissue by allelopathic mechanisms occurs frequently (Harlin 1987). The major cause for low epiphyte densities was identified as the reallocation of protoplasm for new growth through protoplasmic streaming, subsequent blade abandonment and proliferation (Gross 2003). The problems of allocation to defenses are further complicated by spatial or temporal variations in fouling rates. If fouling pressure is constant and predictable, then algae should maintain uniform levels of defense (Harvell and Tollrian 1999).

Several hypotheses can be proposed for antifouling defense: antifouling activity was due to other minor chemical substances secreted by the alga, or there was a synergistic effect between the major compounds and other types of molecule. The inhibition of photosynthesis, the central physiological process of competing primary producers, is an effective defense strategy of many algae. Studies indicate that the majority of the allelochemicals interfere with photosystem PSII (Silkina et al. 2012). Hellio et al. (2002, 2004a) and Bazes et al. (2006, 2008) showed that some

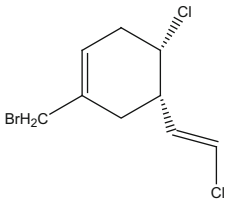

Fig. 1 Chemical structure of halogenated monoterpene from *Plocanium costatum* (König et al. 1999)

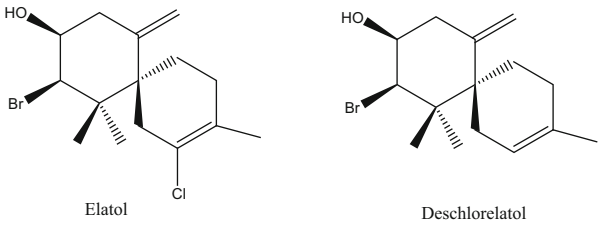

Fig. 2 Metabolites isolated from the genus *Laurencia* (de Nys et al. 1996; König and Wright 1997)

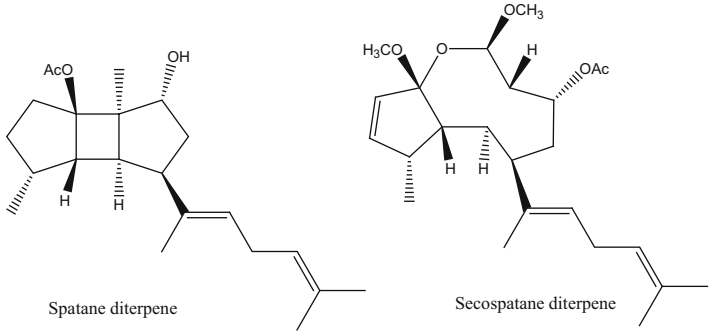

Fig. 3 Metabolites isolated from the brown alga *Rugulopteryx okamurae* (Yamase et al. 1999; Fusetani 2004)

macroalgae possessed efficacy antifungal activity, antimicroalgal and antibacterial activity (Hirschfeld et al. 1973; Archana Pal 2014). A number of red algae produced halogenated terpenoids (Fig. 1).

Antifouling activity against *Balanus Amphitrite* is exhibited by elatol and deschloroelatol (Fig. 2) isolated from the genus *Laurencia* (de Nys et al. 1996; König and Wright 1997). Spatane and secospatane diterpenes (Fig. 3) from the brown alga *Rugulopteryx okamurae* inhibit metamorphosis of abalone larvae at 5–10 ppb (Yamase et al. 1999; Fusetani 2004).

Among green algae, Ulvales are rich in acrylic acid and Caulerpales produce highly bioactive diterpenoids (Amade and Lemée 1998; Dumay et al. 2002; Fusetani 2004). Among brown algae as Fucales and Dictyotales, antimicrobial compounds found include acrylic acid, complex diterpenoids derivates, phlorotannins or phenolic lipids.

Isethionic acid (2-hydroxyethane sulfonic acid) extracted from the red alga, *Grateloupia turuturu* was active for anti-settlement against cyprid larvae of the tropical barnacle, *Balanus amphitrite* but had the disadvantage of being toxic to nauplius larvae. Floridoside (2-O-a-D-galactopyranosylglycerol) was a potent inhibitor of cyprid settlement at non-toxic concentrations to nauplii (0.01 mg ml) (Hellio et al. 2004b). The antifouling properties of *Bifurcaria bifurcata* (Heteroconta, Cystoseiraceae) could be, for example, explained principally by the occurrence of minor diterpenes or phlorotannins (Marechal et al. 2004). These diterpenes have an acyclic structure often with a furan or a lactone moiety, both of which are known to be active groups of many marine natural product antifoulants (Omae 2003). However, their low concentrations and their structural features closely related to those of the major components of the extract were not in agreement with the proposed activity of this type of metabolite. On the other hand, phlorotannins, or more generally speaking polyphenolic compounds, have been widely investigated in the field of chemical ecology (Bourgougnon and Stiger 2011). Phlorotannins are known to deter feeding and inhibit growth in a variety of marine herbivores. These compounds are, however, less likely to be an effective defense against epiphytes compared to nonpolar metabolites, which can adsorb to the surface of the organism that synthesizes them. However, the role of phlorotannins could be greater than expected. The highest antifouling activity was recorded during periods where light intensity and fouling pressure were maximal and several studies have indicated that production of phlorotannins by a number of brown algae is positively affected by these two factors.

2.3 Antioxidant

In marine ecosystems such as rocky shores, seaweeds of the upper intertidal zone are not well protected. They are emerged during long periods in which they are exposed to strong, unfiltered UV radiations which induce the production of active oxygen species and free radicals. The production of phenolic compounds on marine algae have been proved also to be involved in various protection mechanisms such as against oxidative or cytotoxic effects of UV damages. As these compounds are produced in response to the production of reactive oxygen species (ROS), phenolic compounds exhibits anti-ROS, i.e. antioxidative properties which constitute an efficient cytoprotective system. The antioxidant activity of seaweed phenols seems to depend on their structure and especially on the degree of polymerisation of phloroglucinol. Mycosporine-like aminoacids (MAAs) are UV-screen and antioxidant substances found in red algae (Table 1). MAAs are very efficient photoprotectors with high energy dissipation without production of oxidant photoproduct and with high photostability. The presence of MAAs varies throughout the year due to the effect of solar irradiance (Bourgougnon and Stiger 2011; Guinea et al. 2012).

Table 1 MAAs found in red algae

MAAs	Algae	References
Palythine	*Chondrus crispus Palmaria* sp. *Grateloupia lanceola Porphyra columbina Curdiea racovitzae*	Yuan et al. (2009), Huovinen et al. (2006), Karsten and Wiencke (1999), Peinado et al. (2004), Franklin et al. (2001), and Hoyer et al. (2001)
Palythene	*Chondrus crispus Desmarestia menziesii*	Karsten and Wiencke (1999) and Franklin et al. (2001)
Porphyra 334	*Porphyra* sp. *Curdiea racovitzae*	Yuan et al. (2009), Conde et al. (2000), Huovinen et al. (2006), Karsten and Wiencke (1999), Peinado et al. (2004), Hoyer et al. (2001), and Karentz et al. (1991)
Asterina 330	*Palmaria decipiens Palmaria palmata*	Yuan et al. (2009), Karsten and Wiencke (1999), Peinado et al. (2004), Franklin et al. (2001), and Hoyer et al. (2001)
Palythinol	*Grateloupia lanceola Gracilaria cornea Desmarestia menziesii*	Yuan et al. (2009) and Karsten and Wiencke (1999)
Shinorine	*Palmaria palmata Porphyra columbina Chondrus crispus Curdiea racovitzae*	Tsujino et al. (1980), Yuan et al. (2009), Sinha et al. (2000), Peinado et al. (2004), Franklin et al. (2001), Hoyer et al. (2001), and Sinha et al. (1998)
Usujirene	*Palmaria* sp.	Yuan et al. (2009)
Mycosporine-glycine	*Chondrus yendoi Chondrus crispus Gymnogondrus devoniensis Palmaria* sp. *Gracilaria cornea Porphyra columbina Curdiea racovitzae Phyllophora appendiculata*	Huovinen et al. (2006), Karsten and Wiencke (1999), Peinado et al. (2004), Hoyer et al. (2001), and Karentz et al. (1991)

3 Global Mechanisms

Potin et al. (2002) summarize biosynthetic pathways by a model of the integration that results in the production of defense metabolites and putative distance signals in marine algae (Fig. 4). Oxylipins are thought of as endogenous chemical signals that induce the production of phlorotannins in fucoid algae in response to grazing. The isoprenoid pathway is also activated in response to grazing in brown algae.

In red algae, the shikimic pathway and both the octadecanoid and the eicosanoid pathways are activated by elicitor treatment, and are responsive to C20- and C18-fatty-acid-derived oxylipins. In fucoid brown algae, water-borne or air-borne cues that induce phlorotannin accumulation may derive from a lipoxygenase pathway. Alternatively, they may be composed of a bouquet of volatile or non-volatile compounds originating from several biosynthetic pathways. The role of these volatile compounds as chemoattractants for gametes is well established, but the nature of

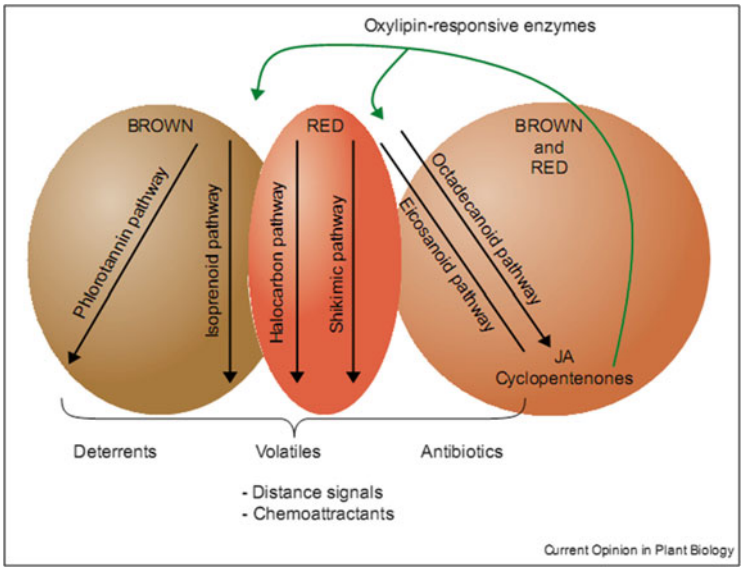

Fig. 4 Model of the integration of the biosynthetic pathways that result in the production of defense metabolites and putative distance signals in marine algae (Potin et al. 2002)

the warning distance signals is not yet elucidated. The function of these bioactive compounds and the nature of specific pathways involved in their synthesis have hardly been investigated in marine algae, which emerged as independent lineages early in the evolution of eukaryotes (Gaquerel et al. 2007). Similarly, since the red alga *C. crispus* both synthesizes and is responsive to hydroperoxides from both eicosanoic and octadecanoic fatty acids, parsimony rules tend to suggest that the oxylipin pathway ancestrally featured these two categories of lipid signals. On the other hand, marine plants also feature defense mechanisms that appear to be phylum- or environment specific. Unlike terrestrial plants they have no tissue-driven system, a difference that is likely to be associated with the absence of vascularization in algae. Also, the occurrence of water-borne defense signals is obviously relevant to the aquatic environment. Another unique feature of the marine environment is the abundance of halides, which are used by marine algae to produce antimicrobial compounds (Potin et al. 2002).

4 Conclusion

Seaweeds are known to produce inhibitory allelochemicals that interfere with epiphytes and other epibiont competitors like bacteria, fungi, diatoms, macroalgae, larvae of mussels or barnacles. These allelochemicals thus constitute a source of natural bioactive products although little has been done to define their ecological

role and to better understand their biosynthesis. The biotic parameters, as well as abiotic factors like seasonality and the geographical localization can influence the level of bioactive extract.

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Marine Algae: Gathered Resource to Global Food Industry

Jeff T. Hafting, M. Lynn Cornish, Amy Deveau, and Alan T. Critchley

1 Introduction

Marine algae (seaweeds) have been gathered for thousands of years as important regional sources of highly nutritious food. The successful development of industrialized large-scale cultivation of seaweeds has led to the distribution of seaweed based foods to markets around the world. Modern aquaculture has advanced to where it is currently the fastest growing sector in food production (FAO 2012), and seaweeds play a major role in this success. Scarcity of supply due to over-harvesting, natural disasters, climate change, etc. has led to market limitations for many seaweeds. Cultivation will continue to be essential for the globalization of seaweed consumption, especially as consumers demand products from sustainable sources. This chapter will focus on the development of the global seaweed food industry, from gathered sustenance, to successful domestication and cultivation, to future development in high value functional markets.

The consumption of seaweed as food is no longer confined regionally to near shore communities with ready access to harvestable raw material, and the distribution and marketing of seaweed-based products has developed into a global enterprise. Food products containing seaweed extracts (e.g., hydrocolloids) were the first to be commoditized and globalized, but whole seaweeds food products are currently increasing in multiple markets. In addition, new processed seaweed food products are being developed based on traditionally utilized species. These include snacks, spices, salt-substitutes, wraps, soups, salads, etc.

Food safety and traceability is critical to the success of the seaweed food industry and particularly future development of nutraceuticals. Seaweeds lend themselves well to dry preservation, and there are few concerns over consumption safety. However, the

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D. Sahoo, J. Seckbach (eds.), *The Algae World*, Cellular Origin, Life in Extreme Habitats and Astrobiology 26, DOI 10.1007/978-94-017-7321-8_15

safety of seaweed foods will come under increased scrutiny by regulators, especially in markets where seaweeds do not have a strong tradition of food usage.

Many major food companies are investing in the development of functional foods, resulting in sustained growth in this sector. Food science is advancing our understanding of the functional properties of many seaweed-based food products, and in many cases traditional healthy benefits of their consumption are being explained by modern food-science. This advancement coupled with the expansion of seaweed cultivation (both on-land and ocean based) bodes well for the continued growth of seaweed markets within the global food chain, and the industry's progression into more refined functional food production.

2 Human Dependence on Seaweed

Coastal human populations have always been dependent upon the harvest from the oceans for sustenance. Seaweeds represent not only a direct food source, but they also provide diverse habitats that support a wealth of other organisms, including fish, shellfish, seabirds, and mammals. Charles Darwin (1909) observed the richness of the great kelp forests of Tierra del Fuego and wrote, "…if in any country, a forest was destroyed, I do not believe nearly so many species of animals would perish as would here, from the destruction of the kelp."

The ocean has not only provided a means for supporting human population and cultural development, but it has also provided support for global migration. "The Kelp Highway Hypothesis" (Erlandson et al. 2007) postulates that the populating of the Americas from Asia at the end of the Pleistocene was facilitated by the presence of continuous cold water kelp forest ecosystems from Japan to Baja California, and along the Pacific coast of South America. Between Baja California and South America are found highly productive mangrove forests and coral reefs. It is the diversity and abundance of food-stuff found in these cold-water kelp forest ecosystems (others in the tropics) that may have sustained migrating populations through the Americas. There is mounting archeological evidence supporting the hypothesis that such a migration occurred, showing the importance that seaweeds have had to human maritime societies from the beginning.

3 Ethnography of Seaweeds as Food

Seaweeds are important sources of everyday foods in China, and are used with a variety of cooking methods, just as vegetables are utilized in the western diet (raw, fried, steamed, boiled, etc.). The cultivation of *Laminaria* and *Porphyra* are huge industrial activities in China. In fact, China utilizes more seaweed species for food than any other ethnic group in the world (Xia and Abbott 1987). There are written records in China which date back at least 2000 years, documenting the medicinal and edible uses of seaweeds. All seaweed groups are utilized by the Chinese (Table 1).

Table 1 Selected seaweed species utilized in China as food (Xia and Abbott 1987)

Chlorophyta (Green algae)	*Ulothrix flacca*	Fresh vegetable
	Monostroma nitidum	Dry condiment
	Ulva lactuca	Fresh vegetable
	Ulva conglobata	Fresh vegetable, tea
	Ulva fasciata	Fresh vegetable, tea
	Enteromorpha linza	Soup
	Enteromorpha intestinalis	Soup, bread
	Enteromorpha compressa	Dry condiment
	Enteromorpha tubulosa	Dry condiment
	Enteromorpha prolifera	Dry condiment
	Caulerpa racemosa	Fried vegetable
Phaeophyta (Brown algae)	*Ishige okamurai*	Dry condiment
	Ishige sinicola	Dry condiment
	Endarachne binghamiae	Dumplings
	Scytosiphon lomentaria	Noodle soup
	Laminaria japonica	Fresh, dry vegetable
	Chorda filum	*Laminaria* substitute
	Hizikia fusiformis (formerly *Sargassum fusiformis*)	Cooked vegetable
Rhodophyta (Red algae)	*Bangia fusco-purpurea*	Fresh, dry rolls
	Porphyra suborbiculata	Fresh, dry vegetable
	Porphyra crispata	Fresh, dry vegetable
	Porphyra dentata	Fresh, dry vegetable
	Porphyra haitanensis	Fresh, dry vegetable
	Porphyra tenera	Fresh, dry vegetable
	Porphyra yezoensis	Fresh, dry vegetable
	Porphyra marginata	Fresh, dry vegetable
	Porphyra monosporangia	Fresh, dry vegetable
	Porphyra oligospermatangia	Fresh, dry vegetable
	Dermonema pulvinatum	Fresh vegetable, often salt cured
	Dermonema frappieri	Fresh vegetable, often salt cured
	Asparagopsis taxiformis	Rinsed, served with pork
	Gloiopeltis spp.	Fried, served hot
	Gratelopia filicina	Boiled, gelatinous liquid consumed
	Gratelopia livida	Boiled, gelatinous liquid consumed
	Gigartina intermedia	Cooked with pork
	Gracilaria spp.	Agar gel extracted and consumed
	Gelidium spp.	Agar gel extracted and consumed
	Pterocladia spp.	Agar gel extracted and consumed
	Hypnea japonica	Stewed with pork, fish

Japan has a traditional use of seaweeds as foods that dates back nearly as long as the Chinese. Indeed there are many similarities between Japan and China in the seaweed species utilized, with 52 species utilized in Japan (Arasaki and Arasaki 1983). The main differences between Japan and China are in the methods used to prepare seaweeds for consumption, with Japanese dishes primarily served cold after blanching and the addition of soy sauce, sugar, and/or vinegar. Japan has a very large *per capita* consumption of seaweed (as much as 1.6 Kg dry *per capita* (Fujiwara-Arasaki et al. 1984)).

Korea has the most diverse seaweed cultivation industry in the world, built mainly around the production of *Porphyra*, *Undaria*, *Hizikia*, *Laminaria*, *Sargassum*, *Enteromorpha*, and *Codium* for Asian markets (Sohn 1998). Over 97 % of all seaweeds produced from Korea are from cultivated sources (Sohn 1998). Traditionally, about 40 seaweed species have been utilized as food, and this continues to the present. Many find their way into Kimchi, a traditional fermented vegetable dish in Korea (Hwang et al. 2007). Korea is a leading nation for the investigation of the functional properties of seaweeds for human health.

In the Philippines, there is a long history of use of seaweed as food. Except for *Caulerpa*, *Kappaphycus*, *Eucheuma*, and *Graciliaria* these seaweeds are wild collected and added to salads (fresh or blanched) or used as flavorings or garnishes in prepared dishes. Commonly collected and consumed genera include, *Acanthophora*, *Codium*, *Enteromorpha*, *Grateloupia*, *Halymenia*, *Scinaia*, *Hydroclathrs*, *Laurencia*, *Porphyra*, and *Gelidiella*. Unlike other places in Asia, seaweeds in the Philippines are usually sold and consumed fresh, and are not pre-packaged in dry format before use (Trono 1999).

In the Hawaiian Islands prior to western contact, seaweed was a regular component of the local diet. It was added to raw and cooked meat and fish dishes, and was used as a spice. Today the local market is provided with fresh seaweeds from four main species, both from wild (w) and cultivated (c) sources: *Asparagopsis taxiformis* (*w*), *Codium reediae* (*w*), *Graciliaria coronopifolia* (*c*) and *Gracilaria parvispora* (*c*). Seaweed is still consumed fresh daily by a diverse Hawaiian population (McDermid and Stuercke 2003). There are a number of other species which are collected and utilized by Hawaiians, and the diversity of seaweed species used as medicine and food is second only to the Chinese (Xia and Abbott 1987).

In the Azores, seaweeds have traditionally been consumed similarly to the Hawaiian Islands. Gathered and consumed species include *Fucus spiralis*, *Porphyra*, *Laurencia*, and *Osmundea*. There is also an industry based on the harvesting of *Pterocladiella capilacea* and *Gelidium microdon* for agar extraction (Patarra et al. 2011).

Ireland has a long history of seaweed use as food, dating back to at least the Middle Ages. Ireland is currently a center for the discovery of human health benefits derived from the consumption of seaweeds, as market value is added to this diverse resource. The main genera utilized as food include, *Alaria*, *Laminaria*, *Himanthalia*, *Palmaria*, *Porphyra*, *Chondrus*, *Ulva*/*Enteromorpha*, and *Codium*. The bulk of this raw material is wild harvested, with some investigation and pilot scale cultivation work underway (Guiry and Hession 1998). Seaweeds are commonly referred to as

"Sea Vegetables" in Ireland, and are finding acceptance as more than just a food consumed during times of famine (there was a stigma attached to seaweed consumption for decades in Ireland as a "poor" family's food).

Along the northwest coast of North America, seaweeds were traditionally valued for medicine, food, and trade. Species harvested as food included *Egregia menziesii*, *Macrocystis pyrifera*, and *Porphyra abbottiae* (Turner and Bell 1973; Compton 1993). In particular, *Porphyra* spp. were frequently harvested and consumed, and continued to be culturally significant foods among several First Nations in British Columbia today (Turner 2003). Historically, dried seaweed was an important barter good between coastal and inland peoples. *Porphyra*, *Fucus distichus*, *Ulva lactuca*, and *U. intestinalis* were used as medicine for various ailments and either eaten or used externally as poultices (Turner and Bell 1973; Compton 1993). Prior to European contact, the long, hollow stipes of *Nereocystis luetkeana* were made into fishing rope or used for storing liquids (Compton 1993; Turner 2003, 2007).

Archaeological evidence strongly suggests the use of algae by Indigenous Peoples in South America beginning thousands of years ago up to the present. At Monte Verde in southern Chile, Dillehay et al. (2008) identified the remains of seven marine algae including *Porphyra*, *Sargassum*, *Durvillaea*, *Gigartina*, *Mazzaella*, *Sarcothalia*, and *Macrocystis* that were dated at approximately 14,000 years B.C. The abundance of seaweed remains in many hearths at the site as well as a close association with the remains of medicinal plants suggests that seaweed was used both as food and medicine. Charred remains of seaweed suggest that the seaweed was dried for preservation and transport from the coast or cooked there on-site. Traces of kelp were found at several sites in Peru dating back to the early Cotton pre-ceramic period (approximately 2500 B.C.) (Moseley 1975). Peruvian Incas had a bridge named *Chaquillcharo*, or 'seaweed bridge,' leading from the mountains toward the Pacific coast that could be used as a trade route (Aaronson 1986). Today, Quechuan peoples in Peru use both marine and freshwater species of algae as part of traditional meals.

In Atlantic Canada, First Nations particularly valued seaweeds as medicine. Raw "dulse" (*Palmaria palmata*) was eaten to treat worm infestations, but also served as food (Wallis 1922; LeVangie and Soto Quenti 2008). Until recently, *Ulva* spp. were used medicinally to treat skin ailments such as warts or jellyfish stings, while *Fucus* and *Laminaria* were used to treat bruises, sore or hoarse throats, and for healing and straightening bones (LeVangie and Soto Quenti 2008). Various macroalgae were also used in food preparation such as pit cooking, smoking fish, obtaining salt, and making tea. Seaweed use has declined among First Nations in eastern North America as a result of drastic changes in socioeconomic factors after European contact (Kuhnlein and Receveur 1996; LeVangie and Soto Quenti 2008). Today *P. palmata* and *Porphyra* spp. are commercially harvested in eastern Canada and New England, and sold to domestic and international markets (Chopin and Ugarte 2006). The harvest of *Palmaria palmata* (dulse) is a long-standing tradition in Eastern Canada, and even today during the seasonal harvest periods, a drive along coastal Nova Scotia will reveal backyards and parking lots (petrol pumps) covered with dulse spread to dry in the sun. It is common to find bags of dulse for sale at gas stations, and at

road-side stands, as it is a coveted seasonal snack food. The harvest of *Chondrus crispus* (Irish Moss) for carrageenan extraction still occurs, although at a reduced scale, when compared to its peak between 1950 and 1970 (Chopin 1989; Collén et al. 2014), when Canada's production of Irish moss ranked first in the world (previously France and Ireland were ranked first, but WWII cut off this supply and Canada took over (Stoloff 1949)). On-land cultivation technology for *Chondrus crispus* production was commercialized first by Marine Colloids, then by Acadian Seaplants Limited in the 1980s, and is still operational today. Today this cultivated *Chondrus crispus* is sold as a high value food marketed as Hana-Tsunomata™ in Japan for the kaiso (seaweed) salad market.

This account is not meant to be exhaustive, and there are a number of geographies that are not mentioned here. This section will give a good overview of the main seaweed "consumer" nations, and some interesting patterns of seaweed food-use globally.

4 Seaweed Food Value

Various seaweeds may contain high levels of carbohydrates, minerals, vitamins and trace elements (MacArtain et al. 2007). Seaweeds differ in their nutritional value species by species, and also geographically and seasonally (Patarra et al. 2011; Stengel et al. 2011). The following overview is meant to provide a generic account of the food value of a variety of seaweeds.

Seaweed polysaccharides occur in soluble and insoluble forms. Seaweeds contain cellulose and hemicellulose as common structural polysaccharides (i.e., insoluble fibre). Soluble dietary fibers include agars, carrageenans and alginates. These carbohydrates are unique to seaweeds and are the basis for the global hydrocolloid industry. Species-specific polysaccharides such as porphyran (*Porphyra*), laminarin (*Laminaria*), ulvan (*Ulva*), xylan (*Palmaria*), floridean starches (red seaweeds), and fucoidans (from various brown seaweeds) can also been found. Seaweed polysaccharides are hydrophilic and often water soluble since they are rich in hydroxyl groups. They are known to form regular inter-chain H-bonds, allowing them to gel and are therefore suitable for use as thickeners and stabilizers (O'Sullivan et al. 2010). Seaweed fibre content compares well with terrestrial-based foods, often with comparatively elevated levels. Soluble seaweed fibres pass through the GI tract largely undigested, therefore increasing the feelings of satiety and aiding digestive transit. There is also evidence for binding of heavy metals, decreasing their bioavailability. Seaweed soluble fibres show fermentative capacity in the lower GI tract (i.e., prebiotic potential). In addition, various seaweeds have a very low glycemic index despite their high polysaccharide content.

Seaweed mineral content is also high and diverse. Sodium and potassium are present at significant levels, although the Na:K ratio is usually below 1:5, thus making seaweeds good candidates as salt substitutes in food (Mouritsen 2012). The calcium content of some seaweeds can be higher than common dairy foods,

and iron and copper are present at higher levels than many terrestrial sources, such as meat and spinach for example. Brown seaweeds are particularly good sources of magnesium, copper, iron and iodine. Consumption of large amounts of iodine may pose some health risks, but seaweeds have been described as ideal food-safe natural sources of this mineral. Trace elements such as zinc are present in seaweeds, as well as some less desirable metals such as arsenic. When considering the potential deleterious health effects of metals such as arsenic, it is important to consider the form present in the specific seaweed. In the case of arsenic, it is the inorganic form which is problematic, and most seaweeds are very low in this type (*Hiziki*, which is consumed in Japan as a high value food may be an exception). For the vast majority of seaweeds, heavy metals are present at concentrations that are well below food safety limits. The presence of metals in seaweed products does not necessarily indicate contamination, as seaweeds can bind and accumulate metals from the environment.

The fatty acid content of seaweeds is typically in the range of 2–4 % of dry weight. Almost half of the lipid content is PUFA, with a good balance of omega-3 and omega-6 lipids. Seaweeds may contain a number of essential fatty acids, and the fatty acid content is preserved well by drying, the platform by which seaweeds are most commonly packaged and transported.

Various seaweeds may also contain many forms of antioxidants, such as vitamins and protective pigments. Vitamins include A, B, C and E. Vitamin E is present in wakame (*Undaria*) at higher levels than is found in peanuts. Vitamin C is present in nori (*Porphyra*) such that an eight dry gram portion may contain 15 % of an individual's recommended daily intake (RDI). Some seaweeds also provide vitamin B12, one of the few vegetable sources and an alternative source of this vitamin for vegetarians and vegans. Beta carotene and lutein may also be found at high levels. Chlorophyll and fucoxanthin can also be present in seaweeds at significant levels.

Protein content differs among seaweed groups with brown seaweeds being generally lower in protein (i.e., maximum content typically up to 15 % dry weight) than the reds and greens (Patarra et al. 2011). Some seaweeds can be very high in protein, with nori having a protein content up to 47 % dry weight. Aspartic and glutamic acids make up a large portion of the amino acid content of these proteins. Glutamic acid is the main component in the "Umami taste", and brown seaweeds typically contain this amino acid at high levels. Dulse (*Palmaria palmata*) also has high levels of glutamic acid and can be used as a base for Umami dishes (Mouritsen et al. 2012). Essential amino acids such as histidine, leucine, isoleucine, and valine are present in a number of seaweeds, at levels that can rival legumes (dulse, *Palmaria*) and eggs (sea lettuce, *Ulva*). Proteins are highly digestible in many red seaweeds, but digestibility is dependent upon the amount of soluble fibre present (i.e., the higher the fibre content the lower the bioavailability of the protein (MacArtain et al. 2007)).

A large number of research articles have identified interesting opportunities for seaweeds as functional foods or ingredients (Holdt and Kraan 2011). Functional foods can be defined those which benefit health when consumed, over and above that of simple delivery of nutrition. Many seaweeds are good dietary sources of

protein, carbohydrate, fibre, minerals, and vitamins. A very small number of seaweeds have some toxicological aspects to their mineral and secondary metabolite concentrations which must be considered before consumption, but in general seaweeds make excellent foodstuff. Interestingly, their protein, carbohydrate, lipid, fiber, and metabolite content can be highly influenced by growing conditions. Seaweeds are excellent bioaccumulators, able to concentrate and provide different types of compounds, depending upon environmental conditions during growth (Plaza et al. 2008). Understanding the effects of environmental conditions upon nutrient content will assist in the improvement of the quality and consistency of consumer products. While there is great potential for seaweeds as sources of functional foods and ingredients, there is much work to be accomplished before their production at industrial scales can be achieved. Investigations into optimal cultivation conditions, extraction, purification, preservation of their activity, and bioavailability in consumer formats must be undertaken by phycologists and food scientists (Day et al. 2009).

5 Seaweed Food Products Currently on the Market

Seaweeds have been consumed globally by coastal peoples since prehistoric times, and have survived into the contemporary cuisine mainly in Asia (especially Japan, Korea, China, and the Philippines (Mouritsen 2012)). Currently the worldwide food market for seaweed is valued near US$10 billion (Mouritsen 2012). The recent globalization of dishes that make obvious use of seaweeds (e.g. Sushi) has raised their profile outside of Asia. There have also been some high profile personalities who have been promoting the food uses of seaweeds in the west. Two of these seaweed "champions" are Dr. Ole Mouritsen (Denmark) and Dr. Prannie Rhatigan (Ireland) who have both published highly successful books illustrating the ease with which seaweeds can be included into non-Asian recipes at home (Mouritsen 2010; Rhatigan 2010). These books describe a wide variety of seaweeds that can be collected locally, or utilize store-bought fresh or dried, whole thallus ingredients.

There have been significant efforts recently to expand the range of seaweed-based packaged foods, where seaweed is front and center (i.e., the main marketing message is that these are seaweed-containing). Most of these new products are found in Asia, but some are making their way to the West, in purely Western formats. For example there are many dry nori-based snacks on the market, using roasted/toasted seaweed along with seasonings, which have been popular in Asia for decades, and which are now using Asian celebrities as spokes-people for their marketing (Jennie 2013). These snacks are currently making waves in North America as chip substitutes (Annie Chun's n.d.). Seaweed marketing in the 1990s was not directed at the mainstream North American market, these were "Asian-style" snacks and were found only in Asian markets. However, a switch to marketing these snacks as potato chip alternatives to North Americans has resulted in their

common-place occurrence on supermarket shelves in the chip/snack section. There has been an explosion of available flavors (combinations of Nori with chocolate, brown sugar, pepper, sesame, wasabi) and formats (chips, sheets, strips) and clever names (e.g., "Dried Zombie Skin" for the school child market).

Seaweed may be found as a main ingredient in western foods such as cereals, granola bars, chips, and crackers (Ocean's Halo n.d.). Miso soup benefits are well known, and there are a number of readily available dried soup mixes which include seaweed (The Vegan Store n.d.). A relatively new "shaved" kelp product which can be added to soups for taste and texture, has been very popular since introduction a few years ago (Japanesefood-Life n.d.). Fresh and dried seaweeds can be found easily in Asia, but there are also now frozen, pickled, and canned products to choose from. Kaiso salads may be found packaged dry or fresh, and seaweed tempura and kimchi are popular processed foods (Daeyang n.d.). Seaweed teas are common in Asia, but they are also finding some acceptance in the west, both in cold and hot formats (Lookfantastic n.d.). Seaweed based noodles are a popular way of including seaweed into western pasta dishes (21food n.d.). It is not difficult to find salt-substitute products which are simply ground seaweed, purchased in a shaker format (Maine Coast Sea Vegetables n.d.).

New seaweed products are usually developed in Asia, and then they eventually find niche markets in the west. Nori sheets for rice based lunch dishes (sushi in Japan, musubi in Hawaii), are also seeing some new marketing strategies come forward. These include the development of novel shapes to entice children, and there is even a hole-punch that can be purchased for making your own shapes from nori sheets to put on top of rice (Lelong.com n.d.). There is a Lays™ potato chip product in Asia that is seaweed flavored (Cebulski 2011), and Dunkin' Donuts™ in China has pork and seaweed stuffed donuts (Smosh.com n.d.)!

It is interesting to note that in Asia, developers use the interest in "western" types of food products (e.g. chips, donuts) to drive seaweed consumption by including this typically Asian ingredient into these new, unfamiliar foods. At the same time, in "western" markets (e.g. North America, Europe), developers use the interest in Asian cuisine to introduce unfamiliar seaweed to a new market. In both cases, Asia is driving the globalization of seaweed foods.

6 Functional Properties of Seaweeds

The recent trend in consumer acceptance towards food products which maintain and improve health, coupled to the convenience of use has driven the food industry towards the development of functional foods. Interest in seaweeds as functional foods has grown over the past decade. To date, investigations demonstrated various bioactivities including antibacterial (Nylund et al. 2010), anti-viral (Kim et al. 2011), apoptotic (Foley et al. 2011), antioxidant (Garcia-Casal et al. 2009), anti-inflammatory (Shin et al. 2006), and anti-coagulant activities (Lee et al. 2008). There are several excellent reviews available in the literature on the functional

properties of seaweeds (Holdt and Kraan 2011; Mendis and Kim 2011; Smit 2004), and the following paragraphs are meant to give broad coverage of the variety of activities that have been investigated with seaweeds to date.

The promotion of digestive health is a relatively recent trend in the food industry where seaweeds will play a major role. Currently, seaweed-based food additives are commonly used in processed foods to provide texture and stability. However due to the generally high dietary fibre content of seaweeds, and the commonly accepted view that a diet rich in fibre can help protect against a number of chronic diseases, seaweeds are an attractive subject for research into digestive health. Seaweeds which contain high concentrations of polysaccharides (dietary fibre) of varying structure, have potential functionality as prebiotics (O'Sullivan et al. 2010). Prebiotic effects may be enhanced with a diet rich in seaweed polysaccharides, and when combined with high-glycemic index foods, the glycemic response is suppressed (i.e., reduction in blood cholesterol and control of blood glucose (Patarra et al. 2011)). There is much *in vitro* and *in vivo* experimental data which demonstrates the potential prebiotic effects of seaweed polysaccharides, however few human studies have been conducted to date. There have been conflicting results from some studies, mainly due to factors including experimental conditions, age and physiology of the animals tested, and variations in the type, dose, purity, and seasonality of the seaweeds studied (O'Sullivan et al. 2010).

Antiviral activity has been shown with sulphated polysaccharides from red seaweeds. Suppression of human immunodeficiency virus (HIV), Herpes simplex virus (HSV), human papilloma virus (HPV), and respiratory syncytial virus (RSV) has been noted. Sulphated polysaccharides are active during the first stage of viral replication, while at the same time these compounds have low cytotoxic activities toward mammalian cells. Carrageenans and fucoidan have also shown anti-viral activities, acting to prevent attachment to the host cells.

Antibiotic activities are common in seaweeds, and a variety of compounds have been associated with these effects, including halogenated compounds such as terpenoids, sterols, and phenolics. There are many potential antiseptic-type products that are currently under development. In the food industry context these antibiotic activities directly associate with enhanced food safety. When included in prepared foods (and cosmetics) microbial counts are reduced, lessening the requirement for the additions of chemical preservatives.

Cytotoxicity against cancer cells has been noted for some sulphated polysaccharides (e.g., fucoidans, and ulvans). Compounds such as chondriamide A, terpenes, and caulerpenyne, among many others have been shown to inhibit mitotic cell division, and therefore may inhibit the growth of cancer cells (Smit 2004).

Seaweeds also contain a wide variety of polyunsaturated fatty acids, especially the red seaweeds. Anti-inflammatory and immune-stimulatory effects have been shown with various fatty acid classes, including eicosapentaenoic (EPA) and arachidonic acids (AA). Phospholipids may be common in some seaweeds, and resist oxidation better than fish oils, making them potentially useful in food applications.

The antioxidant capacity of compounds present in some seaweeds has attracted much interest. There are a wide variety of antioxidants available in seaweeds, since

they are photosynthetic, and are frequently exposed to stressful conditions of high light exposure (including UV/IR) and oxygen production internally (Zubia et al. 2009). Some antioxidants of interest include phlorotannins, polyphenols, ascorbic acid, tocopherols, and carotenoids (Cornish and Garbary 2010). The antioxidant potential of seaweeds for food is two-fold: direct effects on disease prevention, and their stabilization of food ingredients which are prone to oxidation (fish oils and dairy for example, Devi et al. (2008)).

7 Seaweeds as Functional Food and Their Inclusion in Nutraceutical Development

The development of functional food products which contain seaweeds as raw materials (either extracts or whole) is an emerging sector in the food industry. Major food companies have invested heavily in the development of their functional food markets. Success demands investment in nutritional research, as well as in product and market development. Large multi-national food companies typically spend multiple millions of dollars to launch a new food product. These costs can be an order of magnitude greater when a new functional food product is launched, especially if there is a specific health claim associated with the new product. Interestingly, pharmaceutical companies are also attracted to the functional food market because of the shorter product development times and lower costs, as compared to traditional pharmaceuticals! Pharmaceutical companies have considerable expertise in organizing clinical trials for health claim substantiation. The high cost of functional product development limits the success of small and medium-sized food companies (SMEs). In general, these SMEs lack the resources required for intensive R&D activities, and cannot make the financial investments to advertise and promote a new product in order to obtain the market penetration required for success. Some successful SMEs have come from highly innovative companies that supply specific food ingredients. These SMEs have invested in proving the efficacy of specific ingredients, and are then able to market to a wide range of larger food companies. These smaller innovative companies are of high relevance to the functional food industry (Menrad 2003).

It is assumed that the development of functional food markets is sustainable, since socio-demographic trends are favorable. For example, the nutritional sciences have moved towards a better understanding of the links among diet, health and disease prevention. This has driven consumer trends toward healthier eating, especially within populations with aging demographics. A critical success factor for functional food development is the delivery of targeted information to consumers and opinion leaders such as medical doctors and nutritionists. Success depends on correct messaging, and this depends greatly on the intended audience. Regulatory bodies and opinion leaders demand peer-reviewed publication of efficacy data, with human studies recognized as the gold standard, with the mode of action clearly presented. Consumers on the other hand, are generally influenced by the familiarity of a

functional food. They want ingredients which are recognizable, and have clear explanations of their health benefits. Consumer acceptance of specific functional foods is directly linked to knowledge of the health effects attributed to specific ingredients. Therefore, products which are relatively new to consumers, often take a considerable amount of time to become accepted and successful. They start out as something unfamiliar, and the longer these new foods are around, the greater acceptance they gain. Therefore, effective messaging to consumers on efficacy, does not necessarily guarantee success if the new product is not familiar to begin with. This is especially relevant to the seaweed food industry when attempting to penetrate new markets with something as unfamiliar perhaps as seaweed. Consumer messaging must be simple and easily understood. Consumers consider functional properties as "value added" and do not make food purchasing decisions based only on efficacy. Taste is still the most important success factor of any food, functional or otherwise. Success of functional products also heavily depends upon easy access (high volume retail outlets and supermarkets), and not only specialty shops, requiring a special trip to a low-volume vendor.

Since the development costs of functional foods are relatively high, these products require a price premium from consumers. For highly efficacious products supported by convincing published scientific evidence, the price premium is considered to be in the range of 30–50 % (Menrad 2003). This is a limiting factor, since developers need to recoup their R&D development costs, and without a premium, this type of work is not always cost effective.

8 Functional Products Currently on the Market

Before the use of iodized salt became widespread, iodine deficiency was common in human populations in remote inland communities, where no marine foods are consumed, or in mountainous areas were the soil is iodine poor. Iodine deficiency can lead to the development of goiter and cretinism. The problem of iodine deficiency continues today, but is now combated mainly with the development of iodized salt. Before iodized salt was introduced, dried kelp was used to combat goiter in these remote populations, and this may be the first use of seaweed as a functional food.

The nutraceutical industry is currently developing and marketing the bulk of functional seaweed products with associated functional health claims for human consumption. Some examples follow. Fucoidan is available in tablet form from a variety of manufacturers, which can vary in price and purity depending upon the source. In addition, it is often marketed as an immune system booster, and a potent antioxidant. β carotene from seaweeds can also be found on the market in capsule form, sold for its antioxidant effects. Some seaweeds are often sold as low sodium salt replacers for their beneficial effects on blood pressure (i.e., the salty flavor is mainly due to potassium, not sodium). These are ground whole seaweeds, packaged in salt-shaker containers for ease of use. Seaweeds can be found in whole ground

form in a variety of tablets and capsules which make a variety of health claims, mainly based on their mineral content (Irish moss = *Chondrus crispus*, Norwegian Kelp = *Ascophyllum nodosum*).

Alginates (sodium and potassium salts) and some specialty carageenans are now being marketed for their effects in combating obesity. The effects are attributed to their prebiotic fibres, and the benefits seen with higher fibre diets. There is a product on the market that is specifically targeting weight loss. It contains *Fucus vesiculosus* and is sold under the trade name Quantrim™. This product makes a direct claim for weight loss based upon its chelated iodine content, as well as its induction of satiety due to the alginates' hygroscopic properties (gut bulking).

Jamaican Irish Moss drink (marketed by Big Bamboo) is available in canned format in a variety of flavors (e.g., peanut, banana). It is a carrageenan-thickened product that makes the claim of being an aphrodisiac. The product is very high in fat and sodium, and the aphrodisiac claim is dubious at best (no science involved) but the product is very popular in the Caribbean.

9 Global Food Industry Raw Material Requirements/ Regulations

The food industry has become more focused on regulating the source of raw material inputs into consumable products. There have been significant events such as the bovine spongiform encephalopathy (BSE) crisis, *Escherichia coli* contamination of spinach and other vegetables, and considerations such as genetically modified (GM) crops entering the food chain. Consumers are demanding assurances that ingredients and raw materials are safe, and this has focused the food industry on traceability. Traceability relates to the origin of materials, their processing history, and the distribution chain of consumer products. A requirement of industrial food production and distribution is the ability to trace a product's history by means of recorded data (Bertolini et al. 2006).

The European Union (EU) has some of the most stringent requirements for traceability. The development of GM foods and the safety concerns of many consumers around these products, led to the requirement that all GM foods be labeled as such in the EU. The European Food Safety Authority (EFSA), defines "traceability" as the ability to trace or follow a food, feed, food-producing animal or substance intended to be, or expected to be incorporated into a food or feed, through all stages of production, processing and distribution. France was the first European country (1990) to establish specific regulations concerning the use of seaweeds for human consumption (Mabeau and Fleurence 1993). Under these regulations, seaweeds must meet certain consumer safety regulations for maximum allowed levels of toxic minerals (lead, cadmium, tin, mercury, mineral arsenic and iodine), as well as maximum allowed bacterial loads. Within Europe, some countries are not subject to any particular regulations (Ireland, Denmark, The Netherlands), while in others they have not been considered food, and have been strictly forbidden (Greece).

Many food companies now market products in multiple countries, and each country can have its own set of regulations that must be complied with. This is a more powerful driver of food product formulations than it may appear at first glance, since formulators will naturally include ingredients that have widest acceptance. This has implications for seaweed food product regulatory acceptance in non-traditional markets where consumption of seaweed is not common.

Manufacturers frequently incorporate traceability data collection into quality assurance programs, and this practice is often a means to improving product safety. The process as a safety tool is most useful when the system is transparent, and allows for rapid and thorough tracing of a material's history from origin to consumer. A good traceability system allows for a number of competitive advantages including the linkage of end products and raw materials leading to improved process control, reduced occurrences of product recalls, and avoidance of mixing high and low quality materials (McEntire et al. 2010).

Many algal extracts and whole seaweeds are currently in the market (see above), however the road to acceptance of new, non-traditionally utilized species is long and there are many barriers to overcome (Gellenbeck 2011). In order for a new raw material to enter the marketplace, there are multiple viewpoints to consider: product formulators are concerned with efficacy, mechanisms of action and performance of the new material within a specific platform, quality assurance looks for consistency and reliability, regulators require assurance and documentation that a raw material is acceptable in global markets, and procurement requires adequacy of supply to meet market demands. New food materials must satisfy all of these stakeholders in order to enter the marketplace successfully.

In order for a new functional ingredient to achieve commercial success, there are many pieces of information that any food developer requires before launching a new product. Efficacy is fundamental to the success of functional ingredients. In order for a novel ingredient to attract the attention of a formulator, it must demonstrate efficacy within the format in which it will ultimately meet the consumer. Published efficacy data within peer reviewed journals are always the most compelling. From a formulators point of view, the degree of excitement generated by published studies increases as the source of data progresses from chemical activity, bioassay in vitro, gene expression, animal models, to human clinical trials. The elucidation of the underlying mechanism of action is very powerful, and today's competitive market environment demands this level of investigation with any new functional food or ingredient.

The source of raw materials has become very important to the food industry, driven by recent regulatory changes (Gellenbeck 2011). The contamination of raw materials with invading species is a problem that must be considered, especially if efficacies of functional compounds are important. Consistency can vary source to source, and the manufacturing chain of food products cannot survive an interruption due to a shortage of raw material. Environmental sustainability concerns are also front-of-mind for a growing number of consumers. If the seaweed product is cultivated, how does its cultivation affect the natural environment? If the seaweed product is wild collected, how does its removal affect the natural environment? Are there

"fair-trade" practices in place to compensate harvesters or producers in an ethical manner (Nagoya Protocol)? These consumer driven concerns must be addressed by any developer wishing to introduce a new food product.

10 Raw Material Sources

10.1 Gathered/Wild Harvested

Seaweeds have traditionally been gathered along coastlines, or "picked" from near-shore beds. Seasonality of raw material availability has always limited this practice. In the modern world, where the industrialization of natural resources is the norm, reliance upon the availability of wild seaweed populations is problematic on many levels, though raw material costs are typically lowest with these operations. Wild harvested seaweeds can only be commercialized if the seaweed of interest occurs as large individuals, found in dense stands where labor is readily available for collection or harvest. Storm-cast events are unpredictable, and cannot be relied upon to sustain a business. Likewise, storm cast material may comprise multiple species and general flotsam and jetsam, leading to microbial issues. Consistency of quality and efficacy can be variable in wild collected seaweeds, and food safety traceability is lowest with this type of raw material. Sustainability of the harvest must be in place as the resources are developed to avoid catastrophic consequences to the marine ecosystems concerned (large, densely populated seaweeds are typically the main structural/habitat component of ecosystems (Sharp et al. 2006)).

10.2 Cultivated (In-Sea)

The cultivation of seaweeds for food has been highly successful, such that over 93 % of all seaweed biomass that is utilized globally for human products comes from cultivated sources (FAO 2010). The vast majority of cultivated seaweeds are produced from in-sea operations, where seaweeds are tethered to or seeded onto long lines or nets (substrata for attachment) which are anchored in the sea in shallow waters for grow-out. In-sea operations rely on robust seaweeds that must be have some natural resistance to epiphyte attachment, since there is little in terms of management practices that can be implemented to eliminate this difficult and costly problem in seaweed cultivation. Plant or seeding density is relied upon to prevent epiphyte growth on lines and individuals during in-sea cultivation.

In-sea operations must be very large and multi-sited to mitigate crop loss risks. In-sea farms are anchored to the seafloor, and as such are subjected to high risk of crop and equipment losses during storms, and can also conflict with other stakeholders such as shipping and recreation. This latter issue is largely responsible for

the lack of Asian-style, in-sea operations in the near-shore waters of North America and Europe. Regulatory bodies make it exceedingly difficult to license these large farms in waters where recreational stakeholders are not accustomed to seeing large seaweed farms. Seasonal availability of raw materials is also a limitation with this type of cultivation since water temperature and nutrient availability can limit production, and external sources of nutrients (fertilizers) cannot be ethically applied in the sea. Siting seaweed production downstream from fed aquaculture (fin and shellfish) sites allows both the mitigation of dissolved nutrients from excreted sources, and potentially higher seaweed production, and these types of systems are under investigation (Chopin et al. 2001).

When high value food markets, such as those found in Japan, are targeted for the sale of seaweed products, quality is of upmost importance. Though prices can be high for suitable seaweeds, the quality standards are very strong in these premium markets. Raw materials must be clean, epiphyte and foreign material free, have consistent color in all thallus regions, and during all seasons, and must have the correct "mouth-feel" or texture. Therefore it is very difficult to satisfy all these value requirements from wild harvested seaweeds (Bulboa and Macchiavello 2006), and cultivation is necessary.

10.3 Cultivated (On-Land)

Both of the previously mentioned sources of raw materials rely upon dominant, or robust species, which lend themselves well to rope/net attachment or resource development. However, many interesting seaweeds are not these dominant or robust species, and therefore this has limited the number of species used commercially as food, at industrial scales. This is also a major impediment to the development of functional foods and nutraceuticals from seaweeds. When compared to the thousands of land plants utilized in the food and functional industries, the few commonly available seaweed genera currently on the open market do not give industrial formulators a great deal of variety to work with (Table 2).

On-land cultivation typically utilizes tanks or ponds, sited on-land for vegetative propagation of biomass. The biomass is unattached and suspended in the seawater medium with aeration or other means of agitation (e.g., paddlewheels). Since the seaweeds are unattached and housed in controlled on-land conditions, the industrialization of non-dominant species can be successful with this type of system. Species that are diminutive in size, and sparse in population cannot become food or functional ingredients at commercial scales since resource development would be costly (perhaps involving divers and hand-picking) and in-sea development risky (little hope of remaining attached and not overgrown with epiphytes). On-land systems enable the development of these non-dominant seaweeds, and these systems are suitable for all but the largest seaweeds.

On-land cultivation allows for the use of fertilizers when made with effluent control in mind (i.e., little flushing of excess nutrient). The seawater is pumped onshore so particulate and temperature control is possible.

Table 2 Commonly marketed and available seaweed genera (Hafting et al. 2012)

Resource based	*Codium*
	Fucus
	Ascophyllum
	Sargassum
	Gelidium
	Macrocystis
	Chondracanthus
	Solieria
	Lithothamnion
	Chondrus[a]
	Ulva
	Palmaria[a]
Cultivated	*Saccharina/Laminaria*
	Undaria
	Alaria
	Gracilaria
	Eucheuma/Kappaphycus
	Porphyra/Pyropia
	Caulerpa
	Eisenia
	Hizikia

[a]Cultivated at large scale but most material on open market is wild harvested

Seawater can also come from fed aquaculture for nutrient remediation. On-land tanks utilize unattached seaweeds, so they are essentially three dimensional and therefore make efficient use of available area so farms can be smaller than in-sea operations. They also allow for the production of multiple species at single sites, and are on-land so not as risky during storm events. These systems are extremely productive, and highly controlled. Along with these advantages comes their high cost for construction and operations. The cost has been the largest factor in limiting the development of on-land seaweed cultivation, and requires the development of high-value markets for products. So far there are four operations that produce seaweeds this way in large quantities (*Ulva*, (Bolton et al. 2009), *Caulerpa* (Horstmann 1983), *Chondrus* (Acadian Seaplants Limited, Nova Scotia, Canada), and *Palmaria* (Big Island Abalone Corporation, Kona, Hawaii).

11 Market Forces Drive Cultivation Development

The development of industrial-scale cultivation of various seaweed species has been driven by market forces, where traditional sources of raw materials were insufficient to meet consumer demand. Before the development of modern food sales and distribution networks, seaweeds were seasonally gathered and harvested for local consumption. There was little pull to improve the efficiency of raw material sourcing

because local markets were well supplied with wild seaweeds. Investigations into the life histories of seaweeds were the first step toward understanding their biology with the goal of cultivation and domestication.

The great success of the nori (*Porphyra*) industry in Asia can be primarily attributed to studies undertaken by British Phycologist Kathleen Drew (1949); she discovered that a seaweed previously known as *Conchocelis rosea* was actually the alternative life history phase of *Porphyra umbilicalis*. Before this discovery was made, the harvest of nori was unpredictable since it was unknown how the blade phase was established each season. By the early 1950s Drew's discovery was put into practical use in Japan, and artificial seeding techniques were developed. This net seeding technology revolutionized the nori industry and resulted in an enormous boost to the Japanese seaweed industry. This example shows how the combination of market demand and cultivation technology can result in the establishment and growth of an industry.

A similar story can be told with the cultivation of *Laminaria* (kombu in Japan, haidai in China). Once the life history of *Laminaria* was determined with experimental studies in the 1940s, the large scale cultivation of this seaweed was established in the 1950s (Tseng 1993). The success of this industry was assured by the huge market demand for this food product. As early as the eighth century, some wild harvested *Laminaria* was being exported to China from Japan! With the industrialization of its cultivation and development of sales and distribution, China now dominates global *Laminaria* production.

Undaria (wakame) cultivation also began in the 1950s and this seaweed is produced with similar techniques used in *Laminaria* cultivation (seeding of nets with spores from mature fronds; Tseng 1981). The market demand for this edible and popular seaweed was greater than its supply by the 1900s. Again it was market forces that resulted in the establishment and growth of an industry. Korea began *Undaria* cultivation in the 1970s, and now dominates global production. Most of the world's wakame supply is consumed in Japan, Korea, and China and is recently supplying a comparatively small, but growing food market in North America and Europe.

Market demands can result in the depletion of wild seaweed stocks, if regulatory agencies do not oversee their utilization in a sustainable manner. Seaweed resource depletion has occurred a number of times globally, and has led to the development of industrial-scale cultivation of red seaweeds for the extraction of carrageenan. The Chilean carrageenan industry still relies upon wild harvested seaweeds, with no legal harvest regulations in existence (Romo et al. 2001), and it is only very recently that cultivation techniques were developed there. As a consequence there have been multiple "boom and bust" cycles in the production of seaweed biomass (Buschmann et al. 2001) from Chile, with largely unmeasured consequences on the natural environment. It is recognized that the best way of mitigating these cycles is to develop techniques for seaweed cultivation in Chile (Romo et al. 2001).

Before the development of cultivation techniques in the Philippines and Indonesia, approximately 75 % of the world's carrageenan supply came from wild *Eucheuma*, *Kappaphycus* and *Hypnea* and the bulk of the remaining 25 % came

from wild *Chondrus crispus*. In the 1970s the world's demand for carrageenan outpaced the available supply of wild *Eucheuma* and *Kappaphycus* as their stocks began to succumb to over-harvesting. This drove the development of cultivation techniques. These techniques were not put into wide spread use until the cost of harvesting the remaining wild seaweeds became higher than their cultivation cost. Supply/demand cost dynamics favored the development of cultivation, and in the 1970s efforts were put into the development of on-land *Chondrus crispus* cultivation in Massachusetts at Wood's Hole Oceanographic Institution, and in Nova Scotia at the National Research Council – Institute for Marine Biosciences.

The efforts in the Philippines and Indonesia were spectacularly successful at establishing a new supply chain for the production of carrageenan. For a time, economics favoured the production of *Chondrus crispus* on-land for carrageenan extraction, and Acadian Seaplants Limited (ASL) was established in 1981 by Louis Deveau and became the first commercial enterprise to successfully cultivate a seaweed on-land. As *Eucheuma* and *Kappaphycus* cultivation techniques developed, and found widespread adoption (i.e., costs of production were reduced with new techniques and large scale production) in the Philippines and Indonesia in the 1980s, the carrageenan industry focused more and more on these areas and seaweeds for their raw material supply. On-land production of *Chondrus crispus*, with its inherently higher associated costs, found it more difficult to compete. In the late 1980s, ASL's former carageenan production site was completely re-purposed for the cultivation of *Chondrus crispus* for higher value markets. In the 1990s, ASL began production of an entirely new product from on-land cultivated *Chondrus crispus*. Hana Tsunomata™ (the trade name for ASL's cultivated *Chondrus crispus*) is a kaiso salad ingredient for the Japanese market, valued for its food safety, traceability, cleanliness, sustainability and brilliant color (all attributes that are possible with on-land cultivation). It has developed into one of the highest value seaweed food products in Japan, and has large market penetration in the kaiso salad platform. This is another example where market demands allowed for the development of an industry. The higher costs associated with on-land cultivation demanded that higher value products be developed.

12 Future Trends

In general, there is a trend in all marine resource sectors toward value addition. Higher profit margins are possible in markets with high quality standards. The higher standards are being driven by regulatory bodies demanding safety. Recently, better evidence of efficacy in supporting health claims of functional products has also been demanded. As long as the market demand is in place for higher value products that can deliver on safety and efficacy (and have the data to back up claims), food developers will pursue these markets. These high demands on raw materials will lead to the cultivation of biomass as developers seek high quality seaweeds which are sustainably sourced (a regulatory requirement, as well as a

consumer demand). This will be good news for the ecologically minded, since the "boom and bust" cycles of seaweed production will be avoided through expanded cultivation development.

There has been a very recent shift in research focus, away from techniques for the production of commodities (e.g., carrageenan), towards functional products with higher value. This research focus is supporting the development of high value markets for functional products. There are a number of nations focused on applied phycological research such as Korea, France and Ireland, but also a great diversity of bioassay studies on local seaweed flora (not just commercially available seaweeds) which have been undertaken worldwide in an effort to valorize seaweed resources. The number of research efforts within the field of applied phycology on the health benefits of seaweeds has accelerated in recent times and this will result in the development of novel foods from non-traditionally sourced seaweeds (i.e., the diminutive, non-dominant genera which currently cannot be sourced economically). History has shown that within the field of applied phycology, a combination of market demand and biological research has led to the development and implementation of successful seaweed cultivation. The trend will continue in the future, with the development of functional product markets. Market demand will be supplied from cultivated seaweeds, and the importance of on-land cultivation will grow. Since it allows for non-traditional seaweeds to be sourced as functional raw materials. The highest levels of traceability, safety, and consistent efficacy is achieved with on-land cultivation, therefore this technique will become more common for the highest value markets, including food and functional products. The partnering of developers with raw material suppliers will become more common as it is in similar industries (e.g., as with nutra/pharmaceutical developers and suppliers), and the development of a science-based functional seaweed industry will begin to flourish. The future of the advanced seaweed industry lies in the development of high value markets with cultivated, highly traceable, standardized and efficacious seaweeds.

The domestication and subsequent industrial scale cultivation of seaweeds (begun in the 1950s) is most certainly in its infancy when compared to terrestrial agriculture (thousands of years). As such, the seaweed industry currently relies on a very limited number of species and cultivars developed for rapid growth or environmental compatibility with local conditions. As the industry moves toward value-added production, away from commodities, there needs to be more intensive focus on the development of cultivars that not only resist disease, and grow rapidly but also contain elevated levels of bioactive compounds of interest. Over reliance on monoculture of vegetative clones opens the possibility of rapid spread of disease and the study of disease and epiphyte resistance is also an area requiring more focused effort (Loureiro et al. 2012). Genetic improvement can be achieved through traditional selection and breeding programs and this type of research will become important for the improvement of current cultivated production, and for the future (Robinson et al. 2012). Genetic modification of seaweeds, while possible, has the potential to erase the benign public opinion that currently surrounds the cultivation of seaweeds, and should be avoided. Market acceptance of GMO products from

marine organisms is a hill that does not currently need to be climbed at this early stage in the development of seaweed cultivation.

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Metallic Nanoparticle Synthesis by Cyanobacteria: Fundamentals and Applications

Ioan I. Ardelean

1 Introduction

Nanoparticles are materials with different shapes (spherical, triangular, rods etc.) and dimensions between 1 and 100 nm having specific properties which make them very interesting with respect to both fundamental and applicative research. The behaviour of matter at nanoscale is very different from what is familiar and commonly accepted at macroscopic level. Laws relating to physical, chemical, biological, electrical, magnetic and other properties at the nano-scale are different from those that apply at macro scale (Bhat 2003) metals become harder and ceramics become softer, a whole variety of composites and alloys become possible, chemical resistance is increased, interactions with light/other radiations are different (e.g. the absorption of solar radiation in photovoltaic cells is much higher in materials composed of nanoparticles than in thin films of continuous sheets of material, the smaller the particles, the greater the solar absorption) (Bhat 2003; Nalwa 2004, 2011). Furthermore, biologically produced nanoparticles have, sometimes, better applicative properties as compared to not only the bulk material, but also with abiotically produced nanoparticles. For example, the same amount of $Pd^{(0)}$ nanoparticle has a ninefold higher catalytic activity with respect to pollutant removal than commercial $Pd^{(0)}$ powder (De Windt et al. 2005). Nanoparticles studies and applications belong to the Nanotechnology field (Bhattacharya and Gupta 2005), defined as "the understanding and control of matter at dimensions between approximately 1 and 100

This paper is dedicated to the memory of my respected master Professor Gheorghe Zarnea (September 22, 1921–June 16, 2012), from the University of Bucharest and the Institute of Biology Bucharest (Romania). His distinction and intelligence impressed and guided his numerous students, including me, to whom his enthusiasm was lastingly transmitted.

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D. Sahoo, J. Seckbach (eds.), *The Algae World*, Cellular Origin, Life in Extreme Habitats and Astrobiology 26, DOI 10.1007/978-94-017-7321-8_16

nanometers," and technology that "involves imaging, measuring, modeling, and manipulating matter at this length scale." (National Nanotechnology Initiative 2009). The commercial value of nanotechnological industry, including the production of nanoparticles, is expected to increase significantly from $91 billion in 2009 to $1 trillion by 2015 and $3 trillion by 2020 (http://www.nanotechproject.org).

The synthesis of Metallic NanoParticles (MNPs) can be performed, essentially, by physical, chemical and biological methods, each method having advantages and disadvantages (see below). The synthesis of MNPs mediated by living matter, so called biogenic synthesis of MNP, is a relatively new scientific topic, reporting the use of bacteria, cyanobacteria and actinomycetes, fungi, lichens, algae and plants extracts in this process (Gericke and Pinches 2006; Prathna et al. 2010; Rai and Duran 2011). These syntheses, usually occur under normal conditions of temperature and pressure, with no toxic chemicals involved in the process, with the exception of metallic ions to be reduced. Thus, this type of protocol is friendly to the environment, with high potential in developing a green technology. However, so far the processes involved in the biogenic synthesis are poorly understood.

MNPs have great potential for applications in the electronic, chemical, mechanical and life sciences industries. In biology and medicine the main applications are in the following directions: fluorescent biological labels; drug and gene delivery; biodetection of pathogens, detection of proteins, probing of DNA structure, tissue engineering, tumor destruction via heating (hyperthermia), separation and purification of biological molecules and cells (Li et al. 2011; Salata 2004; Nalwa 2004, 2011; Brayner et al. 2013). In few years MNPs with improved catalytic activity have become important also for in situ destroying of organic pollutants (Karn et al. 2009; Hennebel et al. 2009, 2011; Mishra and Malik 2013).

Cyanobacteria are the most diversified, ecologically most successful and evolutionary most important group of prokaryotes, clearly defined by the ability to carry out oxygenic photosynthesis in the thylakoid membranes and respiration both in plasma membrane and thylakoid membrane, in some species the major respiratory activity occurring at plasma membrane whereas in other species the main site of aerobic respiration being thylakoid membrane (Peschek 1996). *Gleobacter violaceus* is the only cyanobacterium found so far which lacks thylakoides, respiration and photosynthesis occurring at the level of cell membrane. Oxygenic photosynthesis, the ability to use the light energy to synthesize glucides from carbon dioxide and water and to evolve oxygen from water molecules is essential for all the other forms of life on earth. Historically, cyanobacteria were the first organisms to perform oxygenic photosynthesis and this metabolic ability of ancient cyanobacteria have converted the early reducing atmosphere of earth (when no free molecular oxygen was available) into an oxidizing one. This process emerged approximately 3.5 billion years and had an essential effect on the evolution of life on our planet. There is a general agreement that the oxic atmosphere allowed the emergence and evolution of aerobic microorganisms, most probably by endosymbiotic association between different types of prokaryotic cells. The ancient cyanobacteria participated to this endosymbiosis thus all photosynthetic organisms on earth have some cyanobacteria as ancestors; together with cyanobacteria all these photosynthetic eukaryotes,

including higher plants, contribute today to the synthesis of organic matter and oxygen production, the basis of all life forms here. Cyanobacteria being very versatile microorganisms can live, freely or attached or in symbiosis with different types of organisms, in very different environments with respect to temperature, pH, salinity, light intensity etc., some of them being able to use atmospheric nitrogen for the synthesis of organic compounds (Bryant 1994; Seckbach 2007; Gault and Marler 2009; Peschek et al. 2011; Govindjee and Shevela 2011; Whitton 2012; Srivastava et al. 2013).

The diversity of cyanobacteria, whose systematics and nomenclature is still a matter of debate (Oren 2011) and their involvement in essential processes occurring in nature (primary production, oxygen evolution, nitrogen fixation etc.) allow them to be very important actors in biotechnology. Important reviews on biotechnological applications and potential of cyanobacteria have been published in the last years the most known topics being the photoproduction of molecular hydrogen or electricity, biomass (and related processes, including valuable products), removal of different pollutants (petroleum hydrocarbon, heavy metals, nitrogen and phosphorus etc.) CO_2 mitigation, biosensors (Becker 1994; Vonshak 1997; Borowitza and Boroworzka 1988; Ardelean and Zarnea 1998; Kuyucak and Volesky 1989; Richmond 2008; Gault and Marler 2009; Grewe and Pulz 2012; Aryal et al. 2013; chapter "Algal Biotechnology" by Borowitzka, this volume). The presentation of these biotechnological applications is very well widespread in academic literatures, some of the very good contributions in the field being freely accessible on internet/academic site/web pages.

2 Cyanobacteria and Nanotechnology

Cyanobacteria are important for nanotechnology mainly for the following topics: (i) model cells to test biocompatibility/cytotoxicity of nanoparticles, (ii) bioenergetics and (iii) model cells for metallic nanoparticle synthesis. Their use as test organisms for measuring the biocompatibility/cytotoxicity of NP takes the advantage of the occurrence of major metabolic processes at different cellular sites- photosynthesis only at the thylakoid level and respiration both at cell membrane and thylakoid membrane level. It is thus possible to measure the interplay between aerobic respiration or oxygenic photosynthesis and different types (chemical nature, size, shape etc.,) of nanoparticles. Some of the results in this field concerns the toxicity of cerium oxide for *Anabaena* CPB4337 (Rodea-Palomares et al. 2011), titanium dioxide nanoparticles (nTiO2) against the nitrogen-fixing cyanobacteria *Anabaena variabilis* (Cherchi et al. 2011), nanosized cadmium sulfide (CdS) against the cyanobacterium *Anabaena flos-aquae* (Brayner et al. 2011) or of CdSe quantum dots against *Synechocystis* PCC 6803 in light or in darkness (Ardelean et al. 2011; Sarchizian et al. 2011). The results show structural and functional disorders induced in cyanobacteria by the interactions with these nanoparticles which could be limiting factor for the synthesis of MNP by cyanobacteria (see Biological significance of MNPs synthesis). In the last years there are several reports on using cyanobacteria

as model systems to develop biomimetic nanodevices for energy conversion. Grimme et al. (2008) used abiotically synthesized platinum/gold nanoparticles which were coupled directly to isolated cyanobacterial photosystem 1 (PSI) particles; this new structure is able to evolve (for short time) molecular hydrogen. In another approach (Iwuchukwu et al. 2010) -directly related to the ability of cyanobacteria to produce MNP, the topic of this chapter- the reduction of platinum ion ("platinization reactions" according to their terminology) took place in a photobioreactor by incubation of cyt c_6, PSI (from *Thermosynechococcus. elongatus* or *Synechocystis* PCC 6803) and platinum ions. The self-organized platinization of the photosystem I nanoparticles allows electron transport from sodium ascorbate to photosystem I via cytochrome-c_6 and finally to the platinum catalyst, where hydrogen gas is formed. Their system produces hydrogen at temperatures up to 55 °C and is temporally stable for >85 days with no decrease in hydrogen yield when tested intermittently. The maximum yield is 5.5 mmol H_2/h/mg chlorophyll and is estimated to be 25-fold greater than current biomass-to-fuel strategies (Iwuchukwu et al. 2010). In another very interesting approach, the thermophilic cyanobacterium *Thermosynechococcus elongatus* was genetically modified in such a way that their photosystem II (PSII) particles can be immobilized in vitro on abiotically synthesized GNPs by orienting the electron acceptor side to the gold surface. The PSII immobilized on GNP (four to five PSII dimmers are bound to a single GNP) retained O_2 evolution activity comparable to that of free PSII. According to the authors, the PSII-GNP conjugate will be a useful nanodevice for the development of artificial systems for light-driven water splitting into O_2 and H_2 (Noji et al. 2011).

3 Cyanobacteria and Metals

The interaction between metals and (cyano)bacteria is very complex in nature, MNPs synthesis by microorganisms, including cyanobacteria, being only one face of these interactions. Other interactions between microorganisms and metals involves biomining – the use of (micro)organisms to extract metals from ores- and bioremediation- the use of (micro)organisms to remove metals from wastes. All these processes are part of the biogeochemical cycles of metals which involve complex biochemical reaction interacting with pure chemical reactions; these reactions induce changes in the physical and chemical properties of the resulting products (Gadd 2010; Mishra and Malik 2013). The recovery of gold, and other metals, from solutions has been actively studied for industrial purposes to process billions of tons of metal waste every year; however, as the conventional methods for removing metals from ore processing solutions are extremely expensive, alternative methods for metal recovery using living or dead microorganisms have been actively investigated (Lengke et al. 2006a).

For our topic it is important to know that the scientists firstly used microorganism for bioremediation and metal recovery and afterwards the interest for biological synthesis of MNP occurs (Lengke et al. 2006a; Gadd 2010; Mishra and Malik

2013). As pointed out by Lengke et al. 2006a, in the case of cyanobacteria, several laboratory experiments have been conducted to understand the interaction of Au ions with cyanobacteria; it was shown that cyanobacteria were able to accumulate Au from $AuCl^{-4}$ by a biosorption mechanism, but the morphology of Au particles resulting from this mechanism has not been investigated (Lengke et al. 2006a).

There are several books and reviews concerning the synthesis of MNPs by microorganisms some of them containing excellent results obtained also with cyanobacteria (Mandal et al. 2006; Gericke and Pinches 2006; Rai and Duran 2011; Prathna et al. 2010; Narayanan and Sakthivel 2010; Li et al. 2011; Lengke et al. 2011; Ghorbani et al. 2011; Tikariha et al. 2012; Liesje et al. 2012) but, up to my best knowledge, this is the first review devoted exclusively to the synthesis of MNPs by cyanobacteria.

The present chapter is focused on the use of cyanobacteria for the synthesis of MNPs which is an upcoming topic and has got lot of attention during last few years because of its basic and applied potential.

4 MNP Synthesis by Cyanobacteria

The first experiments on MNPs synthesis by cyanobacteria were done on *Plectonema boryanum* (Lengke et al. 2006a, b, c). One paper reports two types of experiments to examine the role of cyanobacterium *Plectonema boryanum* UTEX 485 on the accumulation of Au from aqueous solutions of $AuCl^{-}_{4}$ (Lengke et al. 2006b). In type 1, Au solutions (up to 5 mM) were added to the cultures and incubated at different temperature and times (25 °C, 60 °C and 100 °C for up to 1 month, and 200 °C for 1 day). In type 2, Au solutions (up to 5 mM) were added to dead, autoclaved cultures incubated at 25 °C; control experiments were conducted using $AuCl^{-}_{4}$ solutions (2 mM) and no cyanobacteria present (Lengke et al. 2006b). In the type 1 experiments, after addition of $AuCl^{-4}$ all cyanobacteria were killed within several minutes, with concomitant change in color of the culture vessel from (blue) green to purple. Significant increase in precipitation of Au occurred with increase in temperature from 25 to 60–200 °C. In the type 2 experiments, after addition of $AuCl^{-}_{4}$ to the dead, autoclaved cyanobacteria the rate of Au precipitation was faster by approximately three times than that in the type 1 experiments at similar temperature indicating that Au precipitation and formation of octahedral platelets were directly related to degradation of cyanobacteria, clearly showing that increased rates of GNP formation can be obtained in the absence of any metabolic activity (Lengke et al. 2006a). The microscopic images from the type 1 experiments show that at 25 °C, the addition of $AuCl^{-}_{4}$ to the cyanobacteria killed the cells instantly, but the filaments remained intact. Gold particles of irregular to octahedral habit were precipitated on the bacteria cells. Gold particles were also dispersed throughout the interior of the cells. At 60 °C, the cell structure was distorted and cells became rounded, and the filaments were separated into their constituent cells. At 100 °C, the filaments were distorted and encrusted by Au particles and at 200 °C, the cyanobacteria cells

exhibited rounded form, and Au particles were deposited on the individual cells (Lengke et al. 2006b). One can see that, with increasing temperatures, the morphological changes of cyanobacterial filaments become more pronounced; furthermore, in the range of unphysiological temperatures for this strain (above 60 °C), where temperature only can cause cells death, morphology of cells and filaments is far from normality. The authors also showed that the size of Au particles increased with increase in temperature from an approximate diameter of 1.5 μm at 25 °C to 10 μm at 100 °C with a nanometer scale thickness. In the abiotic, chemical control experiments, in the absence of cyanobacteria, the precipitation of Au was observed only at 100–200 °C the pure chemical reaction being promoted by heat. When it comes to the mechanism(s) of gold precipitation both outside and inside the cell, the authors (Lengke et al. 2006b) suggest that Au entered the cells as $AuCl^{-}_{4}$, and then gold ion (Au^{3+}) was reduced to Au° in an unknown manner not directly linked to bacteria metabolism as the cyanobacteria were probably killed by either the gold (III)-chloride or the acidic pH. The results demonstrates two important things: (i) interaction of cyanobacteria with the chemical environment is an important factor controlling the morphology of Au particles and (ii) the reduction of Au(111) is actually two-step, involving an intermediate Au(I)-S phase, with the sulfur being of organic origin (Lengke et al. 2006b).

Another study of this scientific group (Lengke et al. 2006c) was focused on extensive use of laboratory-based cyanobacterial experiments to go deeper in understanding the processes involved in the mechanisms of MNPs formation. The authors clearly stated that the elucidation of the functional groups derived from the cyanobacteria for gold binding is required to understand chemical mechanisms of gold accumulation by cyanobacteria. For the first time, the authors pay a special attention to the effect of interaction between cyanobacteria and the aqueous solutions of gold (III)-chloride on cell viability. Their results showed that at 0.8 mM initial gold chloride concentration the color of the cyanobacteria remained green, but dark purple color was observed in the solution after several hours of reaction. The purple color in the solution is characteristic of formation of colloidal gold. At 1.7 and 7.6 mM initial gold(III)-chloride concentrations change was observed in the color of the cyanobacteria and solutions, from green to reddish brown and yellow to colorless, respectively. Interestingly, the cyanobacteria were killed several minutes after the addition of gold (III)-chloride which argue for a sacrificial gold NP synthesis mechanism (Lengke et al. 2006c). In a larger perspective, the authors discussed their results with respect to geochemical implications for the formation of secondary gold deposits stressing on the fact that the gold concentrations in natural environments are 2–3 orders of magnitude lower than those used in their study, expecting that the reduction of gold (III)-chloride to metallic gold by cyanobacteria is presumably a slow process in nature. The authors put forward a very interesting hypothesis that at low gold concentrations (at ppb level), gold toxicity would not kill all viable bacterial cells, the remaining cells would be able to grow and probably adapt to the presence of gold. To the best of my best knowledge, there are no reports on quantitative relationship between cell viability (either single cells or filamentous cyanobacteria) and gold NP synthesis over a large range of gold chloride concentration.

When it comes to the mechanisms involved in gold NP synthesis, the authors stress on the fact that the formation of an intermediate species, gold (I)-sulfide, shows the importance of organic reduced sulfur species (e.g., cysteine, methionine) in gold binding mechanisms by cyanobacteria. Passive gold accumulation via organic sulfur present in the cyanobacterial outer membrane or periplasm may explain the mechanisms of gold bioaccumulation by cyanobacteria as well as by other living (micro) organisms such as algae, fungi and plants (Lengke et al. 2006b, c, 2007c). As Gram-negative bacterium, *Plectonema boryanum* is able to release vesicles, and this is the case when this cyanobacterium interact with gold ions at high concentration, the interactions being followed by the precipitation of elemental gold (Lengke et al. 2011).

The formation of palladium nanoparticles was investigated by reacting palladium (II) chloride with the filamentous cyanobacterium *Plectonema boryanum*, strain UTEX 485 (Lengke et al. 2007b). The growth of cyanobacteria and experimental design were as in (Lengke et al. 2006a), with the significant exception that palladium ion $PdCl_2$ was used instead of gold ion. After $PdCl_2$ addition all cyanobacteria were killed within several hours at all temperatures investigated (25–100 °C), probably due to the low pH (1.9) and high temperatures (Lengke et al. 2007b). The interaction of cyanobacteria with $PdCl_2$ solutions at 25, 60, and 100 °C resulted in distinctive morphologies for palladium metal nanoparticles which were precipitated outside the cell (equal or smaller than 30 nm). The authors showed that the reaction of aqueous $PdCl_2$ with cyanobacteria caused the precipitation of dispersed palladium metal (equal or smaller than 30 nm) on the bacterial cells, claiming that the release of organic materials from cyanobacteria during their death plays a role in this process.

The same cyanobacterium produce spherical silver nanoparticles and octahedral silver platelets by a mechanism which could involve metabolic processes from the utilization of nitrate at 25 °C and also organics released from the dead cyanobacteria at 25–100 °C (Lengke et al. 2007a). The papers from this group are pioneering work on MNP synthesis by cyanobacteria, having very important contributions to the correlation between the shape and structure of MNP and physical and chemical parameters during their synthesis.

Another important group in this field demonstrated that three filamentous cyanobacterial strains *Anabaena, Calothrix* and *Leptolyngbya* have the capability to reduce Au, Ag, Pd and Pt ions to elemental metal organized as nanoparticles (Brayner et al. 2007). They have reported about the attempts to use cyanobacteria as recyclable bioreactors, showing that the cells recover their full activity (measured as chlorophyll fluorescence and gold nanoparticle production) after a first metal salt addition and reduction (Brayner et al. 2007). These cyanobacteria were cultivated (6–30 days) before the addition of metal as salts at different ions concentration (10^{-3} 10^{-4} 10^{-6} M) in media without added nitrogen, in order to promote nitrogen fixation activity. These strains have different sensibilities against initial ions concentrations; for example, in *Calothrix* grown in culture medium at 10^{-6} M gold the filaments appear in bright field having their normal blue-green color, thus enabling the authors to claim that the cyanobacteria are alive. At higher Au concentrations (10^{-3} M), the

heterocysts color changed from yellow-green to purple-blue and vegetative cells were bleached, this concentration being very close to the lethal dose (Brayner et al. 2007). Contrary to filaments belonging to the genus *Calothrix*, filaments belonging to the genus *Anabaena* remain unbleached, both vegetative cells and heterocysts, indicating that, in this strain, the lethal dose was not reached at 10^{-3} M. The authors put forward the hypothesis that nitrogenase is involved in nanoparticle production, taking into account that most part of these nanoparticles was formed inside the heterocysts where nitrogenase is present. Interesting results were obtained with silver, palladium and platinum salts (Brayner et al. 2007). The authors also reported in vitro enzymatic, nitrogenase mediated, reduction of metallic salts to Au, Ag, Pd, and Pt metallic nanoparticles; the reduction was observed after addition of 10^{-3} M metallic salts under vigorous stirring at 25 °C during 20 min in the presence of nitrogenase and isolated exopolysaccharides from *Calothrix* (Brayner et al. 2007). However, there are no details about the way nitrogenase activity was sustained in their in vitro experimental conditions with respect to ATP need. This is the first report concerning screening of cyanobacteria for nanoparticles synthesis over a large metal ion initial concentrations, paying special attention to the structural and functional integrity of cyanobacteria during MNPs synthesis.

Another report (Govindraju et al. 2008) argue the use of the highly structured physical cells of *Spirulina platensis* for the biosynthesis at room temperature of pure metallic silver, gold as well as Au core/Ag shell nanoparticles. The reduction of aqueous gold and silver ions, in pure solutions or in mixtures, to the corresponding nanoparticles occurs at room temperature and is aided by the polypeptide/proteins of this cyanobacterium; furthermore it is believed that protein might have played an important role in the stabilization of Ag, Au and Au/Ag bimetallic nanoparticles (Govindraju et al. 2008). This is the first report concerning bimetal nanoparticule synthesis by cyanobacteria.

The use of cyanobacteria *Lyngbya majuscula*, *Spirulina subsalsa* both for biorecovery of gold (Au) out of aqueous solution and nanoparticles synthesis is reported (Chakraborty et al. 2009). Interestingly, Au (III) spiked with ^{198}Au was used for the experiment. Gold accumulation by cyanobacterial biomass was 1.93 mg g^{-1} for *L. majuscula* and 1.73 mg g^{-1} for *S. subsalsa*. It was shown that *L. majuscula* biomass exposed in $HAuCl_4$ solution produce <20-nm-sized gold particles located both inside as well as on the surface of the cell. As mechanism, the authors put forward that the first step involves the trapping of the metal ions on the surface of the cells possibly via electrostatic interaction between the ions and the negatively charged carboxylate groups present in the cell surface; this first step occurs quickly and is independent of metabolism. Thereafter, the ions are reduced by the enzymes leading to the formation of nuclei, which subsequently grow through the further reduction of metal ions and accumulation of these nuclei. Most probably the reduction of gold particles occurs due to the presence of cellular reductases (Chakraborty et al. 2009). Following the suggestion concerning that the mechanism of bioaccumulation of gold by sulfate reducing bacteria cultured in the presence of gold (I)-thiosulfate complex (Lengke and Southam 2006), these authors (Chakraborty et al. 2009) put forward that in cyanobacteria, (as in sulphate reducing bacteria) localized reducing

conditions may be produced by bacterial electron transport chain via energy generating reactions within the cells (for more details Chakraborty et al. 2009). This is the first report concerning the use of mixtures of isotopes for nanoparticule synthesis by cyanobacteria.

The extracellular synthesis of copper oxide nanoparticles by *Phormidium cyanobacterium* seems to occur by extracellular hydrolysis of the cationic copper by certain metal chelating anionic proteins/reductases secreted by this cyanobacterium under normal growth conditions (aerobic environment, neutral pH and room temperature); the proteins also play a significant role in stabilization of formed nanoparticles at room temperature (Rahman et al. 2009).

Focsan and co workers (2011) aimed to elucidate the interplay between biomineralization and metabolic activities in the case of the cyanobacterium *Synechocystis* sp. PCC 6803 exposed to gold ions. The authors demonstrated the ability of the cyanobacteria to reduce gold ions, the yield of GNPs synthesis being strongly dependent on the intensity of aerobic respiration and oxygenic photosynthesis. The biosynthesized GNPs (13 ± 2 nm) are localized at the cell wall, plasma membrane and inside the cytoplasm, inclusive at the level thylakoid membranes (Focsan et al. 2011). Interestingly, the authors, alternative to the direct demonstration by TEM of GNP accumulation at the level of thylakoid membranes, surveyed the accumulation of GNPs in cells in real time by making use of a fluorescent signal from *chl a* molecules at room temperature (Focsan et al. 2011). Indeed, they observed a gradual quenching of fluorescence at 681 nm as a function of GNP production inside cells when cyanobacteria are incubated with gold ions, relative to a reference sample of living cyanobacteria which served as control (cyanobacteria with no gold ions). This quenching of fluorescence can be the result of energy transfer from the excited fluorophore to vicinal GNPs in the cyanobacterial membrane (Focsan et al. 2011). If this quenching of chlorophyll *a* fluorescence is the mechanism by which the rate of oxygenic photosynthesis (measured as the production of molecular oxygen) decreases during the synthesis of gold nanoparticles is still an open question. To study the interplay between GNP biosynthesis and metabolic activity, the culture of cyanobacteria was divided into four sub-cultures, one for control and the other three were exposed to similar concentrations of gold ion solution and incubated for 50 h in three different external conditions, respectively: (i) in light at 20 °C, (ii) in dark at 20 °C and (iii) in dark at 4 °C. The results show an inhibition, after 16 h of incubation in the presence of gold ions, of aerobic respiratory (oxygen consumption in darkness) and oxygenic photosynthesis (oxygen production in light); the inhibition is stronger in cultures where the synthesis of MNP is higher (in light at 20 °C) and lower in cultures where the synthesis of MNP is slower (in dark at 4 °C). Interestingly, after the first 4 h of incubation there are no differences in the intensity of either aerobic respiration or oxygenic photosynthesis in all sub-cultures, indicating that MNPs synthesis occurred without any inhibition of respiration or photosynthesis. These results argue the involvement of aerobic respiration and oxygen photosynthesis in MNP synthesis by *Synechocystis* sp. PCC 6803, and the sacrificial nature of this synthesis. When it comes to the electronic mechanisms involved in GNP biosynthesis the authors (Focsan et al. 2011) claim that in their experiments the electron

needed for the reduction of gold ions could come from an intracellular electron donor during both photosynthesis and respiration, pointing out the major role of thylakoids in the synthesis of GNPs determined mainly by the photosynthetic electron transport and, to a lesser extent, by the respiratory electron transport occurring both at the cell membrane and at the thylakoid membrane. A significant factor in the synthesis of GNPs by cyanobacteria could be the $NADPH_2$ (nicotinamide adenine dinucleotide phosphate) or NADH-dependent reductases. The reduced cofactor would be oxidized during the reduction of gold ions and recycled via energy generating reactions within the photosynthetic electron transport, respiratory electron transport and redox reaction of the so-called intermediary metabolism, occurring in the cytoplasm (Focsan et al. 2011). The authors do not exclude that, additionally, the reduction of gold ions can also occur due to exported electrons from the molecular ambient by a membrane transporter system or/and by the presence of carboxyl groups and polysaccharides at the cyanobacterial cell membranes, and due to cysteine- or methionine- containing compounds. This is the first paper on cyanobacteria where surface-enhanced Raman scattering, SERS, use biogenic MNP as reporter structures to analysis their own cellular localization, and where the evolution of respiratory oxygen consumption and photosynthetic oxygen production are quantified during (gold) nanoparticule synthesis, thus arguing the involvement of these catabolic and anabolic processes in MNP synthesis by cyanobacteria, in physiological conditions.

Gold chloride reduction by healthy, exponentially growing filaments of the cyanobacterium *N. ellipsosporum* produce gold nanorod (137–209 nm in length and 33–69 nm in diameter) (Parial et al. 2011). The same group (Parial et al. 2012) further shows that three species of cyanobacteria *Phormidium valderianum*, *P. tenue* and *Microcoleus chthonoplastes* when exposed to hydrogen tetrachloroaurate solution produced gold nanoparticles inside the cell. Interestingly, the shape of NP is dependent on the incubation pH. *Phormidium valderianum* at pH 9 synthesized mostly spherical nanoparticles (around 13.78 nm) whereas at pH 7 mostly spherical particles (around 7.92 nm) were produced, and at pH 5 gold nanorods (411 nm × 32 nm) together with gold nanospheres 15 nm in diameter (more details on other shapes Parial et al. 2012). Moreover, in all the experimental genera cells were poisoned and died after converting Au (III)- to elemental gold; the reduction of gold is associated with cellular metabolism and presumably involves reducing enzymes or synthesis of other metabolites, the results arguing for a suicidal MNP synthesis (Parial et al. 2012).

S. platensis IPPAS B-256 is able to reduce gold ion from Au(III) to Au(0) by proteins and enzymes on the cell surface, and aggregation of the gold nanoparticles in the solution (Kalabegishvili et al. 2012). Interestingly, the size of GNP is dependent on the extracellular initial gold concentration; at 5×10^{-3} M most of the particles are spherical and their sizes are in the range of 15–40 nm, the majority being in the range of 20–30 nm, with different shapes, whereas at concentration of 10^{-2} M the size of formed particles is broader, from nanoscale range to the micrometer range (Kalabegishvili et al. 2012).

In the cyanobacterium *Anabaena flos-aquae* the processes of gold ion incorporation, its intracellular reduction, and Au^0 nanoparticle synthesis has been studied, as compared with similar processes occurring in eukaryotic photosynthetic microorganisms (Dahoumane et al. 2012). In *Anabaena flos-aquae* the synthesized GNP (20 °C, 16 h light/8 h dark) have a disperse location within the bacterial cell, and the dimension depends on the initial concentration of gold ion: at 10^{-3} M gold ions the mean diameter is 10.0 ± 4.7 and at 10^{-4} M gold ions the mean diameter is 8.1 ± 2.1. It is important to point out that in the absence of cells, no gold reduction was observed, even when extracted exopolysaccharides (EPS) were present (Dahoumane et al. 2012). One main concern of the authors is the impact of gold reduction on the physiological state of the cells. Before addition of gold salts, the photosynthetic activity of all the microorganisms remains stable during more than 2 months. The addition of gold ion and the interaction of cyanobacterial cells with gold ion at low (10^{-4} M) or high (10^{-3} M) concentrations causes the inhibition of photosynthesis as measured by monitoring chlorophyll a fluorescence in vivo. The authors claim that, in *Anabaena flos-aquae*, all the observed phenomena related to gold colloids occur when cells are damaged, assuming that the diffusion of gold ions and formed nanoparticles is easier and faster, due to the loss of membrane integrity. The experimental results show that the internal and external particle size is different, with larger particles outside the cells, probably involving extracellular and intracellular reduction combined with easy in/out transport through damaged membranes (Dahoumane et al. 2012). Based on their original results, the authors (Dahoumane et al. 2012), in connection with other papers, conclude that the Au(III) species are first in contact with EPS network, where the reduction take place. However, the authors agree that the reduction can also occur directly on the cell surface, as demonstrated by Lengke and Southam 2006, where an intermediate Au(I) species would be involved, which can be stabilized by some cellular products. These two mechanisms are in good agreement with the data showing gold particle formation even with dead cells (Dahoumane et al. 2012). For the particles observed within the cells, the authors (Dahoumane et al. 2012) suggest that, in the case of dead cells, these particles correspond to pre-formed Au(0) particles penetrating damaged membranes. However, for living cells, the specific localization of these particles in the thylakoids strongly suggests that the gold precursor, being either in the form of Au(III) or stabilized Au(I) reach these specific compartments. For the reduction occurring at the level of thylakoides, the authors claim that NAD(P)H can be involved in GNP formation, hydrogenase or nitrate reductase being, in their opinion, the enzyme fueling this co-factor (Dahoumane et al. 2012). They claim that it would be of high interest to perform in vitro experiments with isolated chloroplasts or artificial membranes, involving hydrogenase enzymes or mimicking systems, the immobilization of the biocatalyst within solid supports representing an important step towards the design of "living" bionanoreactor (Dahoumane et al. 2012). This is also the first paper which comparatively discusses about varied effects of different gold ion concentration on photosynthesis in prokaryote and eukaryote microorganisms during GNP formation.

In another report, *Spirulina platensis* strain PCC 9108 has been shown to convert silver ions to SNP when concentrated biomass from an exponential growth phase is put in contact with 1 mM aqueous AgNO3 solution for 24 h at 25 °C (Mahdieha et al. 2012). The authors claim that, the most probably, the formation of SNPs occurs due to the presence of cellular reductases released by *Spirulina platensis* into the solution. SNPs are in range of 5–35 nm, the majority of them being within three peaks, with a mean value of the size of 11.6 nm. There are no details concerning the structure and functions of cyanobacteria during and after SNP synthesis.

An interesting proposal concerns the in vitro synthesis of CdS nanoparticles by the use of phycoerythrin extracted from the marine cyanobacterium *Phormidium tenue* NTDM05 (MubarakAli et al. 2012). This protein was used for the biosynthesis of CdS nanoparticles by simply mixing the phycoerythrin extract with aqueous $CdCl_2$ and Na_2S in the concentration 0.25 mM and 1 mM, respectively. The reaction mixture was closely monitored for color change over a period of 5 days. The first step in this reaction sequence of nanoparticles synthesis was the formation of C-PE–Cd^{2+} complex, then this complex reacted with Na_2S to produce CdS nanoparticles, when the color reaction mixture changed to orange. The TEM results confirmed the spherical shape of nanoparticles at the size of about 5 nm. Essentially, it was found that the pigment is involved in the synthesis but also in the stabilization of CdS nanoparticles (MubarakAli et al. 2012). According to the authors the simplicity of the procedure presented in this study can be useful in commercial scale production of stable CdS nanoparticles.

The screening of cyanobacteria isolated from muthupet mangrove (A*phanothece sp, Oscillatoria sp, Microcoleus sp, Aphanocapsa sp, Phormidium sp, Lyngbya sp, Gleocapsa sp, Synechococcus sp, Spirulina sp*) showed that only *Microcoleus sp* has the ability to reduce silver ions to silver nanoparticles (Sudha et al. 2013). Silver nanoparticles were spherical shaped, with an average size of about 40–80 nm and well distributed without aggregation in solution. These nanoparticles were further characterized with respect to physical properties and antibacterial activity against test pathogenic bacteria (*Proteus vulgaris, Salmonella typhi, Vibrio cholera, Streptococcus sp., Bacillus subtilis, Staphylococcus aureus*, and *Escherichia coli*) as compared with classical antibiotics (cephotaxime, ampicillin, tetracycline, cephalexin etc.). The results showed that the synthesized silver nanoparticles are effective antimicrobial agent and proved as an alternative for the development of new antimicrobial agents to combat the problem of resistance to classical antibiotics (Sudha et al. 2013). These results argue the need to select cyanobacterial strains able to reduce metallic ions and to understand the mechanism(s) involved in this ability which could help scientists in understanding more deeply the interplay between metallic ions and cyanobacteria.

MubarakAli et al. (2013) reported the synthesis of gold nanoparticles using photosynthetic microorganisms such as *Phormidium* sp. The reaction mixture containing cyanobacterium and chloroauric acid ($HAuCl_4$) was incubated for an hour in dark room condition and it was found that nanotriangles (25 nm) were synthesized by a mechanism involving cellular proteins present in *Phormidium* (MubarakAli et al. 2013).

Rosken et al. (2014) show the ability of the cyanobacterium *Anabaena* sp. (SAG 12.82) to reduce Au^{3+} (at a starting concentration of 0.8 mM HAuCl4•H2O) to Au^0 with the subsequent formation of crystalline Au^0-nanoparticles. Formation of nanoparticles, recorded by X-ray powder diffraction and transmission electron microscopy over a range of several days, starts within the first minutes at the heterocyst polysaccharide layer. After 4 h, the dominating amount of nanoparticles is found in the vegetative cells. The bioproduced nanoparticles are found in both cell types, mainly located along the thylakoid membranes of the vegetative cells, and have a final average size of 9 nm within the examined timescale of a few days. Interestingly, the authors find that the heterocysts are not the favorite place for nanoparticle formation by their strain of *Anabaena* sp (SAG 12.82) the most favorable regions, where nanoparticles are recorded, being the thylakoid membranes, inside the vegetative cells (Rosken et al. 2014). The authors stress that another advantage of their experimental system for the production of gold nanoparticles is the absence of anatoxin *a* production by this strain.

5 Biological Significance of MNP Synthesis by Cyanobacteria

Taking into account the above presented results and different proposed mechanisms of NP synthesis, it seems that the reduction of metal ions to elemental metal organized as nanoparticles when catalyzed/performed by living cyanobacteria is followed by death of cyanobacteria, arguing that MNP bioproduction is detrimental to the cells. However, no experiments are made to check if-at population level- death of some cells during MNP synthesis is related to the chance of other cells to survive because the decrease in metal ion concentration. As suggested by Lengke et al. (2006c) the cyanobacteria reaching low concentration of gold ions could remain active thus having a role in ore formation. Recently, Johnston et al. (2013) has shown that *Delftia acidovorans*, a gold resident non-photosynthetic bacterium, produces a secondary metabolite, a non ribosomal synthesized peptide, that protects this bacterium from soluble gold through the generation of solid gold forms. This finding is the first demonstration that a secreted metabolite can protect against toxic gold and cause gold biomineralization (Johnston et al. 2013). Theoretically this mechanism could act in other bacteria as well, including cyanobacteria. More experiments are needed in order to better understand the biological signification of MNP synthesis by living cyanobacteria, using not only ion over a large range of concentration, but also different ratio between bacterial biomass and amount of ions, more diverse methods to measure the intensity of cyanobacterial metabolism and their structural and functional integrity, as well as genetically modified cyanobacteria.

6 Advantages and Disadvantages of MNP Production Using Cyanobacteria

The large application of nanoparticles is dependent on the ability to synthesize particles with different chemical composition, shape, size, and monodispersity (Ghorbani et al. 2011). Chemical methods are high energy- consuming and unfriendly to the environment as they use (toxic) chemical reducing agents; however, the rate of synthesis is rather high (Ghorbani et al. 2011). In contrast, biological synthesis use reactions which are carried out in normal conditions thus the energy cost are lower; this topic emerges as an eco-friendly and exciting approach which is still in its infancy (Ghorbani et al. 2011). Furthermore, the growth of cells, the biological catalysts, is not very expensive, and the cells are self regenerable. It was shown that, by controlling the temperature and the pH of the bioreactors, the size and the shape of the synthesized MNP can be changed (Rai and Duran 2011).

Besides the evident (potential) advantages of bioproduced nanoparticles, there are some challenges that need to be addressed such as: long production times as compared to chemical production (Hennebel et al. 2009; Ghorbani et al. 2011) optimization for accurate control of the particle size, proper extraction protocols and the search for strains harboring bioprecipitation potential for other unexplored metals (Hennebel et al. 2009). Furthermore the shape, size, and functionalization of the nanoparticles is defined by the biological system used to produce the nanoparticles, hence for every application a specific biological production process needs to be chosen (Sintubin et al. 2012). On the other hand, biogenic nanoparticles need to compete with chemically produced nanoparticles on the market. Hence, the true challenge for biogenic nanoparticles is finding the balance between scalability, price, and applicability (Sintubin et al. 2012). More fundamental research is essential to obtain MNP produced by cyanobacteria which can be really commercialized on a free market for applications.

7 Further Prospects

Here are some ideas for developing the ability of cyanobacteria to synthesize metallic nanoparticles:

1. Isolation of new cyanobacterial populations, including those living in so called extreme environments (high metal concentrations, elevated temperatures, high salinities and/or pH etc.,) and enrichment of these microorganisms in culture media supplemented with different metal ions (Au, Ag, Pt, Pd, etc.,) over a large ion concentrations.
2. Selection from enriched populations of strains with abilities to produce MNP and isolation in pure culture of representatives of the two ways, sacrificial and non –sacrificial, of MNP synthesis.

Table 1 Proposed structures and processes that could be involved in MNPs synthesis by (living) cyanobacteria

Source of electrons	Template	Process	Driving energy
Hydrogen sulphide	Thylakoids	Photosystem 1	Light
Water	Thylakoids	Photosystem 2	Light
Water	Thylakoids	Photosystem 2 + 1	Light
Molecular hydrogen	Thylakoids	Uptake hydrogenase	Chemical energy
Intermediary metabolites	Intracytoplasmic structures	Intermediary metabolism	Chemical energy
Organic molecules	Cell membrane	Aerobic respiration	Chemical energy
Organic or inorganic molecules	External membrane	(Bio)chemical reactions	Chemical energy
Organic or inorganic molecules	Capsule, glycocalix	(Bio)chemical reactions	Chemical energy

3. Optimization of the overall process occurring at laboratory level with respect to: (i) cyanobacterial strain selection and growing conditions before the addition of metal salts; (ii) the nature of metal ion and its initial concentration in the process, and (iii) incubation conditions (pH, temperature, light-dark regime etc.,) during MNP synthesis (in vegetative cells or in heterocysts) at different cellular sites (Table 1), implying different metabolic processes, single enzymatic reaction or pure chemical reaction, having also in mind the need to recover the MNP from the reaction mixtures.
4. Advanced physical and chemical characterization of the MNP produced in each optimized process, in order to understand the interplay between the structure and properties of MNP and biotic and abiotic conditions during their synthesis.
5. Finding of at least one specific utilization for (some of the) produced MNP in order to develop a supply chain production, for scaling up the process and checking its economic viability.
6. A deeper understanding of the processes involved in MNP synthesis by cyanobacteria – via either sacrificial or non sacrificial way- would help the scientists to genetically modify the selected cyanobacteria in order to: (i) change the structure of their cellular components (intraparietal and extraparietal structures etc.,) involved in biomineralisation, and (ii) facilitate the ions reduction at very specific sites, by specific enzymes involved in respiration, photosynthesis, hydrogen uptake, intermediary metabolism etc. (see Table 1 and Fig. 1).
7. The data reported by Focsan et al. (2011) concerning the use of biologically synthesized MNP as (nano)structures reporter inside the (living) cells, taking the advantages in studying living matter using surface-enhanced Raman scattering, represents a highly increasingly topic within the last years (for more details see Premasiri et al. 2005).

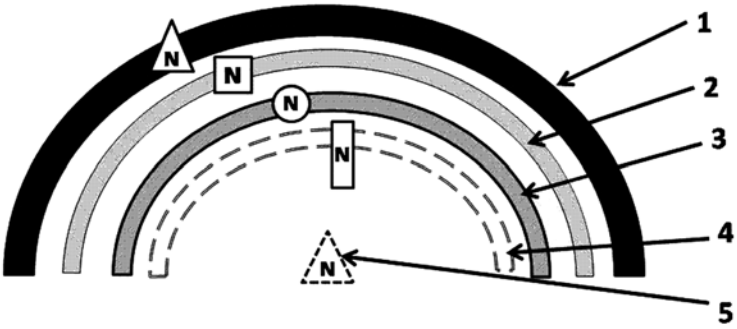

Fig. 1 Schematic representation of the possible cellular sites where MNPs could occur in cyanobacteria: *1* capsule/glycocalix, *2* external membrane, *3* cell membrane, *4* thylakoide, *5* cytoplasm. The figure suggests that, at different sites, the shape of produced nanoparticles (*N*) could be different

8. The MNP synthesis during detoxification and remediation of toxic metals could be important not only for the utilization of selected microorganisms to produce MNP in laboratory or at industrial scale, but also for the in situ degradation of other pollutants. So far, there are no reports concerning the participation of MNP produced in the environment by cyanobacteria to the catalytic degradation of organic pollutants.
9. Cooperation between scientists working on MNP production by cyanobacteria with those focused on other types of MNP producers such as heterotrophic prokaryotes (e.g. *Shewanella*, magnetotactic bacteria etc.,) and photosynthetic eukaryotes. So far, the best understood biomineralisation process in magnetotactic bacteria refers to the synthesis of magnetosome, which are bacterial organelle containing magnetic crystals (magnetite or/and greigite) (Bazylinski and Frankel 2004; Faivre and Schüler 2008; Moisescu et al. 2011, 2014; Lefèvre et al. 2013). Furthermore, on this topic there are some data concerning gold and silver trapping by uncultured magnetotactic cocci (Keim and Farina 2005), as well as on the controlled doping of magnetosome (Staniland et al. 2008).
10. Cooperation between scientists working on MNP production by cyanobacteria with those focused on other aspects of cyanobacterial biology, including other types of biomineralisation (calcium, silica etc.,) carried out by some cyanobacteria (more details see Thompson and Ferris 1990; Benning et al. 2002).

Acknowledgements This work was supported by the Romanian Academy (Grant RO1567-IBB05/2013)

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Blue Green Algae: A Potential Biofertilizer for Rice

Sunil Pabbi

1 Introduction

The demand for food grain is continuously increasing due to the increase in world population in general and India in particular. Since the cropping area is shrinking day by day with increased urbanization, the challenge is to get more yield per unit area to cope up with increased demand leading to intensive cropping. Agriculture involves organized production of crops in near natural systems, which thrives on the fertility and biodiversity existing in nature. Long term sustenance of agriculture is very much dependent upon the preservation of natural resources, without producing any deleterious effect on the delicate balance between biotic and abiotic characteristics of the ecosystem. But the modern day intensive crop cultivation requires the use of mineral fertilizers which are expensive and their excessive use has posed a serious threat to soil health, environment and sustainability of food grain production. Agriculture, however, is not exclusively dependent on chemical fertilizers for external N supplies because the crop can receive sizeable nutrient input from other alternative sources like farm yard manure (FYM), green manures and certain biofertilizers. In this context it is, therefore, worthwhile to use these alternative renewable sources to meet at least part of the nutrient demand for crop. Bio-fertilizer use has become an integral input in intensive agriculture and some of the biofertilizers like *Rhizobium*, *Azotobacter*, *Azospirillum*, P solubilizers and blue green algae are extensively used by the farmers. Unlike the chemical fertilizers, the effect of biofertilizers is not instantaneously visible and these inputs have sustained and cumulative effect on the crop plants and soil health. These are low cost inputs, renewable and pollution free. These have a crucial role in augmenting nutrient supply to crops by

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D. Sahoo, J. Seckbach (eds.), *The Algae World*, Cellular Origin, Life in Extreme Habitats and Astrobiology 26, DOI 10.1007/978-94-017-7321-8_17

increasing the nutrient availability through exploitation of natural processes like biological nitrogen fixation, solubilization of insoluble P and decomposition of organic wastes etc.

Biological nitrogen fixation is especially important in rice field where Blue Green Algae (BGA) or cyanobacteria are recognized as significant contributors to the overall nutrient balance. BGA find a highly favourable abode in the waterlogged conditions of rice fields. They provide cheap nitrogen to plants besides increasing crop yield by making soil vital, fertile and productive. BGA biofertilizer in rice popularly known as 'Algalization' helps in creating an environmentally safe agro- ecosystem that ensures economic viability in paddy cultivation while saving energy intensive inputs. The agricultural importance of these organisms lies in their capacity to metabolize the molecular nitrogen, liberation of part of fixed nitrogen and growth promoting substances as extra metabolites, solubilising the insoluble phosphates, addition of organic matter and improving the physical and chemical nature of soil. Most paddy soils have a natural population of cyanobacteria which thus provides free of cost, a potential source of combined nitrogen. Ever since the importance of these organisms was recognized, considerable amount of research was carried out to evolve methods and means to utilize these organisms effectively (Pabbi 2008).

2 Occurrence and Abundance of Blue Green Algae

Cyanobacteria are oxygen evolving and nitrogen fixing prokaryotes using sunlight as the sole energy source for the fixation of nitrogen (Stewart 1980). Nitrogen fixing blue green algae are present in abundance in rice fields (Fig. 1), their occurrence, however, varies depending upon soil conditions and other climatic factors. In India, on an average, BGA accounted for 33 % of the 2213 samples and some reports showed that up to 50 % of the total algae were BGA in some of the southern and eastern states (Venkataraman 1975; Kaushik 2005). These were most abundant in paddy soil than in other cultivated soils and the predominant N_2 fixing genera are *Anabaena*, *Nostoc*, *Aulosira*, *Calothrix*, *Tolypothrix*, *Aphanothece* and *Gloeotrichia*. Relatively lower occurrence of BGA has also been reported in Australian paddy fields which was attributed to the presence of high levels of copper sulphate and combined nitrogen in the irrigation water (Bunt 1961). Phosphorus nutrition is known to induce the native blue-green algae. Top dressing of (5 kg/ha) single super phosphate (SSP), 10–15 days after transplanting induces the native N_2 fixing algal flora. It not only stimulated the growth of native BGA but also encouraged the growth of inoculated BGA. Interaction of different soil samples from Bengal and Assam states of India with $Ca_3(PO_4)_2$ to find out the nutrients that stimulate algal growth in the paddy fields showed that the nitrogen fixation by cyanobacteria was due to addition of phosphorus either in soluble or insoluble form (De and Sulaiman 1950). They further concluded that addition of bone meal or superphosphate to rice soils would enrich the soil not only with phosphorus but also with nitrogen. Bisoyi and Singh (1988) showed that there exists a good correlation between the level of phosphorus applied and the biomass of cyanobacteria.

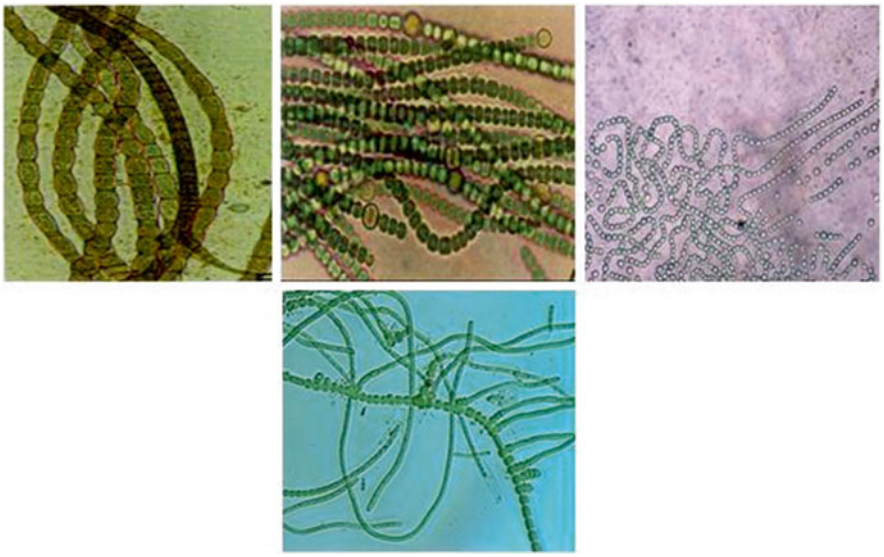

Fig. 1 Photomicrographs of hetrocystous nitrogen fixing blue green algae (*left* to *right*: *Aulosira*, *Anabaena*, *Nostoc* and *Westiellopsis*)

3 N Contribution by Blue Green Algae

Nitrogen fixation is found only in prokaryotic microorganisms. The annual turnover of nitrogen in biosphere varies from estimated 100 to 200 million metric tonnes of which two-third comes from biological sources. The projected global demand of chemically fixed nitrogen in the year 2000 was about 140 million whereas the globally chemically fixed nitrogen was only about 86 million tonnes. Cyanobacteria contribute a considerable build-up of biologically fixed N in rice soils (Roger and Kulasooriya 1980; Roger and Reynaud 1982). Studies have been carried out and it is established that BGA contribute to nitrogen economy of rice fields. A positive role of blue green algae in augmenting the nitrogen levels of rice fields was suggested by De in 1939. Experimental evidence using these organisms resulting in enhanced grain yield and straw yield were provided by De and Mandal (1956). A study carried out at IRRI, Manila (Watanabe et al. 1977) for 23 successive crops grown during 12 years without any added nitrogen fertilizer indicated that biological nitrogen fixation may account for this long term sustained fertility of the flooded rice fields. The removal of algae from paddy field water greatly reduced the *in situ* nitrogen fixing activity. Cyanobacterial contribution to the total nitrogen fixed in paddy fields varies widely and is mainly dependent on physico-chemical properties of soil and many other climatic and biotic factors. Alkalinity of the soil favored N fixation and growth of nitrogen fixing cyanobacteria (Roger and Kulasooriya 1980). The population of cyanobacteria and the extent of N-contribution varies among different soils. Experimental results at the IRRI revealed that the amount of N-accumulation ranged

from a few to 50 kg N per ha per crop among different soils (Roger and Ladha 1992). Nitrogen fixed by cyanobacteria may become available to rice plants only after its release extracellularly into the surroundings, either as extracellular products or by mineralization of their intracellular contents through microbial decomposition after death. In paddy fields the death of algal biomass is associated with alternative desiccation and wetting during cultivation cycle or finally after the harvest of the crop. Release of N from rapid decomposition of fresh or dry algal mass incorporated into the soil has been reported (Saha et al. 1982; Mian and Stewart 1985) and its release in the soil water system may become more useful for crop production during vegetative growth stage of rice plant than at the later stages (Ghosh and Saha 1993; Roger et al. 1993). The gradual build up of cyanobacterial population results in soil fertility with a residual effect of cyanobacterial nitrogen to a different N-pool of soil during the vegetative growth period of rice. In inoculated soil, recovery of cyanobacterial fixed N by rice varied from 13 to 50 % depending upon the nature of inoculum, method of application and the absence of soil fauna (Tirol et al. 1982). The plant available N of rice soils is increased considerably by the growth of N_2 fixing BGA (De and Mandal 1956; Singh 1961; Stewart et al. 1968). The presence of chemical fertilizer or similar sources of nitrogen also affect the extent of cyanobacterial fixed N. Field studies on the indigenous blue green algae in presence of urea super granules and urea (87 kg N/ha), ammonium sulphate (58 kg N/ha), SSP (30 kg/ha), potash (20 kg/ha) and Zn (10 kg/ha) showed that surface application of nitrogenous fertilizers inhibited nitrogen fixation whereas deep placement of urea increased 70 % nitrogen fixing activity over control. Besides this, growth of green algae was also encouraged by surface broadcast which increased the pH of flood water and loss of nitrogen by ammonia volatilization. Algal inoculation results in an addition of 30 kg N/ha and this, however depends upon agro-ecological condition which would regulate the activity and establishment of introduced algae (Venkataraman 1979). These organisms gave a considerable build up of nitrogen fertility in rice soil (Roger and Reynaud 1982). Ghosh and Saha (1997) reported that the inoculation of soil with soil based mixed culture of *Aulosira fertilissima*, *Nostoc muscorum*, *Nostoc commune* and *Anabaena* sp. significantly increased the release of inorganic nitrogen in the soil and the nitrogen content of soil was higher in exposed light incubated soil than unexposed soil due to N gain by blue green algae (Singh and Singh 1987). Application of chemical fertilizers at the recommended level or lower levels stimulated growth of diazotrophic cyanobacterial population and nitrogenase activity in a paddy field whereas higher fertilizer levels proved to be inhibitory (Jha et al. 2001).

As stated earlier, there is an average contribution of 20–30 kg N/ha/season which means that chemical nitrogen fertilizer could be saved to that extent. Since urea, which is the most common inorganic nitrogen fertilizer used in the crop, it contains 46 % nitrogen, a saving of 20–30 kg N/ha/season means that farmer can save roughly 40–60 kg urea with application of BGA. This is especially more relevant to small and marginal farmers who are unable to invest on chemical nitrogen fertilizer. Jha and Prasad (2006) advocated that the saving of 25 kg N per ha can be attained through cyanobacterial fertilization. Application of biofertilizers (BGA & *Azolla*)

along with chemical fertilizers brought about a significant enhancement in chlorophyll accumulation and nitrogenase activity during the mid-crop phase of rice crop. This positive effect on both these parameters further emphasized their contribution to the N-economy of rice fields (Nayak et al. 2004). The cyanobacterial nitrogen fixation has a switch 'on' mechanism which is activated when the level of combined nitrogen falls below a threshold level (~40 ppm). This enables the increased algal biomass to contribute more of the biologically fixed nitrogen when the level of fertilizer nitrogen in the ecosystem is reduced due to progressive utilization and loss. In addition, the cyanobacteria metabolize atmospheric carbon dioxide during photosynthesis. There have been innumerable reports that cyanobacteria give a considerable buildup of N- fertility in rice soils (Roger and Kulasooriya 1980; Saha and Mandal 1980; Roger and Reynaud 1982), but their inoculation has failed to increase rice yields consistently (Watanabe 1986; Roger et al. 1993). Cyanobacterial biofertilizer is much cheaper than the chemical fertilizer. Because of the slow release of the fixed and metabolized nitrogen, the crop plants are able to utilize more nutrients from the soil in presence of algal inoculation (Goyal 2002). The chemical fertilizers provide nutrients in a cyclic manner but the cyanobacteria do so in a gradual and linear manner. This reduces the loss and helps the crop to utilize more of the applied nutrients. It has been established by the ^{15}N tracer technique that about 90 % of the N accumulated by cyanobacteria is derived from the air (Inubushi and Watanabe 1986). The field studies have been equally supported by laboratory studies so that these potential groups of nitrogen fixing organisms may be better exploited for harnessing atmospheric nitrogen so as to increase crop productivity.

4 Blue Green Algae and Soil Fertility

The role of nitrogen fixing cyanobacteria has been shown to be most important in maintaining and improving the productivity of rice fields (Roger et al. 1993). Cyanobacteria are colonizing micro-organisms which remarkably adapt to a wide range of environmental conditions. These are the most abundant microbial constituents of micro-biotic crusts that exert a mechanical effect on soil particles as they form a gluing mesh and bind soil particles on the surface of their polysaccharide sheath material (Belnap 1993; MalamIssa et al. 1999, 2001). Cyanobacteria also excrete extracellular polymeric secretions (EPS) mainly composed of polysaccharides which ensure the role of binding agent of soil particles (Lynch and Bragg 1985). These improve the stability of soil surface and protect it from erosion. Cyanobacterial sheath and EPS also play a significant role in water storage due to the hygroscopic properties of polysaccharides and contribute to increased water retention capacity of the soil. The results have shown that cyanobacterial inoculation can increase the recovery of disturbed soils. Subsequent increase in water stable aggregates as a result of algal growth is important because soil aggregation and their arrangement influence infiltration rate, aeration and soil temperature, thereby improving the physical environment of the crop. Thus, cyanobacteria have been

used as inoculants to improve soil structure, increase soil fertility or recover damaged soil crusts (Rao and Burns 1990a, b; Roger and Burns 1994; Ghosh and Saha 1997; Acea et al. 2001; Hu et al. 2002, 2003; Pandey et al. 2005; Ahmad et al. 2011a, b).

Another important aspect where cyanobacteria can benefit rice plants is by producing Plant Growth Regulators (PGRs). The PGR-like effects of BGA culture were likened to those of Vit. B_{12}, gibberellins and amino acids. Production of vitamins and growth substances by cyanobacteria has been shown to have a positive effect on crop growth and yield (Venkataraman and Neelakantan 1967; Mishra and Kaushik 1989a, b). They also synthesize auxin like substances which stimulate the root growth of seedlings and play an important role in crop growth. They support early recovery of transplanted seedlings and prolong the period of tillering which results in an increased length of ears, number of ears and grains per year. In addition, they add substantial amount of organic matter to the soil and under field conditions, 5–32 % increase in soil organic carbon has been observed as a result of algal inoculations (Singh and Bisoyi 1989). The oxygen liberated during photosynthesis, reduces the oxidizable matter content of the soil, a phenomenon of great importance for areas where more than one crop of rice is taken in a year. Polysachharide sheath binds the soil particles together and increases the soil aggregate size which in turn reduces soil compaction. A 50–70 % increase in soil aggregation capacity due to algal inoculation has been observed. This is attributed to the action of polysaccharides released by the algae and presence of interwoven filamentous BGA growing on soil surface. Production of extracellular polysaccharide by cyanobacteria is known to help soil binding and aggregation. However, these extracellular polysaccharides when occur in the form of thick mat on the soil surface can have equally negative effect by blocking the penetration of water in soil. They also affect the availability of certain trace elements like Mn, Fe and Zn while growing in flooded rice soils by influencing the forms in which they occur in soil. The cyanobacterial sheath also plays an important role in protecting the organism from toxic effects of metal ions (Rai 2001). These inherent attributes have further diversified the application of these algae in harvesting nitrates from the soil, sequestering the 'Green House' effect through scavenging the carbon dioxide and turf treatment.

Phosphorus, another major nutrient required for crop plants which most of the Indian soils are deficient, when applied through phosphatic fertilizers in often fixed and become unavailable to the plants. In organic matter rich soils, the availability of P is greatly enhanced through microbial activity and cyanobacteria play an important role in solubilising phosphorous making it available to plants. *Westiellopsis prolifica* and *Anabaena variabilis* have been shown to solubilize insoluble phosphate sources like Mussorie Rock Phosphate (MRP) and Tricalcium Phosphate (TCP) which can be exploited for the efficient utilization of low cost, low grade rock phosphate fertilizers (Yandigeri and Pabbi 2005; Yandegeri et al. 2010, 2011). Further, BGA inoculation have been shown to increase the P availability in soil. In a field study, native BGA increased P availability by 49, 18 and 13 % during three successive seasons (Singh et al. 1981).

5 Blue Green Algae and Crop Production

Late Prof. G.S. Venkataraman (1964) introduced the term "Algalization" of rice field soils and recommended the use of heterocystous BGA as fertilizer supplement. Cyanobacterial application to rice fields has been found to result in increased grain yield (Venkataraman and Goyal 1968; Singh 1985, 1988; Kannaiyan 1985; Tripathi et al. 2001). The inoculation of soils with BGA or algalization studies carried in India and abroad have shown beneficial effects on grain yield and nitrogen savings (Venkataraman 1972; Singh 1978). Effect of algalization on grain yield under field conditions has been reported from a number of countries like China (Ley 1959), Egypt (El-Nawawy and Hamdi 1975), Japan (Watanabe 1965) and India (Venkataraman 1979; Jha et al. 1965; Singh 1988). The results revealed an average increase of 14 % in rice yield over control (Roger and Kulasooriya 1980). A number of workers have shown an enhancement in grain yield with the application of blue green algae in rice fields (Venkataraman and Goyal 1968; Singh 1985, 1988; Kannaiyan 1985). Inoculation of soil based mixture of four heterocystous species led to an insignificant increase in grain (8 %) and straw (11 %) yield which was however, accompanied by significant increase in total uptake of 15.3 kg N/ha (Ghosh and Saha 1997). Algalisation in presence of nitrogenous fertilizer has shown variable response. Biological nitrogen fixation is known to be suppressed in presence of combined inorganic nitrogen source thus resulting in no response of algalisation under such conditions. Watanabe (1973) pointed out that inoculation with algae was generally fruitless where nitrogen fertilizer was applied to the land. Similarly, Sankaran (1977) indicated that any supply of nitrogen by way of nitrogenous fertilizers (except urea as foliar spray) seems to inactivate the algae in nitrogen fixation. There are, however, numerous reports on the beneficial effect of algalization in the presence of fertilizer-N (Table 1).

Application of BGA along with inorganic fertilizers mostly, show a positive gain in yield over fertilizer application alone. Such gains are higher at lower fertilizer doses and marginal at optimum doses of even up to 100–120 kg N ha^{-1}. Under field conditions, supplementation of urea at 60 kg N/ha with algal inoculation resulted in a grain yield comparable to that obtained with 120 kg N/ha as urea (Singh et al. 1972). Similar observations were made when inoculation of BGA biofertilizer was done along with different chemical N levels (Table 2). A comparison of different times of fertilizer N application (basal or 10 days after transplanting, at tillering and at panicle initiation) showed that grain yield was better with algalisation, irrespective of the time or the method of fertilizer application (Srinivasan 1979). It was also shown that the combined application of BGA and N fertilizer is more effective in increasing the number of tillers and crop yield than the application of blue green algae alone in the rice field (Roychoudhury et al. 1983). Venkataraman (1972) reported an average increase in rice yield by 7.29 % when 100 kg N/ha was applied along with the algae as compared to control where 100 kg N/ha fertilizer was applied alone. The work on algalization trials and its response on grain yield has been reported by large number of other workers also (Mohapatra and Sharma 1988;

Table 1 Influence of Blue Green Algal (BGA) biofertilizers inoculation on rice yield in presence of different levels of chemical N fertilizer

S.No.	Treatment	Rice yield[a] (t/ha)	Increase in yield (kg/ha)	N-saving (kg/ha)
1.	50 N	4.42		
	25 N+BGA	4.46	40	25
2.	60 N	4.15		
	40 N+BGA	4.22	70	20
3.	75 N	5.07		
	50 N+BGA	4.93	−140	25
4.	80 N	4.83		
	50 N+BGA	5.04	210	30
5.	80 N	4.39		
	60 N+BGA	4.61	220	20
6.	100 N	5.36		
	60 N+BGA	5.38	20	40
7.	100 N	4.70		
	75 N+BGA	4.88	180	25

Source: All India Coordinated Project on Algae, 1981
[a]The difference in yield is due to demonstrations at different locations/States

Table 2 Grain yield as affected by various treatment combinations

Nitrogen levels	Grain yield (q ha^{-1})
N1 (30 Kg N ha^{-1})	49.37
N2 (60 Kg N ha^{-1})	53.46
N3 (90 Kg N ha^{-1})	57.20
N4 (120 Kg N ha^{-1})	67.29
SEm±	2.56
CD@ 5 %	6.27
Biofertilizer treatments	
T1 (Control)	53.45
T2 (BGA)	55.81
T3 (*Azolla*)	57.11
T4(BGA+*Azolla*)	60.95
SEm±	2.40
CD@ 5 %	4.96

Singh et al. 1988; Dar et al. 1989; Singh and Singh 1989; Ahmad et al. 2011a, b). A yield improvement of rice between 5 and 25 % was found when fields were inoculated with BGA even in the presence of 100–150 kg-N per ha as fertilizer (Sprent and Sprent 1990; Yanni 1992). Large number of field trial conducted by state departments of agriculture, All India Coordinated Project on Algae (AICPA), Department of Biotechnology, Govt. of India in different parts of the country assessing the effect

of algalization on rice yield suggest that, (a) in absence of chemical-N fertilizer BGA inoculation enhances the yield from a minimum of 4 % to a maximum of 32.8 %; (b) at recommended level of chemical – N, application of BGA increases yield by about 8.85 % and (c) at full recommended dose of chemical – N, the cyanobacterial inoculation gives a net saving of 25–30 kg N ha^{-1} with additional 10–15 % increase in rice yield. Of the 2774 on-farm trials conducted in 32 districts covering 9 agrochemical zones of Uttar Pradesh in different rice cultivars at recommended chemical fertilizer, the increase in yield with application of BGA biofertilizers varied from 1.10 to 5.33 $q.ha^{-1}$ and the cumulative percent increase in yield was 2.51–12.85 (Dwivedi et al. 2000). Field trials conducted at farmers' fields in National Capital Region of Delhi resulted in enhanced yield and increased income to farmers as a consequence of algalization (Table 3). Presence and application of other fertilizers/minerals *viz.* phosphorus, molybdenum, lime etc. has a beneficial effect resulting in rapid growth, establishment and increased metabolic activity of nitrogen fixing BGA and thus improving crop yield. Favourable conditions for biological nitrogen fixation by cyanobacteria is considered to be important factor for the relatively stable yield of rice under flooded conditions. Unlike chemical nitrogen fertilizers, it neither contaminates the environment nor consumes the photosynthates of rice plants (Liu 1979). The results have further demonstrated that inoculation of cyanobacteria improves the N-fertility of the soil, rice yield and N-uptake by the crop.

Algalization of rice crop is beneficial as it has both substituting as well as supplementing effect on the crop yield. Algalization has always shown a perceptible gain in yield in presence of inorganic fertilizer, however, as stated, the gains are higher at lower fertilizer doses and marginal at optimum and higher doses. The results established the advantage of using cyanobacterial inoculation in rice crop and its

Table 3 Use of blue green algal biofertilizer for higher yield of paddy and income to farmers

Name of villages (National Capital region of Delhi)	No. of trials	Treatments	Average yield (q/ha)	% increase in yield	Average gross Income (Rs./ha)	% increase in income
AsodaTodran	7	Control	19.25	–	24,563	–
		Use of BGA	23.00	19.48	29,250	19.08
AsodaShiwan	4	Control	13.66	–	17,575	–
		Use of BGA	15.82	15.81	20,275	15.36
Jakhoda	9	Control	17.93	–	22,913	–
		Use of BGA	20.13	12.26	25,663	12.00
Total	20	Control	17.54	–	22,425	–
		Use of BGA	20.27	15.56	25,838	15.20

Variety used: Local Basmati, Plot size: 8000 m^2 $(2 \times 4000)m^2$
Product price: Grain @ Rs. 1250.00/q Straw@ Rs. 500.00/q

adoption as a management practice. It leads not only to saving on expensive inorganic chemical fertilizer but also sustains productivity by maintaining a continuous supply of crop nutrients.

6 The Production Technology

No doubt that blue green algae are ideal biological system under tropical situation for better nitrogen fixation and conservation in rice soil. Rice cropping system provides favourable aquatic environment for the growth, multiplication and nitrogen fixation by utilizing the solar energy. It has also been demonstrated that use of these organisms as inoculants can be a powerful means of enriching the soil fertility and improving rice crop yields. Use of these cheaper and easily manageable inputs can make the agriculture increasingly ecofriendly and sustainable. In India, considerable progress has been made in the development of BGA based biofertilizers. Because these are self supporting, photoautotrophic microorganisms which can be produced in bulk with ease where water and carbon dioxide is available and temperature is suitable; an inexpensive rural oriented "Algal Biofertilizer Technology for Rice" was developed based on the natural ecology of these organisms mainly for the well being of small and marginal farmers in the country. The adoption of this technology by the farmers was largely due to the simultaneous development of prototypes for mass production of these algae at the farmers' level. Four methods, *viz.* trough/tank method, pit method, field method and nursery-cum-algae method, were developed (Venkataraman 1981), each of which could be adopted by farmers depending on their requirement. The basic method involves mass production of a mixture of cyanobacteria mainly constituting nitrogen fixing *Anabaena*, *Tolypothrix*, *Nostoc* and *Aulosira* species along with soil as a carrier, i.e. in a trough/tank or polythene lined pit and then drying the mixed culture in sun. For each meter square, 2 kg soil, 100 g single super phosphate (SSP) are added and about 5 mL of malathion per unit or equivalent insecticide (carbofuran etc.) is used to prevent breeding of mosquitoes and other insects. The contents are thoroughly mixed and allowed to settle down. BGA starter culture (about 100 g) is sprinkled on the surface. The algae multiply and under favourable conditions form a thick mat over the surface in 10–12 days. The contents are then allowed to dry and the dried flakes are collected, packed and stored for future use in rice field. An inoculum of 10–12 kg is considered sufficient to inoculate one hectare of paddy field 3–4 days after transplantation. The basic advantage of this technology is that farmers after getting the soil-based starter culture can produce the algal biofertilizers on their own and according to their requirement with bare minimum inputs (Venkataraman 1972). Despite all the advantages as mentioned and the simplicity of this easily manageable technology, it has not become popular with the farmers (Pabbi and Kaushik 1997). There are a number of limitations and constraint that confine wider adoption of this technology and problems are multifaceted. Due to open air nature of production it can be produced for only a limited period in a year in northern part of the country (3–4 months

in summer; production has to be stopped during rainy and winter season). This also led to high level of contamination as the production unit is exposed to outside and use of soil as a carrier also results in development of contaminants from soil including algal contaminants that may limit the multiplication of inoculated strains. There is slow production rate and population density is low thus need for heavy inoculum per hectare.

The production technology has been substantially improved with introduction of new and cheap carrier materials that support higher microbial load with longer shelf life thus considerably reducing the quantity of inoculum per unit area. The basic changes the technology has undergone include, (a) indoor production of algal biomass in almost pure state and under semi controlled conditions; (b) a suitable and cheap growth medium for faster growth of the organisms and (c) mixing with a suitable carrier material in desired quantities. The primary method involves the growth of algae in an indoor production unit that may be a polyhouse or glass house (Fig. 2a). The individual unit in the polyhouse can be of either RCC (Fig. 2b), brick

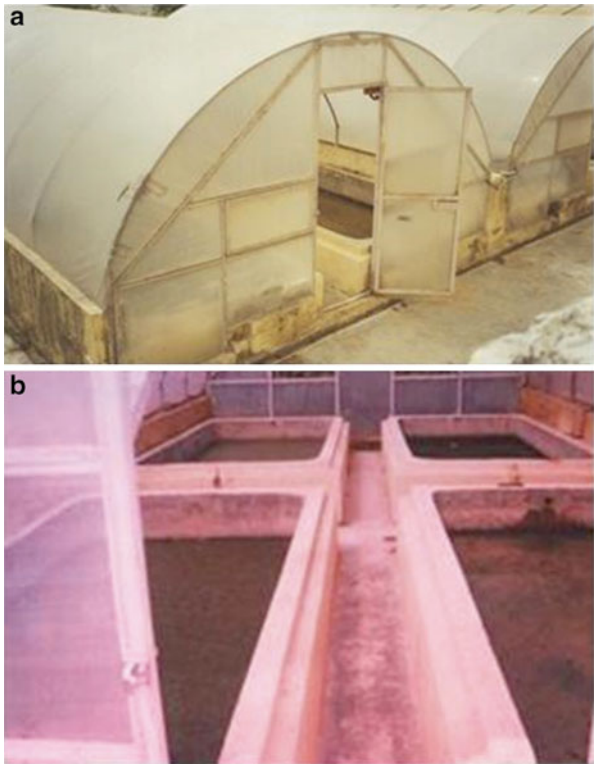

Fig. 2 (**a**) Polyhouse for BGA biofertilizer production and (**b**) Inside view of the polyhouse showing individual production units

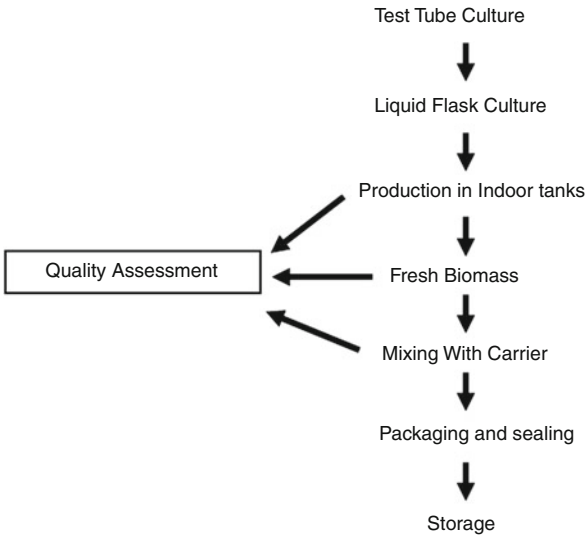

Fig. 3 Flowchart of quality BGA biofertilizer production

and mortar or polythene lined pits on ground and the dimensions may vary so as to utilize the maximum available space with operational management and convenience. The algae may be grown individually or a composite culture in a specially formulated medium inoculated with laboratory grown pure cultures. It has been observed that multiplying unialgal cultures in individual units and then mixing these after harvest along with the carrier ensures desired population of each one in the final product. Once fully grown, the cultures are harvested and mixed with the carrier material. Further processing depends on the kind of product but is normally subjected to sun drying. 'Multani mitti' (Fullers' earth) or clay has been used as a carrier material for the purpose. It has a good water holding capacity, microbially inert, good adhesion property, good buffering capacity and can support higher algal load thus fulfilling all the qualities of a good carrier material. The quality of the produce is maintained by regular checks at different stages of production (Fig. 3).

7 Recommendations for Application

- Broadcast BGA biofertilizer over standing water in the rice field 3–4 days after transplanting of the rice seedlings. Addition of excess algal material is not harmful and will rather accelerate the multiplication and establishment in the field.
- The field should be kept waterlogged for about 10 days after inoculation to allow good growth of BGA.
- Recommended pest control measures and other farm management practices do not normally interfere with the establishment and activity of the BGA in the field, however, one should keep a gap of 4–5 days between application of BGA biofertilizer and any other chemical input.

- If chemical nitrogen fertilizer are not used, apply BGA biofertilizer in order to gain the benefit of 20–30 kg N/ha.
- BGA biofertilizer can be effectively used with lower levels of chemical nitrogen fertilizers also.
- Apply BGA biofertilizer for at least three to four consecutive seasons to have the cumulative and sustained effect.
- Application of a part of phosphorus dose applied to rice after transplanting helps in rapid establishment of BGA.
- The sun dried material can be stored at normal room temperature for about 3 years in a dry state.
- Algal biofertilizer should not be stored in direct contact with fertilizers or other agricultural chemicals.

8 Conclusion

India has been the pioneer in the field of BGA biofertilizers and has generated valuable information on practical utilization of this important input in crop production. Number of field trials conducted with this material have shown promising results both in terms of nitrogen saving as well as crop yield. The results may not be consistent always as the local ecological conditions at every place differ and under all circumstances the inoculum has to work under natural conditions which cannot be modified. Thus, at places where the conditions are appropriate for rapid growth and multiplication of inoculated BGA, it gives desired results but at other places the outcome is not as expected. To overcome this problem to a greater extent, there is a need to develop algal biofertilizer inoculants suitable for different agro climatic regions. This can be possibly achieved by isolating the indigenous organisms from different areas, or create genetically stable strains which can grow quickly under varied soil conditions and efficiently fix atmospheric nitrogen. Such strains combined with capacity to withstand field doses of agrochemicals and ability to excrete N_2-fixed ammonia would be of greater importance for field application. Recent developments of new carrier materials, production under controlled conditions, modified pond designs and growth media to attain rapid cell growth has led to reduction in the inoculum volume, year round production, effective quality control, higher ‘titre value’ and longer shelf life of the product leading to sustained performance. Popularization coupled with success of demonstration trials has led to wider acceptance and adoption at the farmers level. The demand has increased but the gap between demand and production is still wide. Although, these new technologies are economically viable and can be exploited commercially considering the immense market potential, but there is a need to create viable, remunerative and enabling environment for participation of the private sector in order to realize the goal of maximizing BGA biofertilizer production to meet the demand and quality. Formulation of standards/regulations for quality control is very essential and this will go a long way in widening the scope and use of BGA biofertilizer. The effect

of blue-green algae in sustaining productivity and fertility of rice fields is well documented, but we need to develop other BGA based formulations that can be useful for crops like wheat, maize etc. to further boost their role in agriculture. This will go a long way in not only providing economic benefits but also improves and maintains fertility and sustainability in natural ecosystem.

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Polyunsaturated Fatty Acids from Algae

Girish Mishra

Fatty Acid Nomenclature and Abbreviations

AA	arachidic acid (20:0)
ALA	α-linolenic acid (18:3*n*-3)
ARA	arachidonic acid (20:4*n*-6)
DGLA	dihomo-γ-linolenic acid (20:3*n*-6)
DHA	docosahexaenoic acid (20:6*n*-3)
DPA	docosapentaenoic acid (22:5*n*-3)
EDA	eicosadienoic acid (20:2*n*-6)
EPA	eicosapentaenoic acid (20:5*n*-3)
ETA	eicosatetraenoic acid (20:4*n*-3)
FA(s)	fatty acid(s)
GLA	γ-linolenic acid (18:3*n*-6)
HAD	hexadecadienoic acid (16:2*n*-4)
HTA	hexadecatrienoic acid (16:3*n*-4)
LA	linoleic acid (18:2*n*-6)
VLC-PUFA(s)	very long chain polyunsaturated fatty acid(s)
OA	oleic acid (18:1*n*-9)
PA	Palmitic acid (16:0)
PUFA(s)	polyunsaturated fatty acid(s), >C18
SA	stearic acid (18:0)
SDA	stearidonic acid (18:4*n*-3)
AT	acyl transferase
TAG	triacylglycerol

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D. Sahoo, J. Seckbach (eds.), *The Algae World*, Cellular Origin, Life in Extreme Habitats and Astrobiology 26, DOI 10.1007/978-94-017-7321-8_18

1 Introduction

Algae are considered omnipresent as they have been isolated from varying ecosystems such as marine/freshwater environments, deserts, hot springs and snow. These play a vital role as primary producers at the base of the food chain (Van den Hoek et al. 1995). Algae are a very specialized and highly varied group of organisms which have found enormous utility in the modern world. The classification of algae is based on the presence of various photosynthetic pigments as have been discussed in other chapters. The chemical nature of the storage compounds and constituents of its cell wall also play a critical role in defining various algal groups. In accordance with systematic classification, the eukaryotic algal species are classified on the basis of pigments for example Chlorophyceae (green algae), Rhodophyceae (red algae), Pyrrophyceae (dinoflagellates), Phaeophyceae (brown algae) and Chrysophyceae (yellow-green algae).

It is well understood that adaption of algae to various environmental conditions is reflected in their capability to synthesize a number of unusual compounds and formation of unique lipid patterns (Thompson 1996). Moreover, progress in modern analytical techniques of chromatography combined with sensitive detection systems and mass spectrometry, has led to enhance identification and characterization of new fatty acids. The advent of new derivatization processes for unusual fatty acids have helped in analyzing the details of fatty acids from algae. VLC-PUFAs are long-chain hydrocarbon (18 or more carbon atoms) having three or more double bonds between adjacent carbon atoms and a terminal carboxylate group. These fatty acids are named in accordance with position of the first double bond, as counted from their methyl terminus. An *n*-3 PUFA carries the first double bond between 3rd-4th carbons from the methyl terminus. Similarly other PUFA groups are ω-6 and ω-9, which carries the first double bond at sixth carbon and at ninth carbon respectively when counted from the methyl terminus. Chemical structure of several important ω-3 PUFAs are shown in Fig. 1.

Fish oil is currently the key raw material source for commercial extraction of VLC-PUFAs but, due to an increasing demand for purified PUFAs and over fishing activities in natural habitats fresh catch for fisheries have depleted considerably. Furthermore, the chemical composition of fish oil depends on fish species, climate, and the quality of fish feed consumed. In addition, there is a threat of possible contamination by lipid-soluble environmental pollutants. Fish oils extracted from farm raised fishes are found to be low in healthy EPA and DHA. This is because fishes like, all animals have weak expression of enzymes necessary for production of these fatty acids but rather largely depend on their feed to accumulate VLC-PUFAs. Moreover, in order to purify PUFAs from complex low-grade crude fish oils, relatively difficult and expensive techniques such as fractional or molecular distillation, enzymatic splitting, adsorption chromatography, low temperature crystallization and supercritical fluid extraction may be required (Wen and Chen 2003).

Due to these issues alternative resources for efficient production of EPA and DHA are being researched upon. Currently, the production of polyunsaturated fatty acids (PUFA) by marine and fresh water microalgae is the subject of intensive

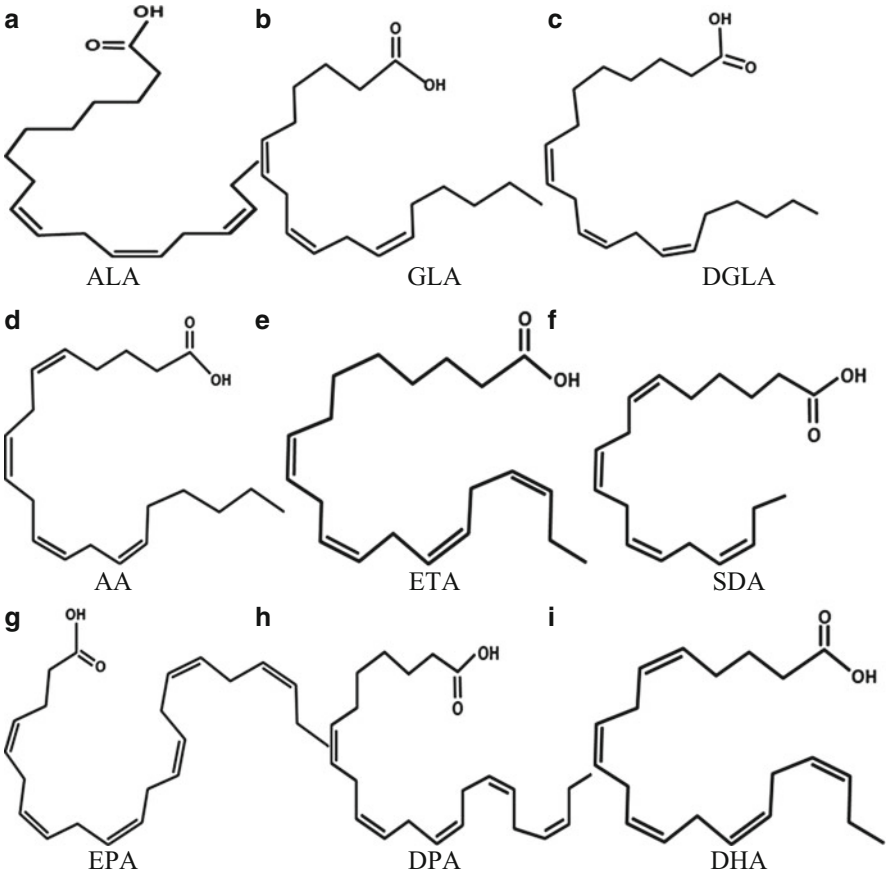

Fig. 1 Polyunsaturated fatty acids (**a**) ALA: Alpha-linolenic acid ($18:3^{\Delta 9,12,15}$), (**b**) GLA: Gama-linolenic acid ($18:3^{\Delta 6,9,12}$), (**c**) DGLA: Dihomo-gama-linolenic acid ($20:3^{\Delta 8,11,14}$), (**d**) AA: Arachidonic acid ($20:4^{\Delta 5,8,11,14}$), (**e**) ETA: Eicosatetraenoic acid ($20:4^{\Delta 8,11,14,17}$), (**f**) SDA: Stearidonic acid ($20:4^{\Delta 6,9,12,15}$), (**g**) EPA: Eicosapentaenoic acid ($20:5^{\Delta 5,8,11,14,17}$), (**h**) DPA: Docosapentaenoic acid ($22:5^{\Delta 7,10,13,16,19}$) and (**i**) DHA: Docosahexaenoic acid ($22:6^{\Delta 4,7,10,13,16,19}$)

research and development at commercial scale (Sijtsma and de Swaaf 2004). Some species of freshwater and marine algae contain large amounts of high-quality PUFAs and are currently used to produce PUFAs for aquaculture operations. These algal species, especially diatoms, can grow heterotrophically on organic substrates, without light, under well-controlled cultivation conditions. Researchers are trying various strategies considered important in order to increase the use of algae for commercial production of PUFAs in the near future. These areas are selection and screening of oleaginous species and further improvement of strains by genetic manipulation for enhanced oil production capacities. Research is being carried out for optimization of culture conditions for rapid growth of these microalgae and to develop newer methods for efficient harvest of healthy VLC-PUFAs.

2 Advantages of Very Long Chain PUFAs (VLC-PUFAs)

Vegetable oils such as soybean, mustard and sunflower are rich sources of polyunsaturated fatty acids (n-6) linolenic acid (LA) but largely lack (ω-3) α-linolenic acid or ALA. These are not only healthy but also essential fatty acids as humans lack the desaturase enzymes required to convert oleic acid (18:1) to LA (18:2) or ALA (18:3). The health benefits of ω-3 FAs, especially n-3 LC-PUFAs, have been proven beyond doubt (Ward and Singh 2005; Kremmyda et al. 2011; Sinn et al. 2012). In humans, as in all animals LA and ALA are further modified using desaturases and elongases to form ARA and EPA (VLC-PUFAs) respectively. These further acts as precursors for the formation of eicosanoid family of metabolites such as prostaglandins, leukotrienes and thromboxanes and thus provide important health benefits. Eicosanoids derived from EPA have been implicated in anti-angiogenic roles, whereas the ones derived through ARA have reverse roles. As ARA (20:4*n*-6) and EPA (20:5*n*-3) synthesis utilize various common enzymes such as desaturases, cyclooxygenases, elongases, and cytochrome P450 the higher concentration of *n*-6 and *n*-3 FAs may lead to large amounts of eicosanoid produced in the cell (Riediger et al. 2009). DHA has gathered great attention in recent past due to various human health benefits associated with it. These FAs are incorporated in cell of various body parts and act as anti-inflammatory agents. DHA is key FA component of brain and retina tissues with more than 50 % of cell membranes are composed of this fatty acid (Giusto et al. 2000).

Humans do have the fatty acid desaturases and elongases responsible for conversion of ALA (18:3*n*-3) to EPA (20:5*n*-3) and DHA (22:6*n*-3) from, but it is not sufficient to provide the amounts required for maintenance of good neural and cardiac tissue development (Plourde and Cunnane 2007; Khozin-Goldberg et al. 2011). Various positive effects including anti-viral, anti-fungal and anti-bacterial properties (Das 1999) have been extensively studied. DHA has also been implicated in reducing risk factors involved in various diseases like cardiovascular diseases (Kang and Leaf 1996) and has shown positive effects on diseases such as thrombosis, hypertension and arteriosclerosis (Horrocks and Yeo 1999). These benefits might be associated with alternations in membrane fluidity due to functional composition of phospholipids and production of eicosanoid (Riediger et al. 2009). Young infants are incapable of synthesizing enough DHA to provide for the rapidly developing brain, thus it is advocated to supplement EPA and DHA in the diet either through breast-feeding (Huisman et al. 1996) or through fortifying infant formulas. Fortification of all infant formulas with EPA and DHA had been recommended (FAO/WHO Expert Committee 1994) and in recent years many EPA/DHA fortified commercial infant formula are seen in the market.

An increase in the dietary intake of healthy fatty acid containing oils have been recommended and may differ largely due to traditional dietary habits (Hibbeln et al. 2006). Along with the absolute intake of PUFAs, the ratio of *n*-3/*n*-6 FAs is an essential factor in gaining proper health benefits. Though the optimal requirement of PUFAs is not established well and will also depend on other food and exercise

habits, a 3 % of the absolute FA intake has been recommended (Gill and Valivety 1997). At present, most consumed PUFAs are of n-6 type and originate from plant oils. A normal western diet contains a ratio of 1/15 *n*-3/*n*-6 PUFAs, whereas studies indicate a diet close to 1:4 or more may limit the damage due many chronic illnesses such as asthma, cancer and cardiovascular disease etc. (Simopoulos 2002). In current scenario, it is highly recommended to reduce the intake of n-6 FAs and eat nutritious supplements containing n-3 PUFAs to counteract this fatty acid imbalance and promote human health.

3 Current VLC-PUFA Sources and the Demand for Alternatives

An extensive study for the content of *n*-6 and *n*-3 PUFAs in various fatty acid sources such as chicken, red meat and oil from seeds reveal that the content of omega-6 fatty acid is far more than omega 3 fatty acids (>4:1) whereas it is reversed in fatty fishes like salmon, mackerel, tuna etc. Marine organisms contain a high percentage of *n*-3 PUFAs while containing less than 15 % *n*-6 FAs (Mühlroth et al. 2013). Fatty acid analysis on various marine microalgal species indicate a very high n-3 fatty acid content as compared to n-6 PUFAs.

As commented earlier, the current major source of LC-PUFAs such as EPA/DHA for human consumption is marine fatty fishes mainly through the fish oils extracted from these fishes. Globally the capacity of wild fish capture industry has remained nearly stable during last two decades, though aquaculture farming has increased substantially. The production of fish oil from wild fishes, which is a major component for aquaculture feed, has decreased by 1/3rd in last quarter of century (FAO fisheries and aquaculture department 2012). To reduce the input cost in aquaculture the fish farming industry has started using plant seed meals and vegetable oils to maintain growth in the fish production instead of fish meal and oil from wild fish sources (Olsen 2011; Nasopoulou and Zabetakis 2012). In other words an *n*-3 fatty acid (EPA/DHA) rich diet for aquaculture is currently being replaced by *n*-6 fatty acid containing oils. It has been reported that fatty fishes such as salmon can grow using 100 % blended vegetable oils in aquaculture feeds, without any negative effect on growth and health (Torstensen et al. 2005). However, such practices cause a severe decline in levels of healthy fatty acids like EPA/DHA in the flesh of fish (Tocher 2010). Meanwhile, there is a high demand for EPA/DHA for fortification of milk, meats, yoghurt etc owing to its health benefits to humans (Tur et al. 2012). This has caused a pressure on wild catch fisheries to overproduce in last few years. As wild catch fisheries are on a decline and cannot increase productions to meet the demand of VLC-PUFAs, there is an extensive search for efficient alternative sources for the future production of EPA/DHA rich oil for both marine aquaculture and human consumptions. Research is in progress to develop genetically modified oil crops to produce EPA and DHA in large quantities but this work is in preliminary

stages and will possess underlying issues related to genetically modified crops. Various microalgae, herbivore copepods and krill are rich in EPA/DHA (Khozin-Goldberg et al. 2011) and are being exploited to produce these n-3 FAs at industrial level.

4 Why Microalgae?

There is a considerable interest in production of VLC-PUFAs due to its action as positive effector in human health. Until recent past, seafood was considered the sole source of *n*-3 LC-PUFAs; however, as marine fishing industry has reached maximal production capacity, alternative sustainable sources for *n*-3 LC-PUFAs must be developed to cater the need of growing human population. Fishes, as in most animals, do not possess an efficient enzymatic mechanism for synthesis of *n*-3 LC-PUFAs and accumulate these in their bodies through consumption of marine algae, the primary producers of the healthy FAs (Kelly et al. 1959). As expected marine microalgae are being extensively studied as potential sources for large scale production of EPA/DHA (Yaguchi et al. 1997; Wan et al. 2013).

VLC-PUFAs are primarily produced by algal species belonging to the Chromista kingdom, a diverse group of microorganisms which consists divisions like cryptomonads, haptophytes and heterokonts (Cavalier-Smith 2010). It is hypothesized that the chloroplasts of chromista have developed from an endosymbiotic event between a red algae and a eukaryote during course of evolution (Cavalier-Smith 1999). Surprisingly, during evolutionary development photosynthetic ability of the heterotrophic thraustochytrid is lost though they do possess a non functional chloroplast and maintain their ability for synthesis and storage of VLC-PUFAs. Closely related algae from Chromista such as photosynthetic microalgae and thraustochytrids are implicated to be a major source of EPA/DHA in future. Additionally, algae have various other advantages to human population such as carbon dioxide sequestration, while growing on marginal land and saline water thus are not competing for agriland or fresh water sources (Hu et al. 2008).

Diatoms (Bacillariophyceae), widely distributed in both freshwater and marine habitats, contain 15–30 % of total fatty acid as EPA with no DHA. Some representative examples are freshwater diatom *Navicula pelliculosa*, or marine diatoms *Nitzschia frustulum, Navicula incerta* and *Biddulphia sinensis* (Renaud et al. 1994; Tan and Johns 1996; Yongmanitchai and Ward 1991b). It has been observed that *Nitzschia laevis* is capable of producing large amounts of EPA even under heterotrophic cultivation (Wen and Chen 2000). Diatom *Phaeodactylum tricornutum*, the only species in genus Phaeodactylum, has been demonstrated to be an excellent algal source of EPA (Yongmanitchai and Ward 1991a) while *Skeletonema costatum* is found to contain up to 30 % EPA of total fatty acids (Blanchemain and Grizeau 1999). Another class of microalgae dinoflagellates (Dinophyceae) consist of high content of DHA ranging from 12–51 % of total fatty acids and are potential source

for DHA production. Some representatives are *Crypthecodinium cohnii* (non-photosynthetic) (Jiang and Gao 2004), *Amphidinium carteri, Gymnodinium simplex* (Yongmanitchai and Ward 1989) and *Gyrodinium cohnii* (Singh and Ward 1997). By using strains of *Crypthecodinium cohnii* from the ATCC and from their own collections, Martek Biosciences has reported oil in range of 150–300 mg/g algal biomass of which DHA made up to 20–35 % (Kyle 1992; Kyle et al. 1991*)*. Thraustochytrids, which were originally thought to be primitive fungi, have recently been assigned to the subclass Thraustochytridae (Cavalier-Smith et al. 1994). They are common marine microheterotrophs, taxonomically aligned with heterokont algae (e.g. brown algae and diatoms) (Lewis et al. 1999). Examples are *Thraustochytrium* and *Schizochytrium* species, which have been shown to produce over 0.5 g/l of DHA in relatively short fermentation times (Ward 1995). Their DHA contents range from 25 to 60 % of total fatty acids. Production of PUFAs by these heterotrophs is predominantly in the form of triglycerides or oils (Kendrick and Ratledge 1992). The strain *Schizochytrium* sp. SR21, gave dry cell biomass of 48 g/l of with 13 g/l of DHA (Yaguchi et al. 1997). Table 1 compares EPA and DHA contents of various microalgae with fish oil.

The levels of *n*-3 LC-PUFAs synthesis from these microalgae has been shown to depend on the growth conditions. It has been reported that exposing the algae to stresses during growth such as nitrogen deprivation, increased salt concentrations of media, intensity of light or media compositional changes in the levels of carbon can enhance lipid accumulation as well as the content of EPA/DHA (Yokochi et al. 1998). For example, subjecting *Nannochloropsis oceanica* to Nitrogen limited conditions combined with high light intensity conditions of increased the EPA content to 2.6 % per dry weight as compared to 1.6 % when grown under mild culture conditions (Hu and Gao 2003; Pal et al. 2011; Sharma et al. 2012). However, inability of this algae to give large biomass densities makes it economically unviable option.

The two most applied strategies to increase the triacylglycerol production from algae can be either through increasing TAG synthesis per unit of biomass or to enhance the density of the selected algal strain. Optimization of algal culture parameters to enhance EPA/DHA synthesis is an arduous task, as TAG/oil accumulates in algal cells under abiotic stress, which is detrimental for the biomass yield. In addition to optimizing the growth/culture conditions various other approaches like metabolic engineering and strain improvements through selection/breeding have been envisaged to increased TAG biosynthesis from various algal species. These approaches have several problems; while modulation of genes in algae needs thorough studies to provide reliable transformation protocols for introduction of plasmids, selection processes require an extensive breeding program (Chepurnov et al. 2008; Radakovits et al. 2010). Recently a combinatorial approach using these two methods have been shown to be successful in land plants where metabolic engineering and classical mutation strategies were used to bypass bottlenecks for formation of VLC-PUFAs (Hoffmann et al. 2008; Ruiz-Lopez et al. 2014). Successful strategies based on identification of genetic factors that influence both quality and quantity of lipids must be adapted.

Table 1 Fatty acids content (in mg/g oil), sterol content (in mg/g oil) of the total lipid extract from several microalgal species and of fish oil

	Isochrysis T-iso	*Nannochloropsis gaditana*	*Nannochloropsis oculata*	*Pavlova lutheri*	*Phaeodactylum tricornutum*	*Porphyridium cruentum*	*Rhodomonas salina*	*Tetraselmis suecica*	*Thalassiosira pseudonoma*	*Fish oil*
ALA (C18:3*n*-3)	29 ± 4	0.3 ± 0.03	0.7 ± 0.1	10.0 ± 0.3	0.8 ± 0.1	1.42 ± 0.01	92 ± 5	68 ± 4	1.9 ± 0.1	7.7 ± 0.2
SDA (C18:4*n*-3)	43 ± 10	0.3 ± 0.1	–	17.0 ± 0.5	1.9 ± 0.1	–	43 ± 2	17.0 ± 0.5	20.4 ± 0.8	29 ± 1
EPA (C20:5*n*-3)	2.8 ± 0.7	175 ± 12	193 ± 24	92 ± 2	111 ± 5	35.6 ± 0.3	18 ± 1	16.3 ± 0.5	81 ± 2	184 ± 5
DPA (22:5*n*-3)	–	–	–	–	1.08 ± 0.01	–	–	–	1.82 ± 0.01	16.8 ± 0.3
DHA (22:6*n*-3)	46 ± 14	–	–	40.9 ± 0.9	8.3 ± 0.5	–	11.1 ± 0.8	0.8 ± 0.1	20.9 ± 0.8	105.2 ± 0.7
Saturated fatty acids	0.10 ± 0.01	0.12 ± 0.01	0.09 ± 0.01	0.101 ± 0.004	0.08 ± 0.01	0.205 ± 0.001	0.059 ± 0.004	0.11 ± 0.01	0.127 ± 0.003	0.26 ± 0.01
n-6/*n*-3 Ratio	0.273 ± 0.001	0.231 ± 0.001	0.208 ± 0.001	0.085 ± 0.003	0.063 ± 0.004	2.0 ± 0.1	0.0531 ± 0.0003	0.33 ± 0.01	0[a]	0.071 ± 0.001
Cholesterol	–	13.8 ± 0.6	20 ± 1	–	–	–	–	–	–	4.4 ± 0.1
Phytosterols	14.9 ± 0.1	17 ± 1	6.1 ± 0.3	97 ± 3	16.5 ± 0.6	26.5 ± 0.2	26 ± 3	10.9 ± 0.2	34 ± 3	–

[a]*Thalassiosira* contains no *n*-6 C_{18}-C_{20} fatty acids (Adapted from Ryckebosch et al. 2014)

5 De Novo Fatty Acid Synthesis

In algae as well as in all plants chloroplast serves as the starting point of fatty acid synthesis. Acetyl-CoA, a three carbon compound derived from breakdown of carbohydrates during glycolysis, is the initial compound required for the FA synthesis. Synthesis of malonyl-CoA is done by condensation of two Acetyl-CoA moieties through acetyl-CoA carboxylase (ACCase). Formation of malonyl-CoA is the rate limiting and committed step for FA synthesis. *De novo* synthesis of fatty acids requires the concerted action of two multiprotein complexes or multifunctional proteins. These are acetyl-CoA (coenzyme A) carboxylase and fatty acid synthase. Acetyl-CoA carboxylase (ACCase) is a type I biotin-containing carboxylase. In eukaryotes, the ACCase is composed of heteromeric subunits known as α-carboxyltransferase domain (α-CT), the β-carboxyltransferase (β-CT) and a central acetyl Co-A carboxylase. Currently there is no evidence for the presences of such subunit in Chromista, instead nuclear encoded homomeric ACCases are present that contain all the three domains. Two homomeric ACCases are present namely ACC1 (plastidic) and ACC2 (cytosolic) though only one homomeric plastidic ACCase has been reported from *I. Galbana*, *P. tricornutum* and *T. pseudonana*, (Huerlimann and Heimann 2013). It is reported that the initial malonyl-CoA conversion is carried out by the plastidic ACC1 while the malonyl-CoA pool for FA elongation is generated through the cytosolic ACC2 (Nikolaü et al. 2003). ACCase is a highly regulated enzyme and an increase in ACC expression did not increase the FA content in plants. FA feeding assays suggested inhibition of plant ACC1. In a similar experiment carried out in diatoms *N. saprophila* and *Cyclotella cryptic* enhanced expression of ACC1 did not result in higher accumulation of FA suggesting the presence of a tight regulatory mechanism in algal system also (Dunahay et al. 1996; Radakovits et al. 2011).

Type II fatty acid synthase (FAS) with distinct, monofunctional enzymes is present in *P. tricornutum* (Ryall et al. 2003). During *de novo* FA synthesis, malonyl-CoA is linked to ACP protein via a reaction catalysed by malonyl-CoA:ACP transacylase or *FAB D*. The ACP-bound acyl chain is the precursor for further elongation of FA chain in a cyclic manner, with two carbons being added in each cycle, till the chain is exported out of chloroplast (Guschina and Harwood 2013). Though no thioesterase (TE) has been characterized in algae, it is predicted that a yet unidentified thioesterase is involved in cleaving and releasing the prolonged acyl-ACP chain for further modification.

6 Fatty Acid Desaturases

In the presence of oxygen, fatty acid desaturases forms a double bond between two adjacent carbons on a Fatty acid chain. Desaturases can be divided into two classes based on the protein structure; membrane associated and soluble desaturases. Further, we can classify membrane associated desaturase into primarily two types (a) ω6/ω3 desaturase which catalyse the formation of a new double bond between a

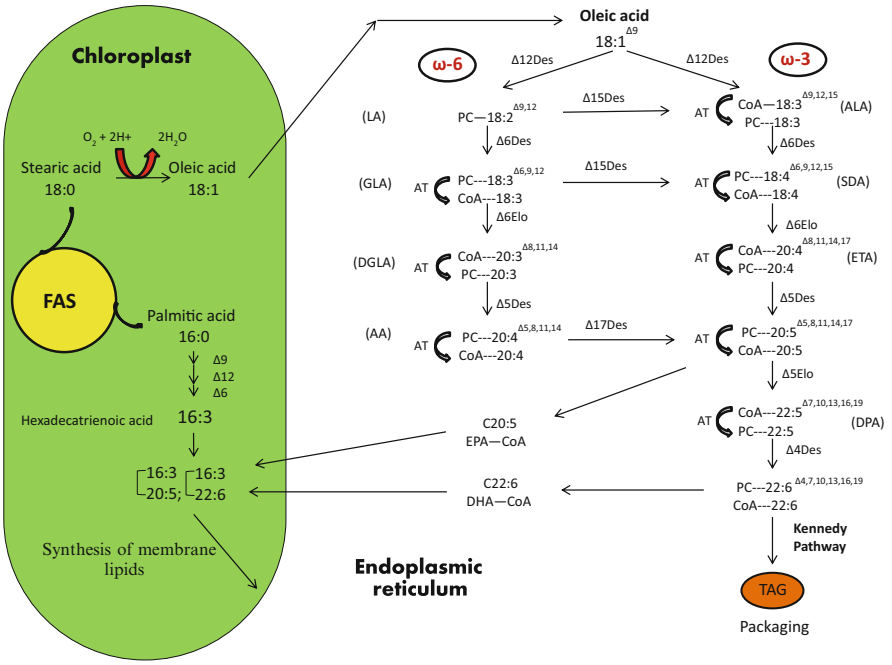

Fig. 2 Schematics diagram of FA and VLC-PUFA synthesis. Shown is *de novo* fatty acid synthesis (FAS) and the formation of EPA (20:5*n*-3) at the ER. Shuffling of fatty acids from the glycerol-backbone to Acyl-coA moiety is indicated with two consecutive arrows (Adapted from Domergue et al. 2003; Guschina and Harwood 2006)

pre-existing double bond and the methyl end of FA hydrocarbon chain and (b) the front-end desaturases, containing a cytochrome b5-domain at *N*-terminal, are capable of catalysing a new double bond between the carboxyl end of FA before an existing double bond. PtFAD6 from *P. tricornutum* is one of many front end desaturases to have been isolated, identified and characterized from algae which acts for synthesis of GLA (γ-Linolenic acid) from HDA (Hexadecadienoic acid). Soluble desaturases are involved in the conversion of 18:0 fatty acid to 18:1*n*9. Figure 2 shows the synthesis of FA in algae, especially the putative EPA (20:5*n*-3), DHA (22:6*n*-3) and HTA (16:3*n*-4). Genes encoding key enzymes involved in the fatty acid biosynthesis have been identified in the model organism *C. reinhardtii* (Shannon et al. 2003), *O. tauri* (Domergue et al. 2005), *T. pseudonana* (Tonon et al. 2005), *P. tricornutum* (Domergue et al. 2002) and several others as listed in Table 2.

7 Conclusions

Polyunsaturated fatty acids are crucial nutrients that act as progenitors of various bioactive metabolites performing diverse physiological functions. Many of the chronic diseases that afflict humans are due to an imbalanced intake of PUFAs. Evidence of the possible medical effects of PUFAs together with the growing

Table 2 List of various fatty acid desaturases isolated from algae and characterized to be involved in EPA/DHA synthesis

Species	Enzymes
Pavlova salina	Δ8, Δ5, Δ4 desaturase
Isochrysis galbana	Δ4 desaturase, Δ9 elongase
Spirulina platensis	Δ6 desaturase
Thalassiosira pseudonana	Δ6, Δ5, Δ4 desaturase and Δ6elongase, Δ5 elongase
Phaeodactylum tricornutum	Δ5, Δ6, Δ12 desaturase
Chlamydomonas reinhardtii	Δ6 desaturase
Ostreococcus tauri	Δ acyl CoA desaturase, Δ6 elongase, Δ5 elongase
Chlorella vulgaris	Δ12 desaturase

acceptance of nutraceuticals by consumers has brought these compounds to the attention of food and pharmaceutical companies. PUFA-based therapies have reached the marketplace and a range of PUFA-fortified foods are now widely available. These applications have led to an increase in demand for purified PUFAs, and the inadequacy of current plant, mammal and fish sources has led to the extensive search for alternatives. Microalgae offer an unlimited and natural resource of PUFAs. However, a greater understanding of the factors that affect PUFA production is required in order to develop a cost-effective process for the commercial production of high-quality PUFAs. As the quest for novel sources of LC-PUFA especially EPA/DHA is increasing, a set of comparative studies will enhance the fundamental understanding of lipid metabolism for algal system. Our understanding of lipid biochemistry and regulation of the FA synthesis has increased rapidly, and will continue to do so in the coming years and will provide new insights into fascinating world of algal lipid production.

Synthesis of LC-PUFAs in Algae has been studied through biochemical analysis such as GC-MS analysis of fatty acids, measurements of activity for various desaturation or elongation reaction through pulse-chase radiolabeling and inhibitor based studies (Guschina and Harwood 2006). Various bottlenecks in metabolism of *n*-3 LC-PUFAs have been identified due to co-expressional network studies using genes encoding for proteins involved in fatty acid/lipid synthesis. Critical analysis of *n*-3 LC-PUFAs synthesis suggests that various cofactors and precursors such as NADPH and Acetyl-CoA pool may play vital roles in these processes. These can possibly be modulated to further enhance the biosynthesis of VLC-PUFA content via various approaches like selective breeding, environmental conditioning and/or genetic engineering. Invariably, genetic factors should be identified and characterized in VLC-PUFA producing algae. This impedes the application of metabolic engineering in algal species. However, due to recent advances in whole genome sequencing of various economically important algal species, we envisage a better understanding of genetic and transcriptional lipid metabolism and provide us a chance for molecular characterization of the identified genetic factors (Fabris et al. 2012). Genetic transformation has been successfully carried out in nearly 40 strains of algae through

controlled expression is via use of endogenous promoters, species-specific codon usage and intron sequences. Current interest for engineering lipid synthesis is for the commercially important genus *Nannochloropsis*, which accumulate higher than 55 % FA per cell dry weight under nitrogen deprived growth with >5 % in the form of EPA (Bondioli et al. 2012). The well-established homologous recombination technique for the haploid genome can help lipid researchers to introduce genes for DHA production in this organism. Furthermore, higher accumulation of lipids and VLC-PUFAs has been shown in genetically bred *Nannochloropsis* and *Pavlova* respectively. With our understanding of various genetic factors and the biochemical pathway for VLC-PUFA synthesis, selective breeding can be improved through selection of triggers that create genetic bottlenecks and further selecting for cells with capability of enhanced lipid production.

Certainly modulation of enzymes involved in desaturation, elongation of FA hydrocarbon chain and acyltransferase action may improve VLC-PUFA content in algal/plant seed oil (Kilian and Vick 2012, 2013). However, the physiology of algal growth may act as an inhibitory rate limiting step for such approach and it remains an enigma. On the basis of current research knowledge in this field, a combined approach using metabolic engineering, culture conditioning and selection will be more appropriate to both increased biomass as well as high VLC-PUFA content in the algal oil.

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Algae as a Source of Biofuel

Savindra Kumar, Dinabandhu Sahoo, and Ira A. Levine

1 Introduction

Food security, fresh water supply, high energy demand and climate change are some of the key global challenges for the twenty first century. Development of any nation is directly dependent upon the kind of available energy resources. Till now, over 1.5 trillion barrels of fuel oil equivalent have been produced since Edwin Drake bored the world's first oil well in 1859. Interestingly, the world will need the same amount of oil to encounter the increasing demand in the next two decades alone (Staff 2007). According to BP Energy Outlook 2035, global energy consumption will rise by 41 % by 2035, with 95 % of that growth coming from rapidly-growing emerging economies (BP 2014). This growth included an increase in the consumption of all major fossil fuels including oil, natural gas, and coal (Jones and Mayfield 2012). The increasing world energy demand for the future is one of the biggest challenges facing governments, policy makers and scientists due to the irreversible depletion of fossil fuel reserves. Current global energy utilization is unsustainable from economic, environmental and societal viewpoint. In addition fossil fuels distributed unevenly in the world and accumulate greenhouse gases in the environment (Sahoo et al. 2012). Therefore, alternative resources have to be found and new technologies developed to meet the above challenges for the ever increasing human population, which is

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D. Sahoo, J. Seckbach (eds.), *The Algae World*, Cellular Origin, Life in Extreme Habitats and Astrobiology 26, DOI 10.1007/978-94-017-7321-8_19

expected to rise from the present 7.1 billion to nearly 10 billion by the year 2050. As concerns on the supply of fossil fuels increase, other renewable energy resources are being sought. Amongst various forms of renewable energy resources, biofuels have been one at the forefront of the discussion which can be produced from living organisms or metabolic byproducts. Over half of the Earth's population uses biomass-based fuels, such as firewood or agricultural materials for cooking and heating purposes (Edwards 2008). Biofuels presently contribute only 0.6 % of total transportation fuel, but estimates of biofuels contributing up to 15 % of the total global energy production by 2050 have been reported (Fischer and Schrattenholzer 2001). Production of low cost commercial biofuels becomes a global priority. Presently, significant work has been made towards reaching this goal. Current biofuel programs are unsustainable from environmental, economic and societal standpoints. The use of food crops for biofuel production has driven the food versus fuels debate because these feedstocks are important components of the human food chain (Mata et al. 2010). Large scale production of biofuels from crop plants harms the environment through the use of destructive pesticides, fertilizer and large requirement of water (Martin et al. 2010). Approximately 500–4000 l of water is required to grow enough feedstock to produce 1 l of bioethanol (Kumar et al. 2013; Faus et al. 2009). Therefore, it is essential to develop new energy resources which can resolve the sustainability challenges.

Over the past decade, global symposia and publications have exposed the potential of algae as a biofuel feedstock. Using algae as biofuel feedstock might elude the economic and environmental challenges which are integral in current terrestrial biomass feedstocks. Algae have several potential advantages as a biofuel feedstock, including: higher area productivity and limited competition with conventional agricultural land. In addition, algae can be grown in a variety of water sources and salinities without using agricultural pesticides. Large diversity of algal cultivars can be used as a possible sources of future bioenergy feedstocks producing a variety of transportation biofuels. Algal-based biofuels have already been utilized in commercial and military demonstration flights (Nair and Paulose 2014).

2 Different Forms of Energy from Algae

The use of algae for biofuel was investigated in United States and Japan as an alternative energy source from 1970s to 1990s in response to the oil crisis. However, these studies discontinued due to oil price stabilization in global market (Bird and Benson 1987; Chynoweth et al. 2001; Yokoyama et al. 2007). The early twenty-first century experienced a resurgence of interests in algal-based biofuels and attention has again turned to algae as a potential feedstock. Algal-based biofuels includes: the use of solid biomass, biohydrogen, biogas and liquid fuels such as bioethanol, biojet and biodiesel. However, biological hydrogen production is still in the developmental stage, therefore bioethanol, biojet, and biodiesel productions from algae are the most commonly envisioned biofuels. Recent research and development efforts have utilized a variety of algal and cyanobacterial forms and environments, i.e. micro and macroalgae; freshwater, brackish, and marine media; and diverse algal taxons.

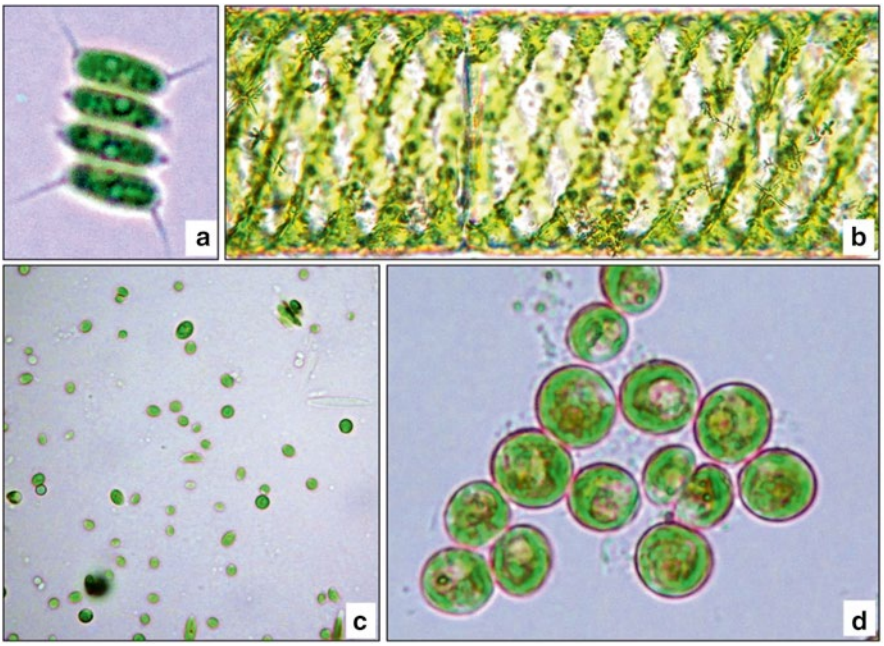

Fig. 1 (**a**–**d**) Some of the potential microalgal species for biodiesel production. (**a**) *Scenedesmus* sp., (**b**) *Spirogyra* sp., (**c**) *Chlorella* sp. and (**d**) *Chlorococcum* sp.

Depending upon their biochemical nature one alga cultivar will be the source only for particular type of biofuel. Several microalgae e.g. *Botryococcus*, *Spirogyra*, *Scenedesmus*, *Chlorococcum*, and *Chlorella* contain significant amount of lipids which make them potential biodiesel feedstock whereas macroalgae e.g. *Ulva*, *Sargassum*, *Laminaria*, *Ascophyllum*, *Gracilaria*, *Kappaphycus*, etc. contain higher carbohydrate concentrations which make them potential bioethanol feedstocks (Figs. 1a–d and 2a–d) (Sahoo et al. 2012). Some strains of microalgae can also be used as a potential feedstock for bioethanol production as they also contain high amount of carbohydrate. Matsumoto et al. (2003) has identified 76 strains with a carbohydrate content ranging from 40 % to 53 %. Productions of biofuels from algae require a biorefinery process. For example the non-soluble and non-extractable cellular components of microalgae can be pyrolyzed to produce a hydrocarbon based fuel (or alkanes). Unlike conventional fatty acid methyl ester (FAME) biodiesel, these hydrocarbon-based fuels can be readily refined to transportation fuel (Ahmed et al. 2012).

2.1 Bioethanol Production from Algae

Bioethanol market is rapidly increasing and using bioethanol as transportation fuel has several advantages over fossil fuels, includes reduction in particulate, nitrogen oxides and carcinogens such as benzene, toluene, ethyl benzene and xylene. The

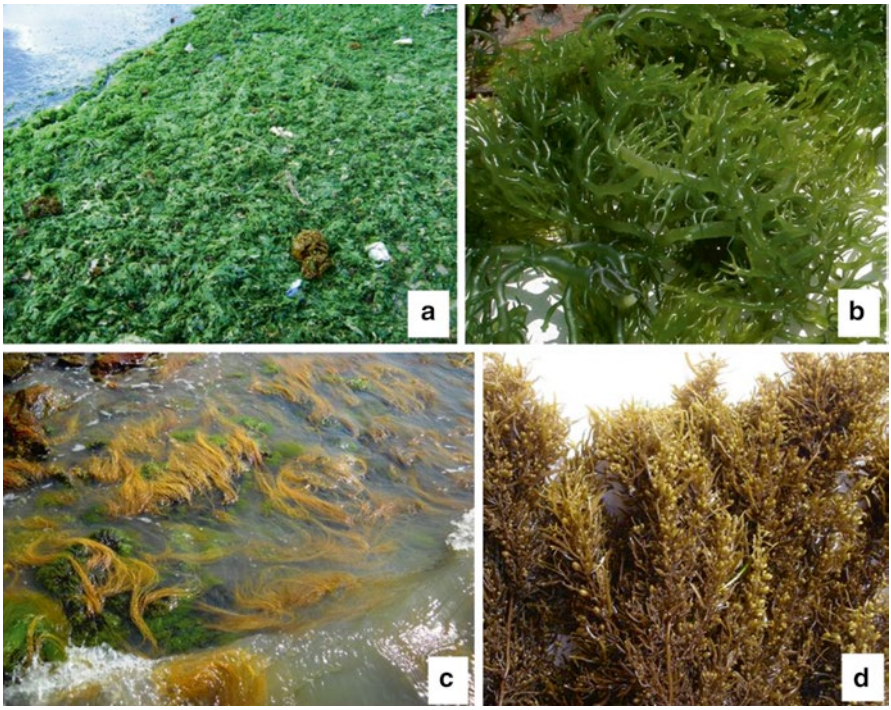

Fig. 2 (**a**–**d**) Some of the potential macroalgal species for bioethanol production. (**a**) *Ulva* sp., (**b**) *Kappaphycus* sp., (**c**) *Gracilaria* sp. and (**d**) *Sargassum* sp.

most important environmental consideration for bioethanol is that the CO_2 released by its combustion can be fixed by the growing plants through photosynthesis (Bastianoni and Marchettini 1996; Wheal et al. 1999). Presently, bioethanol is being produced from various feedstocks i.e. sugarcane, corn, cereal grains, potato, sweet potato, cassava but production of bioethanol from these food crops have raised food prices globally (Ethanol Across America 2007; Singh et al. 2011a, b). Large-scale crop plant production also contributes in GHGs emission, higher humidity and salt invasion (Edwards 2008). The development of cellulosic bioethanol has realized significance progress (pretreatment, enzymatic hydrolysis and fermentation) but commercial production of lignocellulose based bioethanol is still a more complex process, (Galbe and Zacchi 2002; Groenestijn et al. 2007).

The above discussion raises three big questions: Will we have sufficient source for bioethanol to fuel sustained economic development? Will it be safe? And will it be sustainable? Our answer for first question is a unquestionable "Yes". In recent years there are several reports of bioethanol production from various micro and macroalgae. Algenol Biofuels, Inc. of Florida, USA is commercializing a patented "Direct to Ethanol" algae technology through its proprietary cyanobacteria cultivar which secretes ethanol directly into the culture medium. The company has devel-

Table 1 Ethanol yields for various crops and for a red (*Gracilaria*) and a green algae (*Ulva*)

Crop	Ethanol yield (liter/1000 m^2)
Sugar beet (France)	667
Sugarcane (Brazil)	618
Cassava (Nigeria)	381
Sweet sorghum (India)	348
Corn (USA)	329
Wheat (France)	258
Gracilaria starch (Hawaii)	353
Ulva starch (Israel)	727
Ulva carbohydrates (Israel)	4360

Courtesy Reznik and Israel (2012)

oped a second generation, closed bioreactor system resulting in more than 10,000 gallons of bioethanol per acre per year. The cultivar's secreted ethanol is separated and collected without interrupting the culture process.

Similar to higher plants, macroalgae are comprised of rigid cellulose-based cell walls and accumulate various complex polysaccharides, which can be hydrolyzed to sugars and subsequently be fermented to ethanol (Kumar et al. 2013; Goh and Lee 2010). Unlike higher plants, carbohydrates of macroalgae can be converted directly into fermentable sugar and subsequently into bioethanol without going through the expensive pretreatment process as they lack lignin (Carpentier et al. 1988; Haugen et al. 1990; Gacesa 1992; Gunaseelan 1997; Moen et al. 1997; Horn and Østgaard 2001; Yun et al. 2011; Takeda et al. 2011). Kumar et al. (2013) and Reznik and Israel (2012) discussed rationability and feasibility of ethanol yield from seaweeds and interestingly it was found that although bioethanol production from macroalgae is in its very early phase but quite comparable with other well known first and second generation source of bioethanol (Table 1).

Direct use of macroalgae for biofuel production neither will be cost effective nor practical because macroalgae also contain other valuable phycocolloids and bioactive compounds which are very good source of revenue. Since, the future of biofuels depend on the accelerated diffusion of new technologies, with an appropriate and market-friendly regulatory environment, therefore, utilization of macroalgae for ethanol production is probably only of economic interest when integrated with a balanced and total utilization of the macroalgae material, as a biorefinery approach (Horn et al. 2000; Kumar et al. 2013; Takeda et al. 2011). Macroalgal industrial wastes i.e. the remaining pulp after extraction of high value polysaccharides still contain high amount of carbohydrates which may be used as a source of raw material for ethanol production (Kumar and Sahoo 2012; Sahoo et al. 2012). Utilization of algal pulp will reduce organic load where the pulp is deposed of during phycocolloids extraction process (Carpentier et al. 1988; Ge et al. 2011). Macroalgae may be a key link between energy production, local ocean acidification mitigation, and climate change. Production of bioethanol from algae is a sequential process. Following are the main steps for bioethanol production from algae in a biorefinery approach.

2.1.1 Selection of Algae

Algal selection depends upon its availability and accessibility. The following biorefinery approach to bioethanol production includes the following steps. An analysis of potential algal polysaccharides is required prior to cultivar selection for bioethanol production. Two classes of polysaccharides are found in macroalgae: (1) structural and (2) storage. Structural polysaccharides are analogous to that of terrestrial plants, consisting of mainly cellulose, hemicellulose and xylans. Storage polysaccharides, i.e. agar, alginate and carrageenan are more specific to macroalgae which are not found in other land plants. Presence of some compounds such as phenolics in certain algae can act as an inhibitor of hydrolysis and fermentation processes. Thus algae containing high concentration of such compound should not be utilized for bioethanol production.

2.1.2 Collection of Algae

Collection of wild or harvested macroalgae is a coastal activity. Countries such as India which is located in equatorial zone, drying of harvested macroalgae can be done under the sun. Desalination of seaweeds is a necessary step because it can cause problems during purification and processing.

2.1.3 Extraction of Phycocolloids

Phycocolloid extraction from algae is a mature commercial process with annual revenues exceeding USD $ 500 million dollars. Yield of phycocolloids normally range between 20 and 40 % of total dry weight. The extracted phycocolloids are normally purified through filtration and centrifugation processes. Few reports are available on analysis of leftover pulp after extraction of polysaccharides from macroalgae. Processed algal pulp can be dried and used for hydrolysis and fermentation. The dried algal residue and extracted phycocolloids do represent the entire thallus weight due to biomass losses experienced during the phycocolloid extraction process (Davis et al. 2004; Kumar et al. 2013).

2.1.4 Hydrolysis

Carbohydrates found in whole algae or leftover pulp can be converted to fermentable sugars through three basic methods (1) acid hydrolysis, (2) enzymatic hydrolysis, or (3) thermochemical- with variations for each (Badger 2002; Kumar and Sahoo 2012). Although a variety of acids can be utilized, sulfuric acid is the most commonly used due to costs. Similar to higher plant's carbohydrate degradation, algal carbohydrates are also catalysed by endocellulases, exocellulase and β-glucosidase and occurs both under aerobic and anaerobic conditions (Fig. 3).

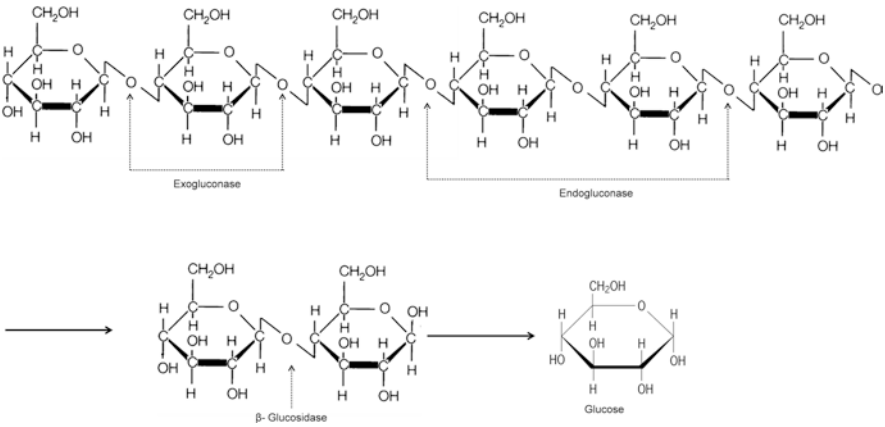

Fig. 3 Enzymatic breakdown of cellulose by exogluconase, endogluconase and β-glucosidase

$$\underset{\text{Glucose (1 kg)}}{C_6H_{12}O_6} \longrightarrow \underset{\text{Ethanol (514g)}}{2CH_3CH_2OH} + \underset{\text{486g}}{2CO_2} + \underset{\text{147Kcal}}{\text{Heat}}$$

Fig. 4 Basic biological reaction in the conversion by fermentation of 1 kg of glucose to ethanol, carbon dioxide, and heat

Algal cell walls contain various compounds which require a suite of enzymes including agarases, alginate lyases, proteases and cellulases necessary to degrade the complete algal cell wall, as seen in the case of protoplast isolation (Butler 1931; Kumar and Sahoo 2012).

2.1.5 Fermentation

Fermentation technology is the oldest of all biotechnological process. The term is derived from the Latin verb *fevere* (to boil). Fermentation is the conversion of a carbohydrate into acid or alcohol or it is a process of deriving energy from the oxidation of organic compounds, using an endogenous electron acceptor. Fermentation includes upstream processing of sugar solution and downstream processing of fermented product. Upstream processing generally accomplished in two steps: (1) preparation of fermentable sugar solution and (2) the fermentation of sugar solution, by appropriate microbial strain into ethanol under controlled and axenic conditions. Downstream processing includes separation and purification of ethanol usually by distillation. Hypothetically, 100 g of glucose produces 51.4 g of ethanol and 48.8 g of carbon dioxide (Fig. 4). However, practically, the microorganisms consume some of the glucose for growth and the actual yield is less. *Saccharomyces cerevisiae*, a cultivar of yeast used in fermentation, is also capable of galactose fermentation. Certain strains of *S. cerevisiae* exhibit exceptional fermentative performance by

completely exhausting the sugars in significantly less time than that typically required by other strains.

S. cerevisiae and the bacterium *Zymomonas mobilis*, two most commonly used microorganisms for ethanol production but have a very narrow substrate range (Dumsday et al. 1997). Yeast lacks transhydrogenase (Van Dijken and Scheffers 1986), so it can't grow on mannitol anaerobically. Compared to glucose, one extra NADH is produced from mannitol, so regeneration of all the NAD+ then requires either oxygen or transhydrogenase to convert NADH to NADPH (Quain and Boulton 1987; Horn et al. 2000). *Zymobacter palmae* a facultatively anaerobic, catalase-positive, oxidase-negative, nonsporeforming and peritrichously flagellated, gram-negative proposed as a new ethanol-fermenting bacterium isolated from palm sap in Okinawa, Japan (Horn et al. 2000). It ferments hexoses, c~-linked di- and tri-saccarides, and sugar alcohols (fructose, galactose, glucose, mannose, maltose, melibiose, saccharose, raffinose, mannitol and sorbitol).

2.2 Biodiesel

Globally biodiesel is the second most common liquid biofuel after bioethanol. Biodiesel can be defined as the monoalkyl esters of vegetable oil and animal fats (ASTM 2009). Generally, biodiesel is produced by the transesterification of triglyceride with monohydric alcohols such as methanol (Fig. 5). Main characteristics, as cetane number, energy content, viscosity and phase changes of biodiesel are similar to petroleum derived diesel (Lin and Teong 2010; Sahoo et al. 2012). Thus, biodiesel can be blended in any proportion with fossil based diesel which is not possible with bioethanol. The global market for biodiesel is poised for explosive growth in the next 20 years. Biodiesel is mainly produced from vegetable oil, including soybean, palm and sunflower oil followed by biomass-based and non-agricultural feedstocks (Demirbas 2009). Production of biodiesel from these sources results in higher food price. All resulting infer scientific community developing an alternate source of biodiesel feedstock.

What makes microalgae one of the main sources of biofuels? It is the required rapid growth of carbon-neutral renewable alternatives. Production of microalgal-based biodiesel has been an area of considerable interest due to its potential commercial feasibility. Microalgae with 30 wt.% oil could produce 12,000 $Lha^{-1}year^{-1}$ compared with 5950 $Lha^{-1}year^{-1}$ from oil palm, and 1892 $Lha^{-1}year^{-1}$ from Jatropha (Adenle et al. 2013; Schenk et al. 2008). However, several challenges need to be

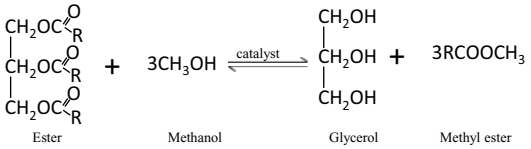

Fig. 5 Chemical transesterification of triglycerides for biodiesel production (R-fatty acid group)

resolved resulting in the commercial-scale production of algal-based biodiesel resulting in significant contributions to transport energy reserves. Many species of microalgae contain higher oil content than conventional oil crops containing more than 50–60 % of their dry weight lipid as their storage products. Microalgae capture solar energy as they are sunlight-driven cell factories that convert carbon dioxide to potential biofuel, food, feeds and bioactive compounds (Metting and Pyne 1986; Spolaore et al. 2006). Microalgal production rates are much higher than land plants and can double their biomass in within 7 days, whereas land based higher plants take many months or years (Vonshak et al. 1982). Chemical composition of microalgae can be controlled through the manipulation of growth medium. CO_2 emitted from power, cement and steel plants etc. can be used as a source of carbon for algal growth (Sahoo et al. 2012). Microalgae limit the competition for resources with conventional agriculture and can be grown in various types of water sources including marine, brackish, or fresh water, utilizing raceway ponds on non-arable land. Similarly microalgal biomass can be harvested throughout the year. Although microalgal biomass is considered as a potential feedstock for biofuel production, their commercial scale cultivation has not presently achieved economically feasibility (Markou and Neraantzis 2013). Similar to bioethanol production, biodiesel from algae also require a sequential process. Following are the main steps for microalgal-based biodiesel production in a biorefinery approach.

2.2.1 Selection of Microalgae

Microalgae cultivar selection is focused on three predominant selection criteria: (1) water type; marine, brackish, and fresh water. (2) cultivation strategy; uni or mixed cultures. (3) Natural or genetically modified cultivars. However, selection of microalgae for the development of algae-based biodiesel is purely based on concentration of lipids, productivity, and its harvest potential. Species of *Chlorella*, *Botryococcus*, *Neochloris*, *Scenedesmus* etc. normally contain high amount of lipids and are considered as potential commercial cultivars.

2.2.2 Cultivation and Recovery of Microalgal Biomass

There are two fundamental methods for microalgae cultivation: (1) open pond systems and (2) closed photo bioreactor systems. Open pond systems normally include raceways ponds, lagoons, and/or troughs; whereas closed systems include various forms of photobioreactors i.e. tubular, flat or columnar etc. Microalgal size and external morphology results in harvesting challenges. The recovery of microalgal biomass is a major step for biodiesel feedstock production. As microalgae contain more than 99 % water (w/w) so dewatering is one of the most important steps in biodiesel production from algae. Microalgal biomass concentration and/or dewatering can be categorized as: physical (e.g. centrifugation, spray drying and filtration), biological (e.g. auto flocculation) or chemical (e.g. chemical flocculants) (Sahoo

et al. 2012). Filtration is the simplest method for microalgal harvest, a function of cultivars sizes. During filtration the pore size of the filter depends on the size of the microalgae and the aggregation rate of microalgae. Centrifugation, an alternative to filtration, is presently used for the harvesting of microalgal cultivars but requires extensive energy and biomass recovery is a function of cell size diversity and settling characteristic (Brennan and Owende 2010). Flocculation is a biological or chemical process where microalgal cells aggregate in order to increase the particle size. Several chemical flocculating agents includes alum, ferric chloride, ammonium sulphate, ferric sulphate, (Brennan and Owende 2010) polyacrylamide polymers, surfactants, chitosan and other man-made fibers are normally used as flocculating agents (Divakaran and Pillai 2002; Lee et al. 2009; Sahoo et al. 2012). The harvested microalgal biomass must be processed rapidly for drying. There are various methods for drying microalgal biomass including: (i) sun drying, the cheapest method taking extended periods and a large surface is required, (ii) most common method for extraction of high value products is spray drying, but it is relatively expensive and can cause significant deterioration of some algal pigments (Sahoo et al. 2012).

2.2.3 Enhancement in Quantity and Quality of Microalgae

Microalgae can grow rapidly and live in harsh conditions, due to their simple unicellular or multicellular structure. Growth rates can be accelerated through careful species selection and culture conditions (e.g. amount of sunlight, water, and nutrients). Biochemical composition of microalgae can be manipulated by modifications in growth medium and changes in physical parameters. Lipid content of *Chlorella* and *Nanochloropsis* species can be influenced by temperature and the concentration of nitrogen and chloride ions in water (DuPont 2013). Algal simple cellular structure also makes them a promising candidate for genetic modification to further improve their yields.

2.2.4 Disruption of Microalgal Biomass

Alternative to drying, oil can be extracted from wet algal biomass, by lysing the cell walls. Cell wall disruption methods include: osmotic shock, explosive decompression, mechanical press, mechanical shear etc. Interestingly, some microalgae degrade through the shearing action of the pumps used in bioreactors, so mechanical shear may also be an option (Sahoo et al. 2012; Shields et al. 2008).

2.2.5 Oil Extraction

Once the cell is ruptured, the lipid fraction, consisting of fatty acids and glycerol, are separated from the remaining cell contents. This can be done by solvent or other extraction processes. Biodiesel is then produce by transesterification in which

triglycerides are reacted with methanol to yield glycerol and methyl esters of fatty acids (Mata et al. 2010). Since the chemical reaction is reversible alcohol is usually overdosed to overcome the biodiesel efficiency and makes it possible to recycle the unreacted alcohol improving biodiesel production rates and economics. Two main products of transesterification i.e. biodiesel and glycerol can be separated through settling, filtration, and decantation (Ahmed et al. 2012).

2.3 Alternative Biofuel

Increasing energy demands and related global climate change phenomenon have resulted in considerable global efforts to discover more sustainable ways to produce bio-based ethanol, jet fuel, and diesel while minimizing carbon dioxide emissions. Photobioreactor hydrogen production is one of the environmentally friendly alternatives. Microalgae are easy to grow in a bioreactor compared to other biological resources which makes them suitable candidate for biohydrogen production. *Chlamydomonas reinhardtii* is an important microalga which is being used as a source of biohydrogen production. Hydrogen Production through direct photolysis according to the reaction:

$$H_2O > PSII > PSI + 4photons > Ferredoxin > H_2ase > H_2$$

has recently been at center of research efforts in biological hydrogen production (Torzillo et al. 2012). Some seaweed such as *Saccharina latissima*, *Fucus serratus*, *Laminaria digitata*, *Alaria esculenta* etc are also being used as a source of biogas production (Vivekanand et al. 2012; Schumacher et al. 2011). Methods used for biogas and biohydrogen production includes gasification, hydrothermal liquefaction, pyrolysis, hydrogenation etc. However, biohydrogen or biogas production from algae is in its incipient phase.

3 Algal-Based Biofuel Constraints

Though algae hold great promise as a potential source for liquid transportation biofuels, some experts still remain skeptical about the economic feasibility of the technology (Hong et al. 2014) and the authors partially concur with their stated conclusions. While the commercial cultivation of algae is already technologically feasible for the small scale production of animal feed and nutraceutical products, the production of algal biofuels on a commercial basis is far from being realized. There are numerous technical challenges and uncertainties associated with large-scale algae biofuel production. The commercial production of algal biofuel is yet to take place, leaving a large degree of uncertainty in existing estimates. This uncertainty is compounded in developing countries as most studies have focused on U.S. or European conditions. The critical question remains as to how easy it will be for

developing countries to take advantage of advanced technologies such as genome manipulation, DNA sequencing, and bioinformatics, in the light of weak biosafety regulatory system and inadequate capacity building and the unknown threats of genetically modified organisms (GMOs) to the environment and long-term impact on human health represents a primary concern in developing countries. For example, in African countries, the issue of possible contamination of conventional crops called "traditional heritage" by GMOs was emphasized among the key stakeholders such as scientists and policymakers (Adenle 2013). Out of the few thousand species kept in culture, only a few have had their chemical content investigated. Extensive work is needed on various aspects of the topic to ensure that any future process is robust and reproducible. Limitations of photosynthetic efficiency, lipid production and storage, interactions of algae with other organism (e.g. other algae, bacteria and microorganism), and grazing problems represent significant constraints to commercial production (Day and Stanley 2012). Land availability is another potential algal production constraint. In order to satisfy global oil demand, arable land estimates of 20.5 % would be required to convert agriculture to algae production but interestingly this could be reduced to zero if ponds and bio-reactors are situated on non-arable land (Adenle et al. 2013; Schenk et al. 2008). Presently it appears that the sustainable production of algal-based biofuels requires a leap of faith, but there are grounds for optimism. The diversity of algal species is such that it is highly likely that new applications and products will be found. As experience with algal cultivation increases and modern technologies are applied, algal-based biofuels may achieve sustainable, commercial production scale (Slade and Bauen 2013).

4 Advance Technologies and Algal Biofuel

A multidisciplinary approach is needed to achieve the full potential of algal-based biofuel production. Use of various advance technologies can enhance algal-based biofuel production several fold. Flow cytometry is a rapid method for quantitative measurement of individual cells in a moving fluid. Microalgae are ideal for flow cytometric analysis because they are single celled and contain photosynthetic pigments, such as chlorophyll *a*, which auto fluorescence when excited by blue light. In 2003, de la Jara et al. reported a method for *Crycodinium cohnii* lipid composition assessment by flow cytometry. Multi-staining flow cytometry is proving to be a useful technique for microalgal lipid production process optimization, providing important physiological information, at the individual cell level, about process efficiency that is difficult to obtaining in any other way (Silva and Reis 2012). Modern biotechnology, particularly genetic engineering and conventional methods of strain selection have great potential to improve the production efficiency, and reduce the costs that are associated with algal-based biofuel. Genetic modification (GM) of algae can provide the important breakthrough needed through gene manipulation while unraveling the barrier to understand the metabolic pathway of the algal

genome (Adenle et al. 2013; Beer et al. 2009). Advance technologies can help to solve the following challenges for commercialization of algal-based biofuels:

- Algal strain development
- Enhanced algal culture productivities and yields
- Increased harvesting technology efficiencies
- Lipid and carbohydrate extraction and processing advancements

Nanotechnology can potentially provide solutions to many of the challenges faced in commercialization of algal-based fuels. Pattarkine and Pattarkine (2012) discussed various roles of nanotechnology in algal biofuel production including:

- Bioreactor design
- Culture illumination
- Growth of algal culture (role of nanobubbles)
- Conversion of biomass to biofuel products
    1. *Nanotechnology for biomass transformation*
    2. *Nanocatalysts for cracking/hydrocracking*
    3. *Nanocatalysts for transesterification*
    4. *Metal nanocatalysts for biogasification of wet biomass*
    5. *Zeolites*
    6. *Nanohybrid catalysts as emulsion stabilizers*
- Nanotechnology and biofuel additive

5 Algal Biorefinery: Common Solution for Different Challenges

Considering the increase demand and availability of suitable feedstocks, sustainable production of third generation biofuels have a very bright future. Production of third generation biofuels will surely contribute in the reduction of Green House Gasses (GHGs) to reduce global warming. With concerns of cost efficiency, the economics of algal biofuel production can be significantly improved by using a biorefinery based production strategy where all the components of the biomass raw material are used to produce useful byproducts. Algae can yield many co-products from the same biomass and this multiproduct paradigm makes it a perfect candidate for the biorefinery concept. A conceptual biorefinery system (Fig. 6) can be constructed to understand the production of various industrial co-product as well as biofuels simultaneously. Products from integrated algal biorefinery can service various industries such as pharmaceutical, paper and food production sectors (Subhadra and Grinson-George 2011). A wide array of algal-based products can be developed including food supplements, livestock feeds, fine organic chemicals for pharmaceuticals, pigments, various other applications and bioethanol production along with biogas and biodiesel (Singh and Gu 2010; Kumar et al. 2013). Apart from these various

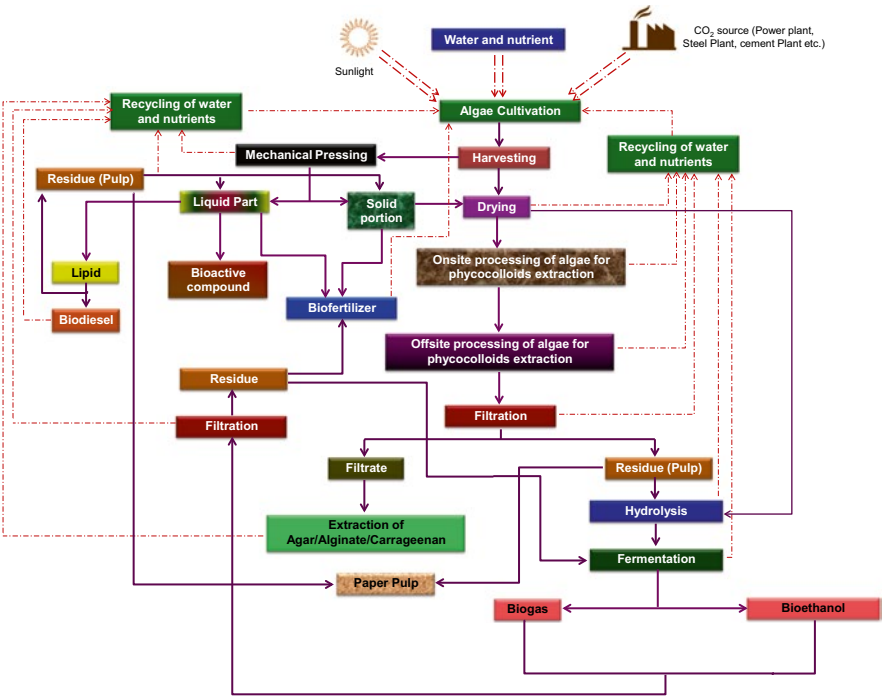

Fig. 6 Schematic representation of conceptual algae-based biorefinery

products, algae-based biorefinery will also contribute in waste water treatment and carbon dioxide sequestration. Modern day algal biorefineries will start shining like a beacon of hope for the development of renewable energy for the upcoming generations if the scientific and financial investments being currently made are suitably and realistically targeted.

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Phylogenomics in Algal Research: Current Trends and Future Perspectives

Cheong Xin Chan

1 Introduction

Phylogenomics is a widely adopted approach for assessing the evolutionary histories among organismal lineages based on comparative analysis of genome-scale data. Extending from phylogenetic analysis at the gene level, phylogenomic inference is commonly observed based on gene-by-gene (Beiko et al. 2005; Puigbò et al. 2010), concatenated multi-genes (Nozaki et al. 2007; Baurain et al. 2010) or whole-genome (Rannala and Yang 2008) comparisons. Current standard for phylogenomics involves the identification of homologous gene/protein sequences, multiply aligned these sequences in a multiple sequence alignment framework, from which phylogenies would be inferred. Using phylogenomics, we can gain a better understanding of how a genome has evolved relative to other species.

The power of phylogenomics relies on the availability of high-quality genome data. The earlier phylogenomics studies focused on prokaryotes (Beiko et al. 2005; Puigbò et al. 2010; Bansal et al. 2013). These typically small, simple genomes (mostly <10 Mb in size; little intergenic regions) can be obtained at lower cost than eukaryote genomes. As of 25 April 2014, there are 24,349 prokaryote genomes available on NCBI (http://ncbi.nlm.nih.gov/genome), compared to 2775 eukaryote genomes. Moreover, sequencing decisions have long been biased towards species of economic and medical importance. As the costs of sequencing decrease in recent years, other biological aspects e.g. evolution and phyletic positions can now drive sequencing decision, enabling sequences from taxa that are evolutionarily important (but not necessarily of medical or economic importance) to be generated at scale that was previously unimaginable.

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D. Sahoo, J. Seckbach (eds.), *The Algae World*, Cellular Origin, Life in Extreme Habitats and Astrobiology 26, DOI 10.1007/978-94-017-7321-8_20

The following sections highlight the importance of phylogenomics in algal research, what have we learned from algal phylogenomics, and its limitations. The future perspectives of algal phylogenomics are discussed in light of the on-going deluge of sequencing data.

2 Why Do We Need Phylogenomics in Algal Research?

For decades, algal research has largely been driven by (a) biotechnology and fisheries, particularly the production of biomass and secondary metabolites (De Ruiter and Rudolph 1997; Chopin and Swahney 2009; Bixler and Porse 2011), and (b) taxonomy and systematics, in which identification of a species, particularly of seaweeds, is complicated by the presence of multiple physiological appearances and ploidies across different life history stages (Blouin et al. 2011). The algal hydrocolloids (e.g. carrageenan, alginate, agar and agarose) are key thickening, gelling and emulsifying agents that are widely used in the industries of food, animal feed, pharmaceuticals and cosmetics. The value of seaweed hydrocolloids is estimated between US$ 0.65 to 1.02 billion (Chopin and Swahney 2009; Bixler and Porse 2011), and the global seaweed industry valued at about US$ 6 billion (Chopin and Swahney 2009; Soto 2009). Lipid production has also been highlighted due to the worldwide attention on biofuel as an alternative to petroleum (Dismukes et al. 2008; Mata et al. 2010). The availability of genome data allows us to address more-fundamental biological questions from the evolutionary perspective.

2.1 Algal Diversity

Algae are a diverse group of simple photosynthetic organisms. Growing almost exclusively in aquatic environments ranging from the freshwaters, estuaries, ocean surface to coral reefs, algae are the most important primary producers on Earth (Amante and Eakins 2009). The diversity of algae has been conservatively estimated at about 300,000 species, with a rough estimate of >1 million species (Guiry 2012). Figure 1 shows the evolutionary relationships among eukaryote lineages based on current systematics (Adl et al. 2012). Photosynthetic lineages in eukaryotes are broadly distributed across different supergroups within Diaphoretickes. These photosynthetic eukaryotes, except plants, are loosely defined as algae (note that colloquially the prokaryotic cyanobacteria are known as the blue-green algae). The supergroup Archaeplastida (Cavalier-Smith 1981; Rodríguez-Ezpeleta et al. 2005), also known as Plantae, represents the most primitive lineages of photosynthetic eukaryotes, which include Glaucophyta (glaucophyte algae), Rhodophyta (red algae), and Chloroplastida (green algae and plants; also known as Viridiplantae). Well-known examples of these taxa include the red seaweeds that are biotechnologically important e.g. *Porphyra* and *Gracilaria*, and the green alga *Chlamydomonas*

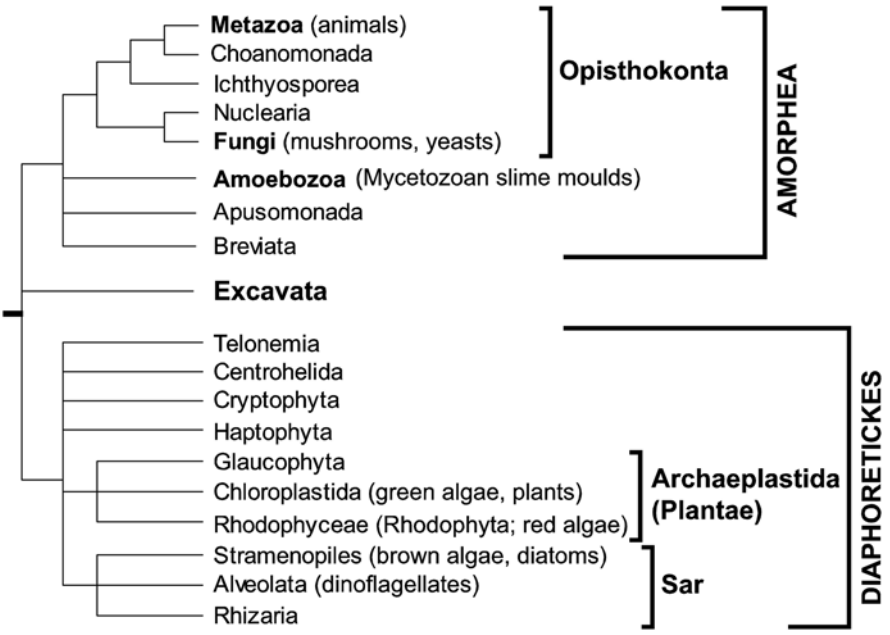

Fig. 1 Current classification of eukaryote lineages and their evolutionary relationships based on Adl et al. (2012)

reinhardtii. These algae possess the simple, two-membrane-bound primary plastids (Cavalier-Smith 1981; Rodríguez-Ezpeleta et al. 2005). In comparison, the other eukaryotic algae possess the more structurally complex, secondary (or tertiary) plastids, bound by three or four membranes. These taxa are sometimes loosely grouped as the "chromalveolates", which include the stramenopiles (e.g. diatoms, brown algae), alveolates (e.g. dinoflagellates), and the haptophytes (e.g. *Emiliania*). Some dinoflagellates cause "red tides", which have a huge impact on global economy and human health (Hallegraeff 1993; Anderson et al. 2008).

2.2 Algal Evolution and Plastid Origin

The origin of algae and plastids (hence photosynthesis) among eukaryotes, are critical to our understanding of the geological and atmospheric histories of planet Earth, e.g. the Great Oxygenation Event ca. 2.4 billion years ago (Scott et al. 2008). Current understanding of plastid origins has been extensively reviewed (Reyes-Prieto et al. 2007; Howe et al. 2008; Keeling 2010; Chan et al. 2011a). Figure 2 shows the current understanding of plastid evolution in eukaryotic algae. The origin of primary plastids among the Archaeplastida lineages (and the known example of the rhizarian *Paulinella*) traced back to a cyanobacterial source, in which a

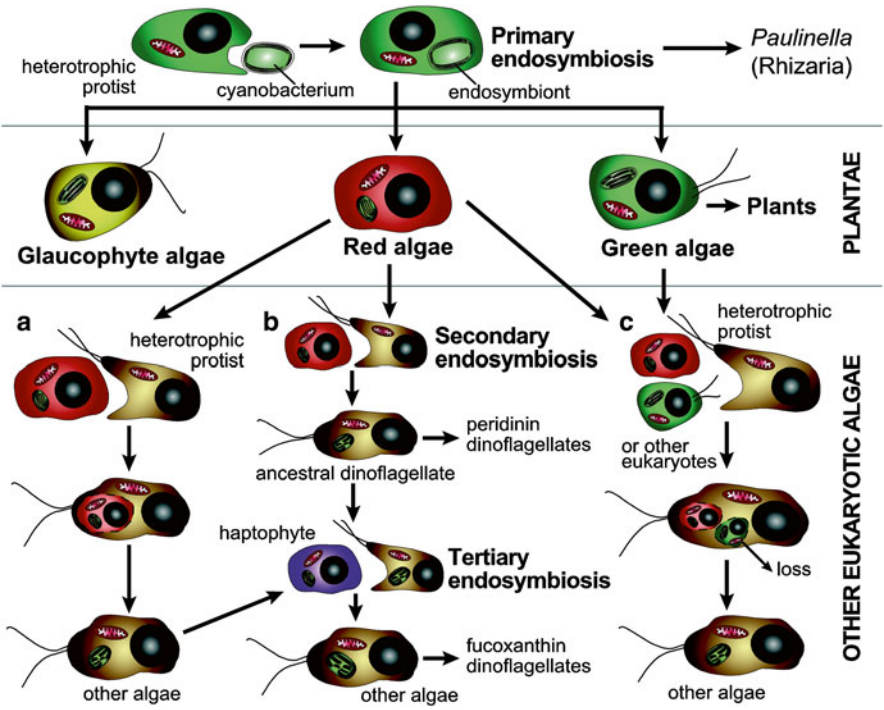

Fig. 2 Current understanding of plastid evolution in photosynthetic eukaryotes, for those with primary plastids (Archaeplastida/Plantae), and others with plastids that are more structurally complex, based on three major hypotheses (**a**, **b** and **c**) in plastid evolution

cyanobacterium was engulfed by and retained within a heterotrophic host (i.e. primary endosymbiosis) (Margulis 1970), estimated to have occurred around 1–1.5 billion years ago (Douzery et al. 2004; Yoon et al. 2004). This process induced genetic transfer from the endosymbiont to the host nucleus, and the engulfed endosymbiont gradually became the extant plastids.

On the other hand, the evolutionary history of the more-complex plastids (e.g. in brown algae, diatoms and dinoflagellates) is complicated by multiple, serial events of endosymbiosis involving already plastid-bearing endosymbionts (Yoon et al. 2005; Reyes-Prieto et al. 2007). Published studies suggest three possible paths from which the secondary/tertiary plastids could have arisen: (a) secondary endosymbiosis involving an ancestral red algal cell, i.e. the chromalveolate hypothesis (Cavalier-Smith 1998, 1999) (Fig. 2A); (b) secondary red algal endosymbiosis followed by tertiary endosymbiosis involving an ancestral haptophyte-like cell, as postulated for fucoxanthin-containing dinoflagellates (Ishida and Green 2002; Yoon et al. 2005) (Fig. 2B); and (c) a secondary endosymbiosis involving both ancestral red and green algal cells (Moustafa et al. 2009), or other eukaryote-eukaryote endosymbioses (Archibald 2009; Bodył et al. 2009; Baurain et al. 2010; Stiller et al. 2014) (Fig. 2C). These hypotheses remain to be investigated further as more genome data become available.

Table 1 Non-exhaustive list of key published algal genomes as of 1 January 2014, sorted by estimated genome size in an ascending order

Genome	Group	Estimated size (Mbp)	References/remarks
Paulinella chromatophora	Thecate amoeba (Rhizaria)	4–7	Bhattacharya et al. (2012)
Galdieria sulphuraria	Red alga (Rhodophyta)	10.8	Schönknecht et al. (2013)
Ostreococcus tauri	Green alga (Chloroplastida)	12.6	Derelle et al. (2006)
Cyanidioschyzon merolae	Red alga (Rhodophyta)	17.6	Matsuzaki et al. (2004)
Porphyridium purpureum	Red alga (Rhodophyta)	19.7	Bhattacharya et al. (2013)
Micromonas sp.	Green alga (Chloroplastida)	21	Worden et al. (2009)
Phaeodactylum tricornutum	Diatom (Stramenopile)	27.4	Bowler et al. (2008)
Thalassiosira pseudonana	Diatom (Stramenopile)	34	Armbrust et al. (2004)
*Pyropia yezoensis**	Red alga (Rhodophyta)	43.5	Nakamura et al. (2013)
Chlorella variabilis	Green alga (Chloroplastida)	46.2	Blanc et al. (2010)
Cyanophora paradoxa	Glaucophyte (Glaucophyta)	70	Price et al. (2012)
Guillardia theta	Cryptophyte (Cryptophyta)	87	Curtis et al. (2012)
Bigelowiella natans	Chlorarachniophyte (Rhizaria)	95	Curtis et al. (2012)
*Chondrus crispus**	Red alga (Rhodophyta)	105	Collén et al. (2013)
Chlamydomonas reinhardtii	Green alga (Chloroplastida)	120	Merchant et al. (2007)
*Volvox carteri**	Green alga (Chloroplastida)	138	Prochnik et al. (2010)
Ectocarpus siliculosus	Brown alga (Stramenopile)	214	Cock et al. (2010)
Symbiodinium minutum	Dinoflagellate (Alveolata)	1500	Shoguchi et al. (2013)

Taxa in multicellular form are denoted with an asterisk (*)

3 What Have We Learned from Algal Phylogenomics?

Table 1 shows a number of key published algal genomes as of 1 January 2014. For years, biased taxon sampling in algal phylogenomics has been attributed to inadequacy of red algal genome data. The availability of red algal genomes in recent years therefore represents a significant milestone in algal research. Given the important role of red algal lineages in algal evolution (Fig. 2), these genomes provide an excellent analysis platform for addressing many outstanding questions in algal evolution and endosymbiosis.

3.1 Origin of Photosynthetic Eukaryotes

Archaeplastida supergroup represents the primitive lineages of photosynthetic eukaryotes. These taxa bear the primary plastids, and are expected to share a common ancestry. However, the initial phylogenetic support for this hypothesis had been limited to a handful of genes (Rodríguez-Ezpeleta et al. 2005; Nozaki et al. 2007). This is partly due to lack of gene repertoires for glaucophyte (Glaucophyta) and red algae (Rhodophyta), which are scarce in comparison to those available for green algae and plants (Chloroplastida). Not until recently, no glaucophyte genome was available, and the only available red algal genome was the highly reduced genome from the hyperthermophile *Cyanidioschyzon merolae*. Enriching available data using novel data of mesophilic red algal species, i.e. *Porphyridium purpureum* and *Calliarthron tuberculosum*, an earlier study (Chan et al. 2011c) demonstrated a strong support for Archaeplastida (by proxy of strongly supported clades of reds and greens) across hundreds (~50 %) of the analyzed protein phylogenies. These findings are further reinforced by a later study incorporating the novel genome data of *Cyanophora paradoxa* (Price et al. 2012), the first of any glaucophyte algae. This work completes the missing link that unifies all three major groups under Archaeplastida, thus evidence for a single origin of all primary plastids in eukaryotes. These studies also demonstrate that the earlier difficulty in resolving the supergroup using phylogenetic approaches is likely due to the extent of lateral genetic transfer among microbial lineages.

3.2 Endosymbiosis and Algal Evolution

Owing to endosymbiosis (Fig. 2), the complication of genetic transfer in algal evolution is expected, especially among taxa that possess secondary (and tertiary) plastids, e.g. the "chromalveolates". The positions of these lineages on the eukaryote tree of life are far from being resolved, as demonstrated in a number of studies based on phylogenies of select genes (Burki et al. 2007, 2012b; Baurain et al. 2010; Parfrey et al. 2010). Key examples of these taxa include the ubiquitous diatoms (stramenopiles) and dinoflagellates (alveolates). In a phylogenomic analysis (Moustafa et al. 2009) using two completely sequenced diatom genomes (Armbrust et al. 2004; Bowler et al. 2008), hundreds of diatom genes are found to be of red or green algal origin, suggesting a putative cryptic endosymbiosis involving an ancestral (prasinophyte-like) green alga in the course of diatom evolution. Later studies of algal genes encoding functions of membrane transport (Chan et al. 2011b) and fatty acid biosynthesis (Chan et al. 2013; Wang et al. 2014) revealed red and/or green algal prominence in these genes, demonstrating that algal genetic transfer as a key factor to environmental adaptation in microbial eukaryotes.

The extent of genetic transfer in prokaryotes is known to be rampant (Beiko et al. 2005; Zhaxybayeva et al. 2006; Dagan and Martin 2007; Puigbò et al. 2010). In a

recent transcriptome analysis of the dinoflagellate *Alexandrium tamarense* (Chan et al. 2012b), the extent of genetic transfer in microbial eukaryotes is shown to be comparable to that in prokaryotes, despite more-complex coding capacity in eukaryotes. The dinoflagellates can be considered as the worst-case scenario in terms of the complexity of algal evolution, because tertiary (and likely quaternary) endosymbiosis events involving other eukaryotic (e.g. haptophyte-like) cells have been postulated (Hackett et al. 2004; Yoon et al. 2005; Wisecaver and Hackett 2011) in addition to the presence of bacterial derived genes (Nosenko and Bhattacharya 2007; Slamovits et al. 2011). Such an evolutionary complexity is against the backdrop of mysteriously immense genome sizes, with the largest dinoflagellate genome (of *Prorocentrum micans*) estimated to exceed 210 Gbp (Hackett et al. 2004; LaJeunesse et al. 2005).

Some have argued that these findings could in part be an artifact due to inadequacy of red algal genes at the time (Burki et al. 2012a; Deschamps and Moreira 2012) and to technical biases (Dagan et al. 2013). Nevertheless, all these studies demonstrate algal and bacterial genetic transfer as key contributing factors to the adaption and survival of microbial species in fluctuating marine environments.

3.3 Algal Biology and Physiology

Recently available algal genomes also provide an interesting analysis platform for assessing biological features that would inform us about genome innovation relative to physiological and/or environmental changes. The red algal genomes, for instance, are found to be highly compact with few intronic regions across unicellular (Bhattacharya et al. 2013) and multicellular species (Collén et al. 2013; Nakamura et al. 2013), with only about 0.3 introns per gene.

In cases where genome data are not yet available, e.g. for the economically important *Porphyra* (Gantt et al. 2010; Blouin et al. 2011), studies of transcriptomes are already providing clues to key physiological characteristics in red algae, e.g. new fatty acid biosynthesis and trafficking pathways (Chan et al. 2012a), and differential expression of genes involved in key development processes (Stiller et al. 2012). Studies of other red algal genomes (Bhattacharya et al. 2013; Collén et al. 2013) are generating novel insights into the origin and evolution of carbohydrate metabolism and biosynthesis of secondary metabolites e.g. starch and isoprenoid compounds.

Algal epigenomes (Zhao et al. 2007; Gross et al. 2013) are providing first clues about genetic regulation in these organisms by non-coding elements. Other studies have demonstrated that green algal derived genes in microbial eukaryotes are important for the function of light-harvesting complex superfamily (Peers et al. 2009), and for protection from oxidative damage (Frommolt et al. 2008). Genetic transfer has recently been demonstrated in the cryptophyte *Guillardia theta* and chlorarachniophyte *Bigelowiella natans* (Curtis et al. 2012), implicating their respective relict endosymbiont nucleus within the cell, i.e. the nucleomorph (Archibald 2007). All these findings are barely the tip of an iceberg in algal biology.

Recent phylogenomic studies clearly demonstrate the critical role of lateral genetic transfer and endosymbiosis in shaping genomes of algae and all other microbial eukaryotes. Although many of the implicated genes and/or pathways remain to be experimentally validated, findings from these studies provide a knowledgebase of interesting biological and ecophysiological aspects that one could hone in on, e.g. the development of multicellularity in algae (Cock et al. 2010).

3.4 Uncovering Hidden Biodiversity

Phylogenomic methods have recently been used to uncover hidden biodiversity and physiological stages of unculturable microbes. This approach plays to the strength of single-cell genomics (Lasken 2007; Woyke et al. 2009), which allows for capturing snapshots of genome from individual cells, and genomic variation within a population. In an analysis of three single-cell genomes of an unculturable marine "algal" species, picobiliphytes (Yoon et al. 2011), each genome content reveals distinct physiological stage for each cell: normal, actively feeding, and severely infected by a marine virus. These cells were collected from the same 50-mL seawater sample from a single location, suggesting that marine biodiversity is greater than what one would expect, and that it extends beyond the conventional scope at species level.

Interestingly, the authors described a complete absence of chloroplast- or photosynthesis-related genes across these genomes, suggesting that picobiliphytes, previously described as algae (Not et al. 2007), are more likely heterotrophs than photoautotroph. Therefore, the plastid nucleomorph observed in these cells could be a result of kleptoplasty whereby the plastid could have been "stolen" from another algal source (Trench 1969), or simply from an ingested cell, i.e. the plastid was within an algal cell that was engulfed by the picobiliphyte. In this case, phylogenomics using single-cell genome data has uncovered hidden marine biodiversity that would have been overlooked using the conventional genomic approaches based on cultured cells. Incorporating this approach into other means of capturing genome snapshots *in situ* (Bhattacharya et al. 2012), e.g. across multiple time points via experimental evolution (Sniegowski et al. 1997; Ebert 1998), allows for systematic assessment of diverse aspects of ecology and evolution for specific organisms/cells, as well as their interactions with one another and with the environments.

4 Limitations of Algal Phylogenomics

Given that most of algal genomes available to date are sequenced *de novo*, the quality of the genome assembly and annotation remains to be improved as more data become available. In addition to large genome sizes (e.g. for dinoflagellates) (LaJeunesse et al. 2005; Hackett and Bhattacharya 2008), yet-to-be identified

genome features could be a hurdle for data assembly in algal genome projects. Phylogenomics (and phylogenetics) is a working hypothesis, and one needs to be aware that such an approach yields only clues, not the absolute truth, about how genomes have evolved. Over- and under-interpreting phylogenomic results could yield biased, inaccurate conclusions.

The technical limitations of phylogenetic approaches have been reviewed extensively in the literature (Philippe et al. 2004, 2005, 2011; Stiller 2011). The quality of sequence data *vis-à-vis* stochastic sequence variation, convergence, long-branch attraction, incomplete sequence data (e.g. transcriptome, gene fragments) in addition to contaminations, represents the biggest hurdle in phylogenomics, and any sequence analysis. Perhaps the most relevant, longstanding issue in the study of algal phylogenomics is the biased or unbalanced taxon sampling, which has significant impact on any phylogenetic inferences and how the results are interpreted. For instance, the opposing views of the algal contribution to the evolution of diatoms *vis-à-vis* endosymbiosis (Moustafa et al. 2009; Burki et al. 2012a; Deschamps and Moreira 2012) have been largely attributed to the limited red algal gene repertoire. Certainly, the biases of taxon sampling will diminish as more genome data are becoming available. Given the vast diversity of algal species, however, to what extent will such biases of taxon sampling be tolerable remains an open question.

5 Future Perspectives and Conclusions

As the application of phylogenomics in algal research is becoming more common as more data are becoming available, a key question remains: are current state-of-the-art phylogenomic approaches sufficient, or should we spend more time in developing one that is better? In other words, where is the balance between extracting as much information as we can from the rapidly growing data using our current know-how, *versus* exploring approaches that would take us perhaps closer to the truth? This question has no easy answer.

Given the on-going deluge of sequencing data, the limitation of computational and human resources in data management, interpretation and analysis cannot be overstated. Where genome data is unavailable, the use of transcriptome data in phylogenomic analysis is not uncommon (Struck et al. 2011). However, assembled transcriptome data, e.g. mostly of expressed sequence tags, contain partial gene transcripts and could be biased by environmental conditions during which genetic materials were harvested. Multiple sequence alignment of these sequences alongside with other (putatively homologous) full-length sequences inevitably creates undesirable "gappy" aligned positions (i.e. phylogenetically non-informative sites) that would affect subsequent phylogenetic inferences, against the backdrop of genome rearrangement, genetic recombination, and lateral genetic transfer. An alternative strategy is to use the so-called *alignment-free* methods in calculating sequence distances (e.g. using *k*-mers) (Vinga and Almeida 2003; Höhl and Ragan 2007; Reinert et al. 2009; Chan and Ragan 2013; Bonham-Carter et al. 2014; Ragan et al. 2014),

which does not require contiguity of homologous sequences to be conserved. However, the application of these approaches and their scalability in phylogenomics remain to be systematically investigated (Posada 2013; Ragan and Chan 2013; Chan et al. 2014). Besides, alternative phylogenetic representations independent from the tree-like structure, e.g. the use of networks (Dagan 2011; Huson and Scornavacca 2011) also provide a fresh perspective into genome evolution.

Given current limitations in phylogenomics (as highlighted in the above section), one could argue that current approaches would yield biased inferences that would be of little use. One could always improve the phylogenetic framework, e.g. in "perfecting" sequence alignments (Edgar 2004; Sievers et al. 2011), identification of homologous groups (Li et al. 2001; Harlow et al. 2004) or phylogenetic algorithms (Neuwald 2009; Liu et al. 2012; Nelesen et al. 2012) to reduce inaccurate inferences. On the other end of the spectrum, providing more-efficient scalability, higher computing capacity, better implementations and sampling strategies among existing data, phylogenomic studies could yield valuable insights into algal biology and evolution. A common ground between the two schools of thoughts is crucial for the field to move forward.

Phylogenomics is a powerful tool for delineating organismal evolution from the genomic perspective, yielding novel, high-level biological hypotheses that would guide experimental designs for further genetic, biochemical or physiological studies at greater depth and in a more-refined focus. As algal research shifts towards a multidisciplinary framework, projects involving large international collaborative networks that combine expertise from various research areas are desirable, as demonstrated in a number of recent studies (Chan et al. 2012a; Price et al. 2012; Collén et al. 2013). With a positive outlook on the forthcoming algal genome data, phylogenomics remains a highly powerful and relevant tool in algal research, especially when we are now at the juncture that we can address interesting biological questions at scale that was unimaginable just a few years ago.

Acknowledgement The author is supported by a University of Queensland Early Career Researcher Grant.

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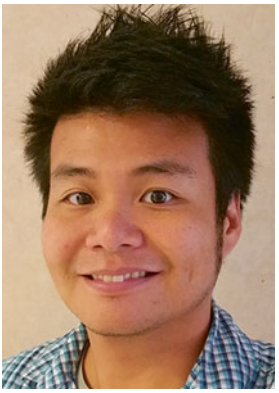

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Concepts and Techniques for the Study of Algae

Mani Arora and Dinabandhu Sahoo

1 Introduction

Algae can be studied in the field or in the laboratory (Fig. 1). A variety of conventional and modern techniques are available for isolation, characterization and identification of different species of algae. An alga is generally characterized on the basis of its size, colour, shape, form or growth habit. Some algae are visible to the naked eye whereas others can be seen with the help of a magnifying glass or a microscope. Algae are divided into two groups based on size: macroalgae and microalgae. Algae growing in the form of large, plant-like structures are called 'macroalgae' (e.g. Seaweeds and kelps). 'Microalgae' is a very broad term for all microscopic, photosynthetic protists. Microalgae that swim or drift within the well-lit regions of the water bodies are collectively termed "phytoplankton". Microalgae that inhabit the surfaces such as sea floor or river bottoms are called "benthic microalgae". The common unit for measuring microalgae is micrometer (μm). Phytoplankton can be differentiated on the basis of cell size into micro-phytoplankton (200–20 μm), nano-phytoplankton (20–2 μm) and pico-phytoplankton (2–0.2 μm). While the microphytoplankton and nanophytoplankton are easily identified with conventional techniques for instance light microscopy, the picophytoplankton can only be distinguished by fluorescence techniques.

Microscopes are essential tools for studying algae. Now, an incredible array of microscopes are available. Use of microscopes for the examination of algal cells requires the preparation of cells by appropriate methods for the particular type of

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D. Sahoo, J. Seckbach (eds.), *The Algae World*, Cellular Origin, Life in Extreme Habitats and Astrobiology 26, DOI 10.1007/978-94-017-7321-8_21

Fig. 1 Studying algae in the field (**a**, **b**) Magnifying glass, (**c**) Portable spectrometer, (**d**) AlgaeTorch, a field instrument used to measure chlorophyll directly in the water body, from live algae (**e**) FluoroProbe, a multiwavelength field fluorometer (**f**) FlowCytobot, an in-situ automated submersible imaging flow cytometer (**g**) Images of algae obtained through FlowCam, an imaging cytometer (**h**) CytoBuoy, a real time monitoring flow cytometer

microscopy. Compound light microscopes provide enough magnification to see the cells of microalgae and macroalgae, but not the ultrastructure or many of the structures inside those cells. For that, we need an electron microscope. In the past few decades, transmission electron microscopy has added an entirely new aspect to our knowledge of the algae and has helped in understanding the structure and functions of cells and organelles. Analysis of a statistically significant quantity of cells requires some level of automation to be brought to the characterization. This job is fulfilled by flow cytometry. Innovation in fluorescent probe technology has led to diversification of the technique, allowing development of additional methods

for analysing labelled cells which incorporate the feature of automation. Various instruments have been devised for the automation of cell counting which go beyond the limitations presented by microscopy.

Algae contain different combination of pigments such as chlorophylls, carotenoids and phycobiliproteins which make them appear colourful. Pigment analysis offers deep insights into the taxonomic composition, distribution and abundance of natural algal populations in oceans. In addition, the signature pigments present in specific algae can be utilized as "fingerprints" in the study of harmful algal blooms (HAB). The spectrophotometric and fluorometric methods are routinely used to study the pigment composition in various algal species. In vivo fluorescence measurements allow real-time monitoring of phytoplankton distribution. HPLC provides detailed information about the composition of phytoplankton communities based on diagnostic signature or marker pigments which have been selected as taxonomical pigments for different algal groups such as zeaxanthin (for cyanobacteria) and prasinoxanthin (for Prasinophyceae), fucoxanthin (for diatoms), and chlorophyll *b* (for green algae) (Stauber and Jeffrey 1988; Millie et al. 1993; Jeffrey and Vest 1997). High precision liquid chromatography (HPLC) has been used to discriminate and quantify pigments directly from water samples.

2 Commonly Used Detection Tools for the Quantitative and Qualitative Analysis of Algae

Various tools provide distinctly different information and are chosen according to the application. Cells can be subjected to analysis of morphological changes and taxonomic diversity using light microscopy. For over a century light microscopy was the basic tool to observe phytoplankton. It is still invaluable to determine species composition. Since the late 1960s, both transmission and scanning electron microscopes have proved extremely useful in establishing accurate algal taxonomy using ultrastructural features such as ornamentation of body scales or architecture of flagellar roots. Later, fluorescence techniques such as epifluorescence microscopy and flow cytometry played a key role in the discovery of picoplankton. Flow cytometry, initially borrowed from the biomedical field, has become the method of choice to estimate cell abundance in the field, since it permits the counting and classification of several thousand cells per minute. Fluorescence microscopy, epifluorescence microscopy and flow cytometry are generally used for the detection of picophytoplankton and for the documentation of viable cells after staining. Quantification of cells can be easily achieved by flow cytometry, but ample species discrimination is difficult to obtain. Automated microscope based image cytometry can be used to analyse cells over time, a technique which is not possible using flow cytometry. Important criteria to consider for the selection of instrument include application, accuracy and speed of analysis, light sources etc.

2.1 Light Microscopy

Light microscopy is used to study most algae. It is the fundamental tool employed in an algae laboratory. In light microscope, visible light passes directly through the lenses and specimen. The simplest type of light microscope consists of only one glass lens, a magnifying glass (Fig. 1a, b). Microscope composed of more than one glass lens is called a compound microscope. Compound microscope includes condenser lens, objective lens and eye piece lens. Condenser lens focuses light from the light source onto the specimen. The objective lens is responsible for producing the magnifying image. Objective lens is commonly available in 4×, 10×, 20×, 40×, 60× and 100× magnifications. Light microscopes are available in two orientations: upright microscope and inverted microscope (Fig. 2a, b). Upright microscope is suitable for viewing microscope slides whereas inverted microscope is appropriate for examining culture vessels such as multi-well plates and petri dishes. Using an inverted microscope cells can be isolated straight from the well plates. It is also used in efficiently monitoring the enrichment cultures and single-cell isolates in the multi-well plates.

The type of microscope and observation method may differ depending on the specimen to be observed and the purpose of microscopic observations.

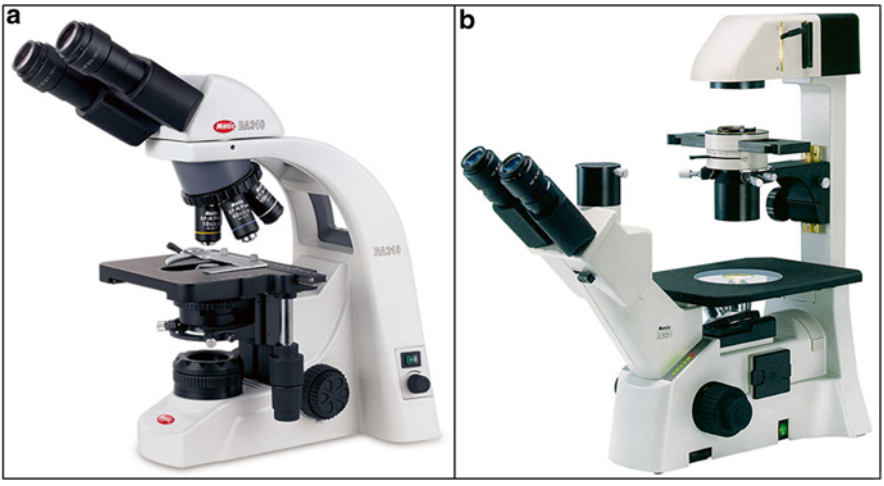

Fig. 2 Light microscope (**a**) Upright, (**b**) Inverted

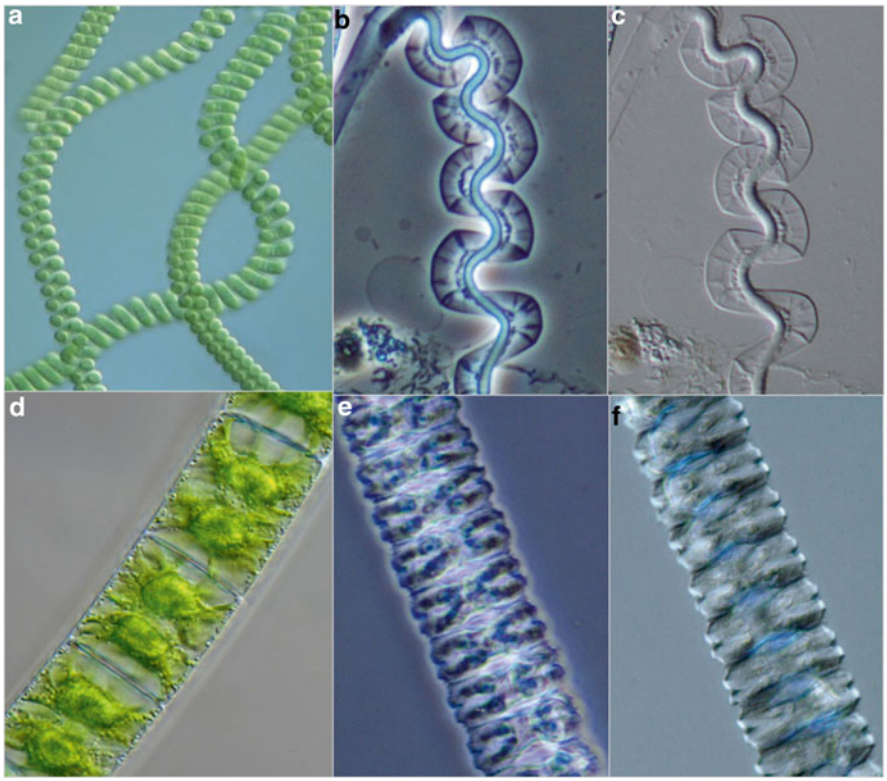

Fig. 3 (**a**–**c**) *Spirulina* and (**d**-**f**) *Zygnema* as observed by bright field, phase contrast and DIC microscopy (Image courtesy: Michael W. Davidson, The Florida State University)

2.1.1 Bright Field (BF) Microscopy

It is the most common observation method to observe an unstained or stained algal specimen. In a bright field microscope, the entire field of observation is illuminated and appears bright. The algal specimen is visible in the light path because of its natural pigmentation or stains that absorb light differentially (Fig. 3a, d).

2.1.2 Phase Contrast Microscopy

This method is appropriate for viewing live cells and colourless, transparent specimens. It exploits the difference between the phase of light passing through a relatively thinner region of the specimen and the phase of light passing through a denser or thicker region of the specimen. Hence the phase of light wave changes according to the refractive index of various regions of the specimen. The specimen appears as varying levels of brightness and contrast. The microscope is

built-in with a phase-contrast objective lens and a condenser lens. In positive phase contrast, the background light is phase shifted by +90° and hence specimens appear dark against a bright background. In negative phase contrast, the background light is phase shifted by −90° and hence specimens appear bright against a dark background. The contours of images are delimited by a distinctively bright diffraction halo (Fig. 3b, e).

2.1.3 Differential Interference Contrast (DIC) Microscopy

Differential interference contrast microscopy also known as Nomarski microscopy is used to enhance the contrast in transparent unstained specimens. For DIC observations, the microscope is equipped with a DIC prism or Nomarski prism and a polarizing plate. The image obtained using DIC is almost similar to that obtained through phase contrast microscopy but the edges of image appear shadowed, giving the image a three-dimensional appearance (Fig. 3c, f). DIC utilizes the phase difference in areas of the specimen when light passes through it to add contrast to images of transparent specimens.

Specimen preparation for light microscopy: Specimens to be observed with the light microscope are broadly divided into two categories: whole mounts and sections.

(i) Whole mount

A wealth of structural detail can be seen in wholemounts of living unicellular and filamentous algae (Fig. 4). Such preparations allow observations over time so that developmental phenomena can be followed. Phase contrast and DIC have been most useful for the observation of living cells. Sample preparation involve mounting the specimen in a mounting medium (water, tissue culture medium or glycerol) on a glass slide and covering it with a glass coverslip. The slide is then positioned on the specimen stage of the microscope and examined through the ocular lens, or camera. To take maximum advantage of the resolving power of the light microscope, specimens are usually prepared in a way designed to enhance contrast (difference in the darkness or colour of the structures being examined). A regular means of enhancing contrast is to apply particular dyes that colour or otherwise adjust the light transmitting properties of cell constituents.

(ii) Sections

Anatomical examination of macroalgal specimens are based on a very thin cross section either using a razor blade or a device called microtome.

Hand sections: Hand sections of macroalgae are useful in the study of cell walls, extracellular matrices and other anatomical details. Hand sectioning requires sharp and flexible razor blades. Sections are kept in sea water or freshwater for about a minute and then mounted in glycerine or examined directly. Sections can be stained with aniline blue or methylene blue to give good morphological description. Sections can also be stained histochemically using Toluidine blue, Alcian blue,

Fig. 4 Whole mounts of *Polysiphonia* (red algae) showing marine diatoms attached to it (Picture courtesy: Mr. Charles Krebs)

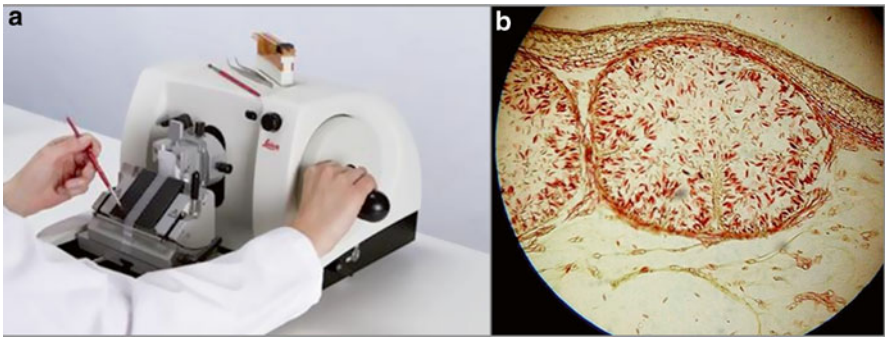

Fig. 5 (**a**) Microtome, (**b**) Section through a male conceptacle of *Fucus*, showing many antheridia borne on branched hairs (Picture courtesy: (**b**) Dr. Christopher Skilbeck)

Alcian yellow or PAS procedure (involving the use of Schiff's reagent) to identify the chemical components of cellular and subcellular structures. Algae are usually sensitive to dehydration hence a small drop of glycerine (mounting medium) is placed on the section. The sections are carefully covered with a cover glass. These glycerine mounts are sealed by applying clear nail polish to the edges of the cover glass.

Microtome sections: A microtome is a tool used to cut extremely thin sections (Fig. 5a, b). Specimen needs to be processed to avoid cell damage before sections

are prepared. The processing involves a sequence of steps: fixation, dehydration, embedding and the consequent sectioning using a microtome.

Fixation and dehydration: Fixation preserves the cells in their original state. Specimens are treated with a solution containing chemicals that penetrate the cell membrane and cross-link most nucleic acids, proteins and lipids. This cross-linking maintains the structural integrity of the cell. The most widely used fixatives are acids and aldehydes, for example, acetic acid, picric acid, formaldehyde and glutaraldehyde. Formaldehyde is the most commonly used primary fixative for light microscopy which cross-links amino groups on adjacent molecules, resulting in the formation of DNA- protein cross-links (DPCs). Other common fixative is glutaraldehyde which readily cross-links proteins. After fixation, the specimen is dehydrated through a series of alcohols or acetones.

Embedding: The specimen is then embedded in an embedding medium of liquefied epoxy resins, methacrylates or paraffin sand allowed to harden at appropriate temperature. Embedding media surround the specimen and infiltrates into it, harden upon cooling, hence provide mechanical support to the specimen both internally as well as externally during sectioning. The resulting blocks containing the specimen provide a stable base for fixing in the microtome and are ready for sectioning.

Sectioning: The block is mounted on the microtome holder. Microtome arm progresses the specimen towards the knife. The sections are cut with the knife and are picked up by a corner using fine forceps. The microtome knives are generally made up of polished steel or glass for light microscopy. The sections are then transferred to small drops of distilled water on a glass slide.

Before staining, sections are immersed in toluene or xylene, which removes the paraffin and wax, leaving the thin section attached to the slide. Sections embedded in epoxy resins and methacrylates (plastics) can be stained without the removal of the plastics.

Staining: Dewaxed sections are then stained with any of the morphological or histochemical stains, antibodies or enzymes showing affinity for a particular kind of cellular component. Most commonly used morphological stains are aniline blue or methylene blue. A few specific stains for histochemical studies are Bromophenol blue, Fast green, Acid fuchsin, Feulgen stain, Toluidine blue, Alcian blue, Alcian yellow or PAS procedure (involving the use of Schiff's reagent). A glass coverslip is mounted over the section using an appropriate mounting medium.

2.2 Fluorescence Microscopy

Fluorescence microscopy is a rapidly expanding technique in the field of phycology (Fig. 6). Fluorescent stains or fluorochromes absorb light at one particular wavelength and fluoresce (emit light) at a longer wavelength. In fluorescence microscopes, only fluorescent light emitted by the fluorochrome is utilized to form an image. Fluorescence microscopy techniques are most commonly used for seeing structures of cells, for observing physiological and biochemical events in the live algal cells, and for the enumeration of live/dead cells.

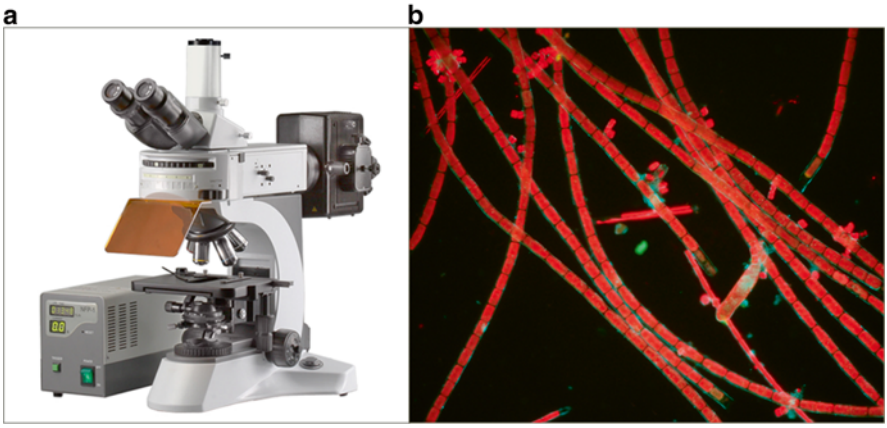

Fig. 6 (**a**) Fluorescence microscope, (**b**) Fluorescence micrograph of a filamentous alga (Picture Courtesy: (**b**) David W. Walker, Micscape)

2.2.1 Widefield Epifluorescence Microscopy

It is most commonly used to locate or detect specific proteins or other molecules in cells. In this technique the whole sample is simultaneously illuminated using a light source, usually a mercury lamp. Excitation filters are used to select the excitation wavelength. Excitation light is focused on the sample using an objective lens. The fluorescence emitted by the sample is directed to the same objective.

All algae contain chlorophyll *a* and exhibit fluorescence. Blue excitation causes chlorophyll *a* to emit or fluoresce red, green excitation causes phycoerythrin to fluoresce bright orange-red and phycocyanin to fluoresce orange yellow. An inverted microscope including epifluorescence illumination is a useful tool to detect and isolate cysts of many bloom forming algae (e.g., raphidophytes, diatoms, and dinoflagellates) from sediments. Cysts provide a fluorescent signal because of their cell wall and chlorophyll. Isolated cyst can be used for the cultivation of a particular alga.

Automated microscope based digital image cytometry, is where a fluorescence microscope is integrated with systems for image acquisition and analysis, and can be used to collect measurements of fluorescence per cell over time. In this type of microscopy, slow scan cameras are combined with a microscope interface and software for digital image acquisition and analysis. This type of microscopy is useful in single cell quantitative analysis. It is also useful in understanding the sources of variation in cell fluorescence which mainly arises due to differences in cell cycle position, variations in gene expression, the metabolic status of cells, and micro environmental differences.

2.2.2 Confocal Laser Scanning Microscopy (CLSM)

CLSM is a computerised microscope which involves the coupling of a laser light source to a light microscope and results in the generation of 3-D digital images of the microorganism (Fig. 7). Confocal means having the same focus. Confocal

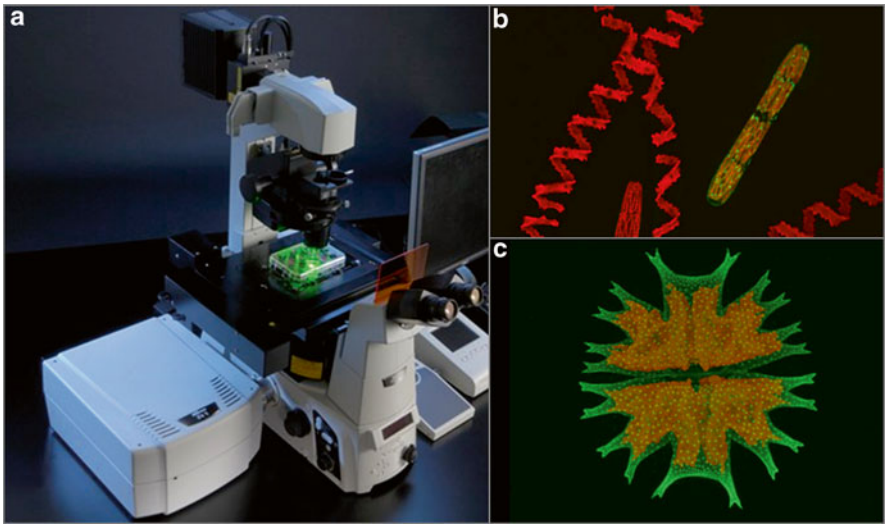

Fig. 7 (**a**) Confocal Microscope, (**b**) Confocal micrographs of *Penium margaritaceum* and *Spirogyra* algae. (**c**) *Micrasterias* (Image credits: (**b**) A. Andreas (**c**) J. Ochs'14)

microscopy solves one major problem, out-of-focus blur associated with regular fluorescence microscopy. Normally when a sample is illuminated by excitation light in the fluorescence microscope, full thickness of the specimen produces fluorescent signal at the same time. This adds to a background haze which does not permit most of the image to be in focus. Confocal microscopy is used to increase contrast and optical resolution of an image by filtering out the out-of-focus light from above and below the point of focus in the specimen. The confocal microscope filters out the out-of-focus light by means of a confocal pinhole situated in front of the image plane which acts as a spatial filter and allows only the in-focus portion of the light to be imaged. Light from above and below the plane of focus is eliminated from the final image. The ability of confocal microscope to produce sharp images of focal planes deep within a specimen by removing the involvement of out-of-focus light without any physical sectioning of the tissue, is termed optical sectioning. This method has revolutionized the ability to gather images from thick specimens. The confocal microscope uses a laser beam to illuminate a fluorescently stained or auto-fluorescent specimen.

Confocal microscopy combined with the use of fluorescent probes has been used in almost all cell based studies in phytoplankton. This technique has been used for assessing the ecotoxicity of chlorine and an oxidizing biocide on a marine diatom, *Cocconeis scutellum* Ehrenb (Nanchariah et al. 2007). It has also proven useful for the analysis of the three dimensional distribution of total and active cells in solid structures such as biofilms. In a confocal microscope, the illumination and light collection optics are configured to minimize the contributions from out-of-focus regions, providing a high resolution image from a very thin slice of specimen. This

resolution is further improved in multiphoton confocal microscopy, in which the fluorescence is excited by the nearly simultaneous observation of two or more photons of lower energy than would normally be needed for excitation (Shapiro 2003).

2.2.3 Inwater and Holographic Microscopy

In water microscopes are appropriate for real time imaging of microalgae in all aquatic environments (Fig. 8). These microscopes use a laser and camera to image, count, size and characterize objects in any body of water within the sample area. Resolution extends to objects as small as 0.5 μm and as large as 3 mm. Holography is a technique in which live three-dimensional and four dimensional holograms are

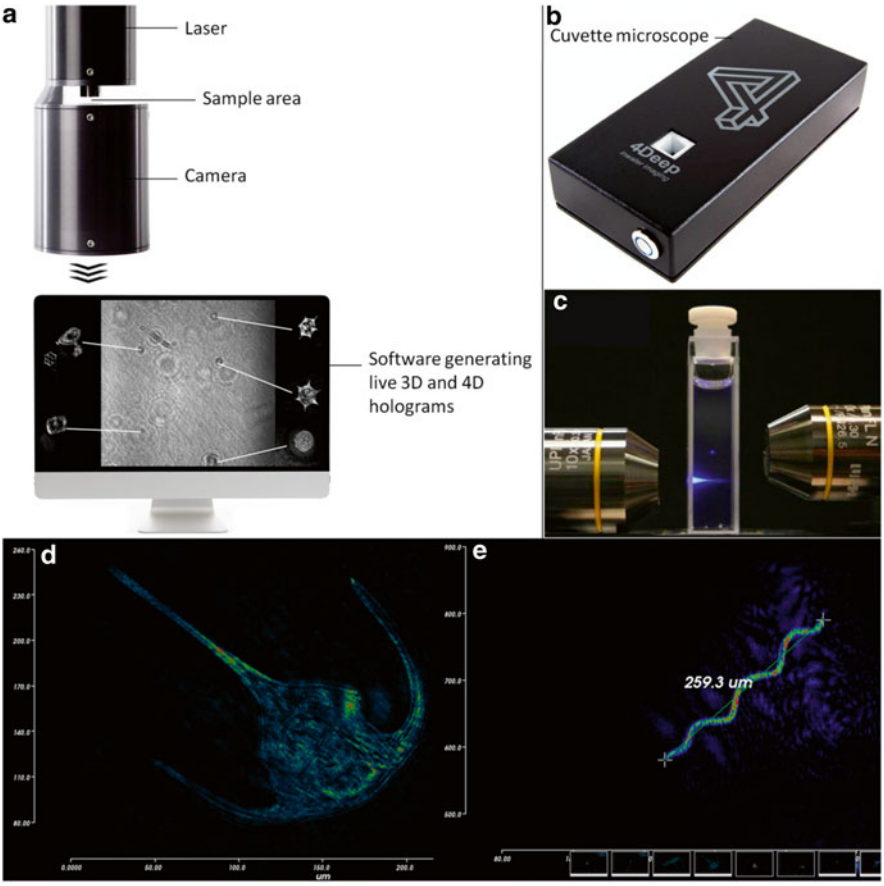

Fig. 8 In water microscopes: (**a**) Submersible microscope, (**b**, **c**) Cuvette microscope, (**d**, **e**) *Ceratium* (a diatom) and *Spirulina* as observed through submersible microscope (Image courtesy: 4Deep Inwater Imaging)

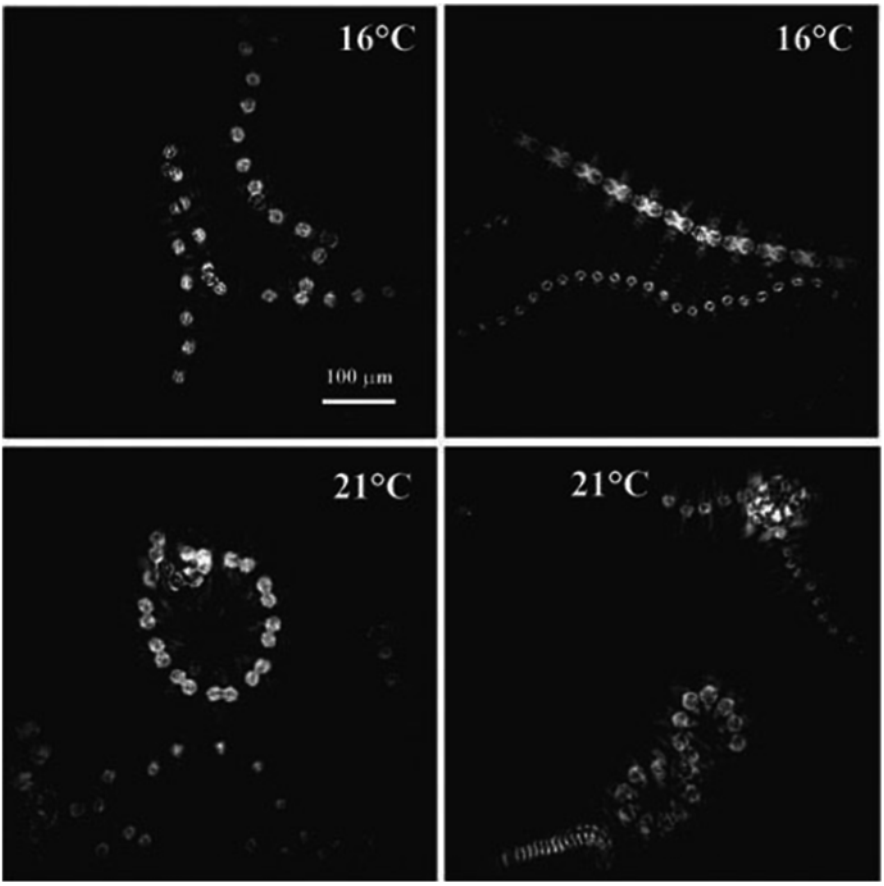

Fig. 9 Reconstructed images from holograms showing the effect of temperature on the motion of algae (*Alexandrium*). Circular motion at the elevated temperature (*bottom* pictures) is due to loss of rear flagella (Image courtesy: Prof. Hans Juergen Kreuzer, Department of Physics and Atmospheric Science, Dalhousie University, Halifax)

generated. It involves the use of a laser, interference, diffraction, light intensity recording and suitable illumination of the recording. The image changes as the position and orientation of the viewing system changes in exactly the same way as if the object were still present, thus making the image appear three-dimensional (Fig. 9).

2.3 Electron Microscopy

Electron microscopes use a focused stream of highly energetic electrons to see an object. This examination gives information regarding surface topography, morphology, ultrastructure and crystallographic structure (arrangement of atoms in an

object). The Electron microscopes have higher resolution and therefore can achieve higher magnification of up to two million times. This increase in resolution is possible since the electron beams have shorter wavelengths than that of the wavelengths of light. The drawback of the electron microscope is that the live cells cannot be observed.

Electron microscopes use electromagnetic lenses, instead of optical lenses to focus a beam of electrons instead of visible light to image the specimen. The fundamental steps implicated in all Electron microscopes are the following: A stream of electrons is produced by electron guns under high vacuum. This stream is focused into a monochromatic beam with the help of metal apertures and magnetic lenses. The accelerated electron beam impinges upon the sample and a variety of electron-specimen interactions take place. These interactions make electron microscopy possible. The products of electron-specimen interactions are detected and can be transformed into an image. There are different types of electron microscopy: Scanning electron microscopy, Transmission electron microscopy, Scanning tunneling microscopy, Atomic force electron microscopy, Immunoelectron microscopy.

2.3.1 Scanning Electron Microscopy

Scanning electron microscope (Fig. 10) permits the researcher to observe the external features of intact microorganisms. It gives information about the topography of a specimen surface. SEM can provide a three dimensional topographical information about the specimen surface (Fig. 11).

In scanning electron microscopy, primary electron beam moves over the surface of the specimen coated with a thin film of heavy metal such as gold or palladium.

Fig. 10 Scanning electron microscope

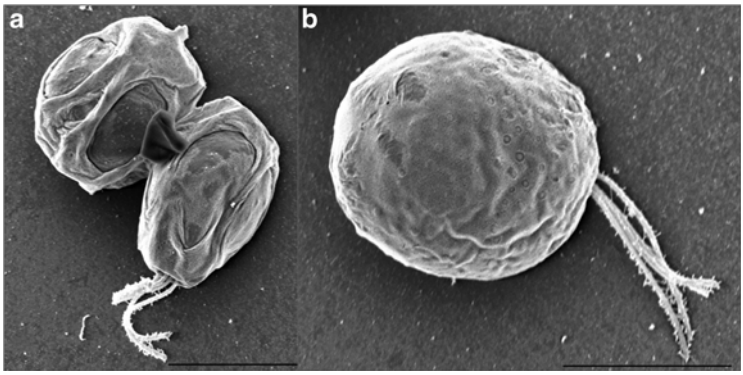

Fig. 11 Scanning electron micrograph of a green alga, *Tetraselmis indica*

This causes the emission of secondary electrons from the surface of the specimen. Detectors collect the secondary electrons and convert them into an electronic signal. These signals are then processed to generate an image on a cathode ray tube (CRT) screen.

Sample preparation for SEM

(i) Fixation and dehydration
Fixation preserves the algae in their original state by crosslinking nucleic acids, proteins and lipids. Specimens are treated with a chemical fixative such as glutaraldehyde and osmium tetroxide. The sample is usually immersed in 2–5 % glutaraldehyde in a buffer (phosphate buffer, cacodylate buffer or HEPES buffer) that maintains physiological pH. The sample is incubated overnight at 4 °C. The specimen is then rinsed a few times in their particular buffer. Following fixation, the specimen is dehydrated through a graded series of ethanol. The concentrations of ethanol usually start at 25 % and proceed at 25 %, 50–70 % followed by 85 %, 95–100 % ethanol. Usually three final changes in 100 % ethanol (EM grade) are used.

(ii) Specimen Drying
Specimens go through a procedure known as "critical point drying," in which all of the ethanol in the specimen is replaced with liquid carbon dioxide under pressure. This process removes moisture from the specimen without altering its features. A critical point drier (CPD) is used for this purpose. It is an automated process that takes around 40 min to complete. Once the dried specimen is taken out, it needs to be kept in a desiccated environment.

(iii) Specimen Mounting
Specimens are mounted on circular metallic stubs with the help of adhesives. An adhesive should be electrically conductive. One may use double-sided sticky carbon tape, copper tape, colloidal silver paste or colloidal graphite.

(iv) Sputter coating
In conventional Scanning electron microscopy specimen is sputter-coated with a metal such as gold or gold/palladium alloy prior to examination in the microscope. Sputter coating is a sputter deposition process which involves erosion of atoms from a target and their subsequent deposition onto the specimen. Sputter coating is performed using an automated sputter coater. Sputter coating is required to prevent the charging of specimen with electron beam and it also increases signal to noise ratio as heavy metals are good emitters of secondary electrons.

2.3.2 Transmission Electron Microscopy

Algal cells have membrane enclosed organelles and a rigid cell wall composed of polysaccharides. Cellular structures such as organelles, which allow the cell to function properly within its specified environment, can be examined at the ultrastructural level using Transmission electron microscope (TEM) (Fig. 12a). Ultrastructure has often been a reliable means of classifying microalgae. Transmission Electron microscopy provides detailed images of intracellular structures at a very high resolution and magnification (Fig. 13d).

Special techniques of thin sectioning are required to prepare a specimen for TEM, as electron beams have a poor penetrating power. In Transmission Electron Microscope (TEM), a focused beam of electrons is transmitted through a thin section of the specimen (around 60 nm). The electron beam then goes through the specimen. Depending on the specimen density, some of the electrons get scattered and disappear from the electron beam. When the electron beam emerges from the specimen, it carries information on the structure of the specimen. The unscattered electrons are magnified by a series of magnetic lenses and are recorded by hitting a

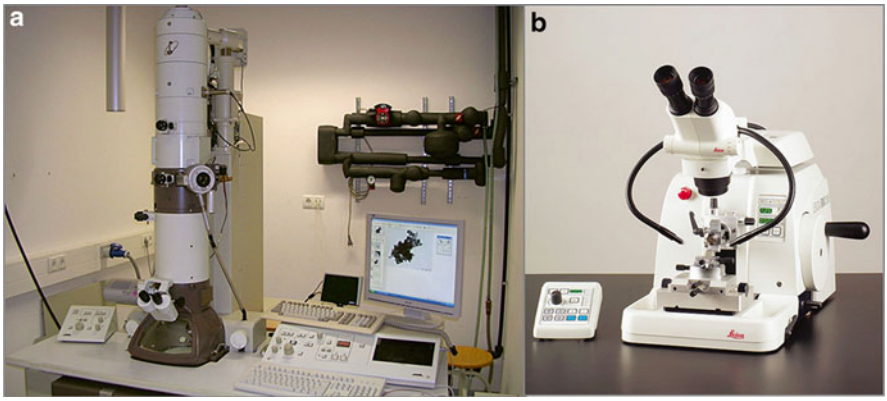

Fig. 12 (**a**) Transmission Electron Microscope, (**b**) An ultramicrotome

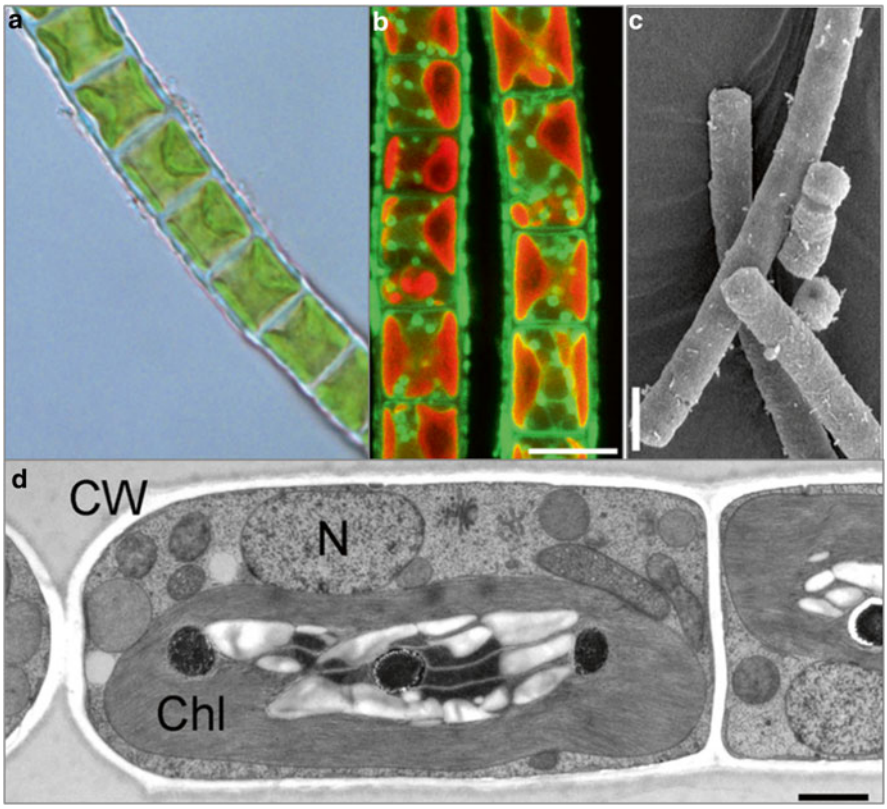

Fig. 13 Comparison of morphology and ultrastructure of an alga *Klebsormidium* as observed by (**a**) Light microscopy, (**b**) Confocal laser scanning microscopy, (**c**) Scanning electron microscopy, (**d**) Transmission electron microscopy (Image courtesy: Prof. Andreas Holzinger, University of Innsbruck, Austria)

CCD (charge-coupled device) camera or fluorescent screen, which produces an image of the specimen with varied regions of darkness depending on the density. To attain adequate contrast, the specimens are treated with compounds of heavy metals such as osmium, uranium, lanthanum, lead or gold (in immunogold labeling). These stains scatter electrons adequately and hence improve contrast.

Sample preparation in TEM

(i) Fixation and dehydration
Specimens are usually treated with 2–5 % glutaraldehyde in cacodylate buffer for primary fixation. The sample is incubated overnight at 4 °C. The specimen is then rinsed a few times in cacodylate buffer. Following primary fixation, the specimen is treated with 1 % osmium tetroxide in cacodylate buffer for secondary fixation. Glutaraldehyde cross links proteins during primary fixation, and lipids tend to be cross linked by osmium tetroxide during secondary

fixation. After fixation, samples are stained with 2 % aqueous uranyl acetate or phosphotungstic acid. The sample then undergoes dehydration through a graded series of ethanol, starting from 50 % followed by 50, 70, 85, 95 to 100 % ethanol. Usually three final changes in acetonitrile are used.

(ii) Infiltration
Infiltration is the replacement of the dehydrating fluid or transition solvent with plastic resin. In the case of Spurr's resin, changes of 1/2 resin (50/50 acetonitrile/spurr's resin), 1/3 resin, 2/3 resin and 100 % resin are used in progression. The purpose of infiltration is basically the complete penetration or infiltration of resin into the specimen. Slow rotation facilitates the penetration of the resin into the specimen.

(iii) Embedding
After infiltration, sample is embedded so that it can be sectioned. Embedding involves placing the sample in liquid plastic formulations or spurr's resin followed by its subsequent polymerization by heat or UV light. Special plastic BEEM capsules or gelatin capsules can be used as molds in embedding. After the polymerization (hardening) of plastic, the sample is sectioned into ultrathin sections and stained.

(iv) Sectioning
Sections are cut with a diamond knife mounted on an ultramicrotome (Fig. 12b). The sections are stretched with chloroform to eliminate compression, and mounted on pioloform filmed copper grids. Sections are stained with uranyl acetate and lead citrate. The grids are examined using a Transmission electron microscope (TEM) and digital images are collected using a CCD camera.

Advances in microscopes and microscopic techniques continue to be introduced to study cells, molecules, and even atoms. Among these are the scanning tunneling microscope, atomic force microscope and immunoelectron microscopy. These are particularly significant for studies of microorganisms at the molecular level.

2.3.3 Scanning Tunneling Microscope

The scanning tunneling microscope (STM) is used for studying the structure of an electrically conductive sample surface at atomic resolution (Fig. 14). STM can image individual atoms on a surface. STM integrates scanning capacity into vacuum tunneling capability. STM works by scanning a conducting tip over the surface to be examined, at a constant spacing. A voltage difference (bias) is applied between the tip and sample surface, which allows the electrons to tunnel through the vacuum between them. Information is obtained by monitoring the resulting tunneling current as the tip's position scan across the sample surface, and is displayed in the form of a STM image. Scanning tunneling microscopy has been used to acquire images at molecular resolution for the kappa- and iota-carrageenan algal polysaccharides, and to investigate the three-dimensional structure of C-phycocyanin (C-PC) isolated from blue-green alga *Spirulina platensis*.

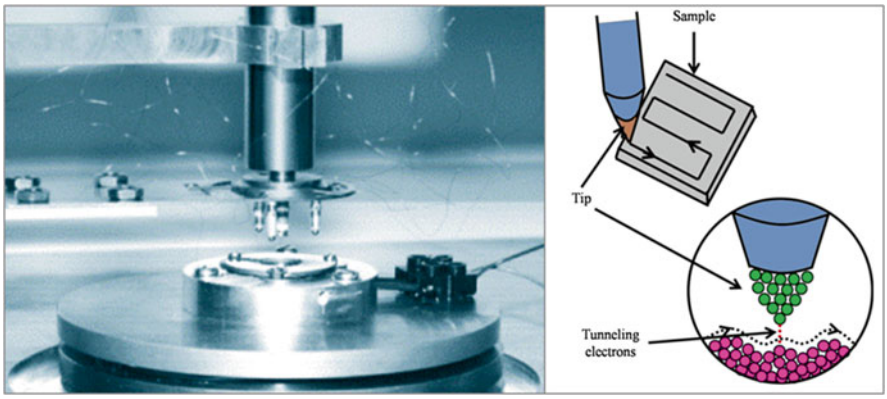

Fig. 14 Scanning tunneling microscope

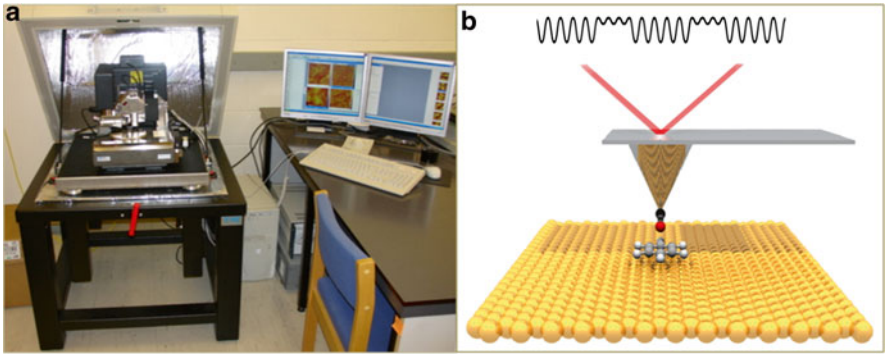

Fig. 15 (**a**) Atomic force microscope (**b**) The tip of atomic force microscope senses the changes in electronic forces as it moves across the sample surface at a constant height. Consequential movements of the AFM stylus are perceived by a laser beam to form images (Picture courtesy: (**a**) Stan Zurek, CC-BY-SA-3.0, Wikimedia Commons)

Atomic force microscopy (AFM) or scanning force microscopy (SFM) was developed as a variant of Scanning Tunneling Microscopy (STM), which makes it possible to study nonconductive or insulating samples (Fig. 15). Atomic force microscopy (AFM) allows the topographic structure of cells to be resolved under physiological conditions, without any fixation and dehydration artifacts. AFM is one of the leading tools for imaging sample at nanoscale (Fig. 16). Light and electron microscopes can produce two dimensional (X-Y) images of a sample surface, with a magnification ~1000× for an optical microscope and ~100,000× for an electron microscope. Nevertheless, these microscopes cannot determine the vertical dimension (z-direction) of the sample, e.g. the height (of particles) or depth (of holes, pits) of the surface topographic features. AFM, which exploits a sharp tip to

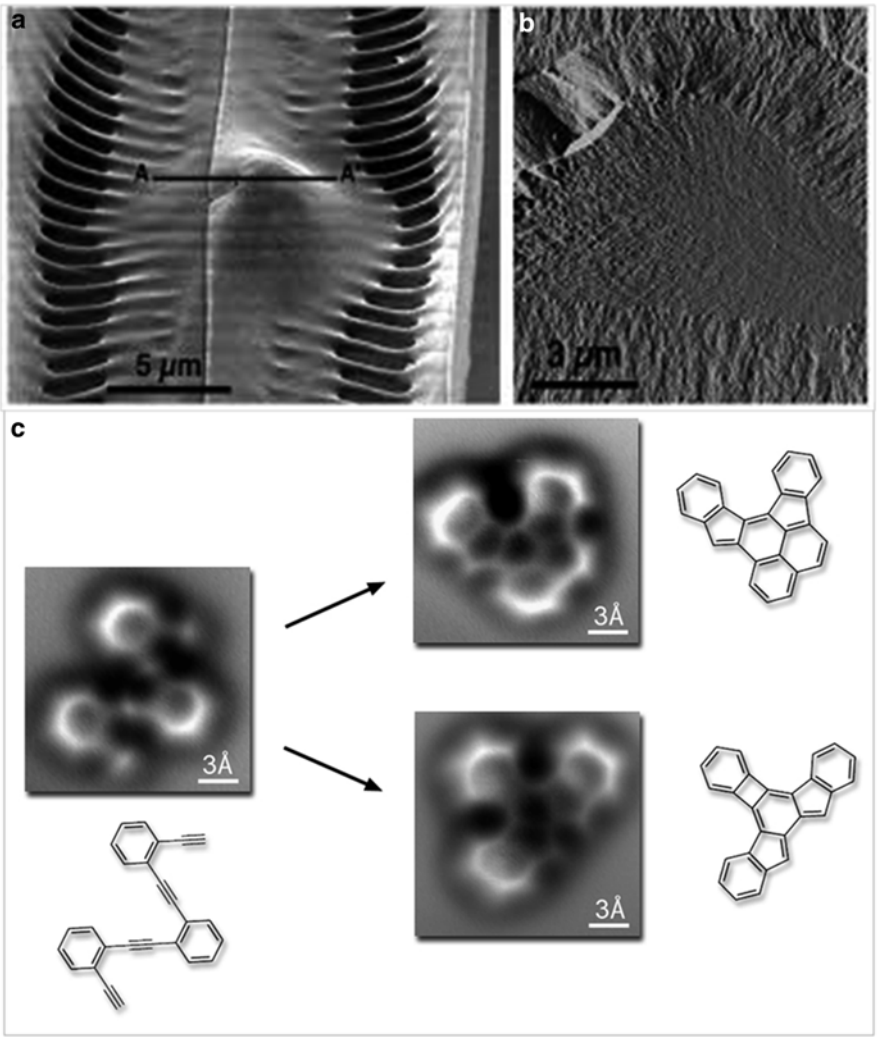

Fig. 16 (**a**, **b**) Comparison of a SEM micrograph and an atomic force microscope image of the diatom *Pinnularia viridis* valve, (**c**) The original reactant molecule imaged through AFM both before and after the reaction. The two most common final products of the reaction are shown (Image courtesy: (**a**, **b**) Dr. Simon A. Crawford, The University of Melbourne, Australia. (**c**) Lawrence Berkeley National Laboratory)

probe the surface features by raster scanning, can image the surface topography with extremely high magnifications, up to 1,000,000×. AFM measurements are made in three dimensions, X-Y, the horizontal plane and Z dimension, the vertical plane. Resolution at Z-direction is usually higher than X-Y. AFM has been used for the imaging of algal cells and for the characterization of the mechanical properties of glycoproteins that have potential utility as adhesives.

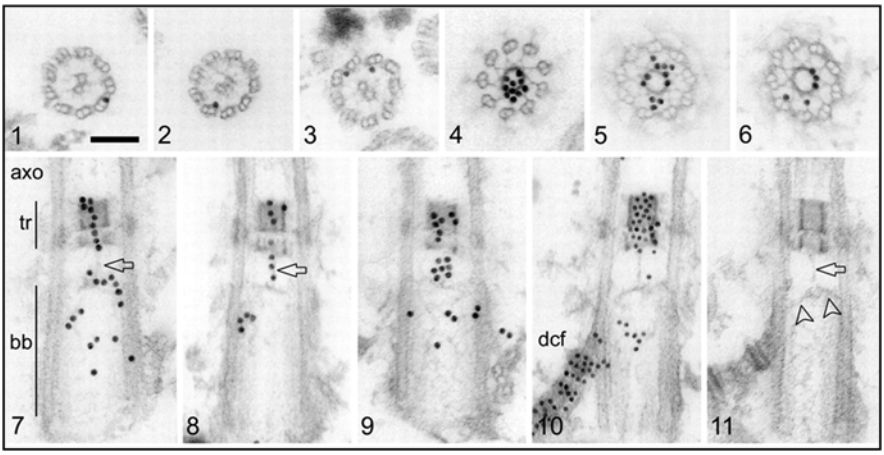

Fig. 17 Immunoelectron micrograph showing labeling of centrin using 15-nm gold particles in the cytoskeletons of *Chlamydomonas reinhardtii* (Image Courtesy: Dr. Stephan Geimer)

2.3.4 Immunoelectron Microscopy

This technique employs the use of antibodies to identify the intracellular position of particular proteins through electron microscopy (Fig. 17). Ultra thin sections of the specimen are labeled with antibodies (produced against the required antigen) conjugated with gold particles. The gold particles make the antibody markers electron-dense. Thus, immunoelectron microscopy appears as a technique that associates biochemistry and molecular biology with ultrastructural studies, by putting macromolecular functions into a cellular perspective. For immunoelectron microscopy cells are fixed and embedded in plastic. The plastic blocks are sectioned into ultrathin sections. The ultrathin serial (sequential) sections are labeled with a monospecific antibody raised against the protein of interest, and subsequently with protein-A gold, creating the antigen–antibody complex which is visible in the electron microscope. The sections are then imaged in the electron microscope.

2.4 Flow Cytometry

Cytometry is the measurement of physical and chemical characteristics of cells or other biological particles (e.g., nuclei or individual chromosomes). Flow cytometry involves the measurement of cells or particles in a flow system or fluid stream as they pass through a laser beam. Flow cytometry achieves multiparametric analysis of cells or other microscopic objects at high speed. Parameters are various physical or chemical characteristics of a cell (e.g. cytoplasmic granularity, cell size, nuclear DNA content, light scattering or fluorescence). Algal cells are inherently fluorescent

Fig. 18 Flow cytometer

but usually different fluorescent chemicals are used to label specific components of a cell which are subsequently excited by a laser to emit light at particular wavelengths. Flow cytometry integrates fluidics, optics, electronics and computational components (Fig. 18).

2.4.1 Basic components of a flow cytometer

(i) Fluidics System Fluidics aligns and carries the cells to laser interrogation point and takes away the waste. It utilizes hydrodynamic focusing to produce a stable particle stream within which cells or particles are aligned in a single file, to facilitate the proper analysis and sorting of cells and particles.

(ii) Optical System In flow cytometry, particles travel one at a time through a laser beam interrogation point. Optical components allow the microscopic particles to be illuminated by one or more lasers (Fig. 19a, b). As a particle passes through the focused laser beam it gives out optical signals or analogue signals (scattered light and fluorescence signals). Optical components resolve and route the optical signals to their individual detectors (photomultiplier tubes) after passing them through suitable filters.

(iii) Electronics Detectors or photomultiplier tubes (PMTs) convert optical signal into electronic signals (voltage pulses). These electronic signals are then converted to channel numbers (a digital value) by Analog-to-Digital Converter (ADC), so that they can be stored for display and analysis on a computer screen. Electronics are involved in signal acquisition, signal processing, computer display and analysis. For flow cytometers equipped with a sorting command (Fig. 20), electronics are involved in the formation, charging and deflection of individual droplets and to perform sorting of objects.

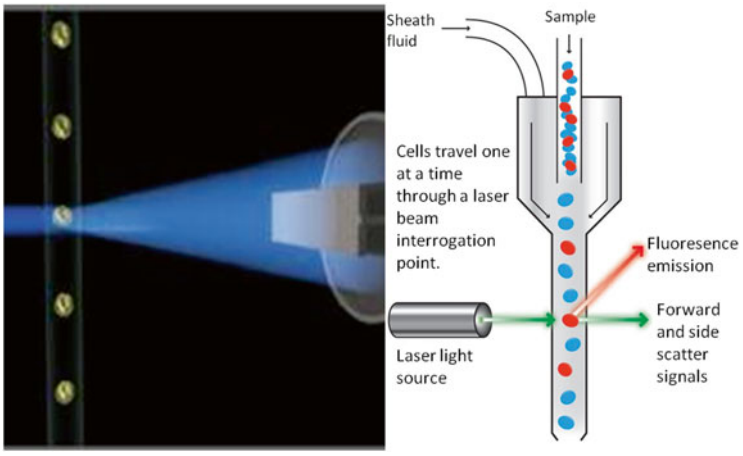

Fig. 19 The fluid stream is illuminated by a laser beam and the fluorescence and light signals scattered by each cell are detected by detectors after passing through filters

(iv) Computational Components Data collection process from samples using the FCM is termed 'acquisition'. Acquisition is performed through a computer coupled to the flow cytometer, and the software which regulates the digital interface with the flow cytometer. Acquired data comprises of individual measurements on thousands of signals, each corresponding to a particle flowing through the flow system. This data is stored according to the flow cytometry standard (FCS) format. Computational components take part in storage, display and analysis of data. A data storage file can be studied to obtain information on diverse cellular properties. These properties include the relative cell size, cell surface properties, granularity (internal complexity), autofluorescence intensity and relative fluorescence intensity of the object. Several commercial softwares for analysis of cell cycle and DNA content etc. are also available.

2.4.2 Flow Cytometry with Activated Cell Sorting (FACS)

Flow cytometry with activated cell sorting (FACS) is a method that allows separation or sorting of an object of interest from a heterogenous population (Fig. 20). Sorting of objects is achieved by electrostatic deflection. In this process a slight vibration is applied on the nozzle to produce small waves on the stream of cells, causing it to break into individual droplets after passing through the laser intersection. An electric charge is placed on the droplets having the object of interest. These droplets are then deflected or diverted by an electric field and collected into tubes based upon their charge, whereas the remaining uncharged droplets go into a waste stream.

Flow cytometry allows rapid automated characterization and classification of phytoplankton assemblages by measuring fluorescence characteristics (emission and light scatter) of individual cells at high speed (Phinney and Cucci 1989). The use of flow cytometry is very promising in the study of cell viability since it is a very

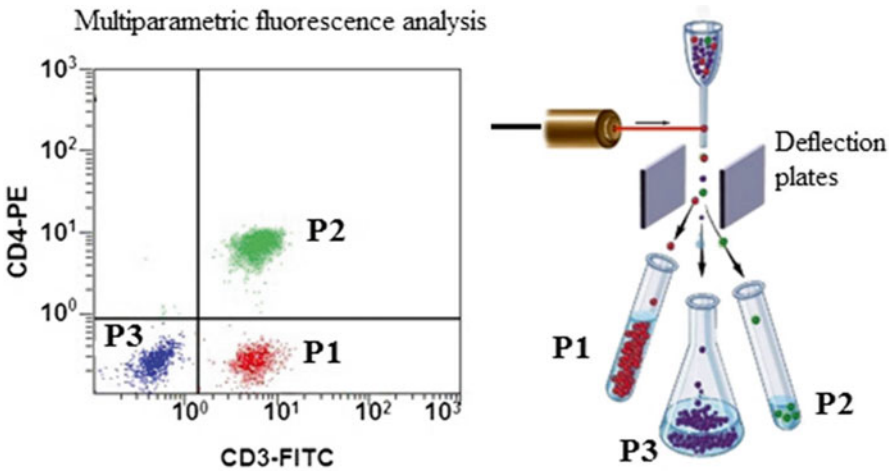

Fig. 20 Flow cytometry with activated cell sorting (FACS)

sensitive method for the quantitative examination of many cellular functions including membrane potential and enzyme activity. Using a flow cytometer it is possible to analyse and quantify a large sample of cells, thereby giving more statistically significant results. It also allows multi-colour fluorescence detection, increased sophistication and speedier data analysis. A flow cytometer can also evaluate the heterogeneity of a cell population due to the different levels of autofluorescence or stain intensity in single cells. Recently, flow cytometers have also been applied to determine dinoflagellate cyst viability in ballast water studies, which was traditionally assessed by microscopic examination of cyst germination over a period of at least 4 weeks (Binet and Stauber 2006). The results obtained using flow cytometry showed a high test precision and were in excellent agreement with the standard germination method.

Conventional flow cytometry analyzes single cells at a high rate but cannot analyse larger cells or chains, and to cover the full-size range of phytoplankton (~1 to several 100 μm) new instruments have been designed. These systems employ the use of an imaging flow cytometer to count, image and analyse the organisms as they pass through the instrument. This new generation of flow cytometers includes FlowCytoBot, which is coupled to an image in-flow system that covers the size range of 10 to ~100 μm (Fig. 1f). The cytometer, CytoBuoy, (Fig. 1h) has been used for the enumeration of cells per diatom chain and the results correlated well, and were much more precise and time efficient, than microscopic quantification (Takabayashi et al. 2006). Another instrument named FlowCAM (Fluid Imaging Technologies; Sieracki et al. 1998), is a portable plankton image analyser which combines the capabilities of both flow and a high resolution digital image cytometer (Fig. 1g). This instrument is of great interest since the analysis time when using FlowCAM is much less than that of microscopic enumeration and it does not require the sample to be fixed.

3 Isolation and Separation of Microalgae

Isolation is usually performed to obtain a pure strain of microalgae or to save a cross contaminated culture of algae. Algal cultures are usually unialgal (containing only one type of alga) clonal populations (a group of genetically identical cells). These cultures may be non axenic (have bacteria, fungi, or protozoa), or axenic (no bacteria, fungi or protozoa). There are five major techniques for achieving unialgal clonal isolates: streaking, spraying, serial dilution, single-cell isolations through capillary pipette and sorting with flow cytometry.

3.1 Streaking

This method is used for the isolation of small species of algae (<10 mm) that grow well on a substrate. Petriplates containing solidified agar growth medium are prepared, and the mixed phytoplankton sample is streaked with the help of a flame sterilized wire loop (Fig. 21). Plates are then covered, sealed and incubated under appropriate light and temperature conditions. Algal colonies are selected and removed from the plate with the help of a sterilized wire loop. These colonies are observed under the microscope to check that the colony is unialgal and the desired algal species has been isolated. Selected colonies are then transferred to liquid or agar growth medium.

3.2 Spraying

In this technique, a fine spray of cells is used for the inoculation of agar plates. Cells in a liquid suspension are sprayed using atomized sterile air so that they get dispersed onto the plate. These plates are incubated under appropriate conditions. Once the colonies have formed, cells are selected, removed and inoculated further.

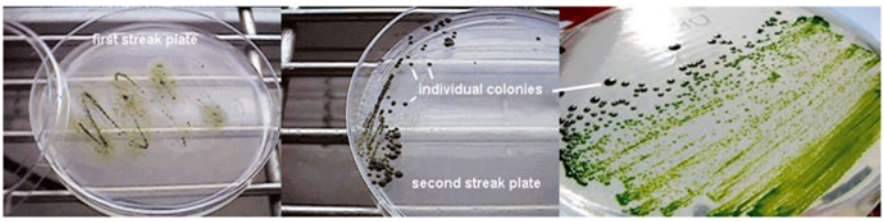

Fig. 21 Isolation of microalgae using streaking

Streaking and spraying techniques are useful for single-celled, colonial or filamentous algae that can easily grow on agar surface. Cultures of phytoflagellates such as *Cryptomonas* and *Chlamydomonas* can also be attained by these methods. Many algae are isolated by single-cell isolations or serial-dilution techniques. The most extensively used technique of single cell isolation is capillary pipette removal.

3.3 Micromanipulation or Capillary Pipette Removal

Capillary pipette removal or micropipette isolation is the most common method for single-cell isolation (Fig. 22). It is generally performed with a glass capillary or a Pasteur pipette. A capillary tube is heated in a fine flame, drawn out or extended, and then broken. The narrow end of the capillary should be approximately twofold the diameter of the algal cell to be isolated. Algal cell to be isolated is located in the drop of enrichment sample using an inverted microscope. While viewing, the cell is sucked up into the micropipette. The cell is then transferred to a drop of sterile medium on agar plate or glass slide.

3.4 Serial Dilution

A serial dilution is a step wise dilution technique which is useful for the organisms that are abundant in water sample. The dilution factor on each and every step is kept constant. This dilution technique is widely used to isolate random algal species

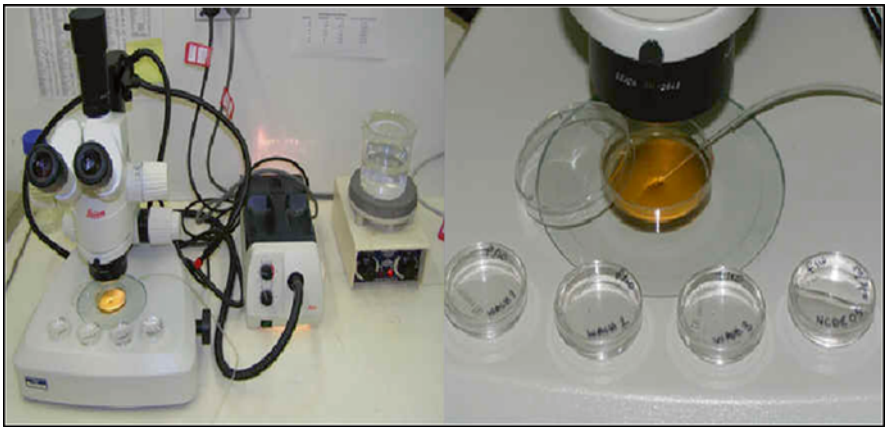

Fig. 22 Capillary pipette removal

present in field samples and consequently new algal species can be discovered. The dilution can be performed with distilled water, culture medium, seawater or filtered water from the field sample.

To perform a tenfold dilution (1 mL scale), 900 μL of diluting media is added into 100 μL of the algal sample by thorough mixing subsequent to each dilution step. Test tubes containing diluted samples are incubated under appropriate temperature and light conditions. Cultures are observed microscopically after 1–2 weeks. Concentrations having higher dilution e.g. 10^{-6} to 10^{-10} are expected to contain unialgal isolates. If the tubes have two or three different algal species, then capillary pipette removal may be used to achieve unialgal cultures.

3.5 Automated Single Cell Isolation

Flow cytometry with activated cell sorting (FACS) is an automated single cell isolation technique that is extensively used for cell sorting. This technique has been effectively used for sorting microalgae from water containing different strains of algae. Sorting is principally based on chlorophyll autofluorescence (CAF) and green autofluorescence (GAF) to discriminate algae such as phytoflagellates, dinoflagellates, diatoms or prokaryotic phytoplankton. FACS is a proficient and sensible tool for the isolation of microalgae from a field sample. Samples are acquired by FACS and several 2-D plots record the distribution of cells in forward scatter or FSC (measures the size of the cell), side scatter or SSC (measures the granularity of the cell) and in all fluorescence channels. Usually the sorting traits are achieved using a dot plot (Fig. 23) which combines inner cell complexity or granularity (SSC) and the chlorophyll autofluorescence of cells.

4 Growth Measurements of Microalgae

Under favorable conditions, microalgae grow constantly by cell division and yield an exponentially growing culture. Growth of microalgae slows down because of the depletion of nutrients and decreased light penetration as the culture becomes more crowded. After reaching their stationary phase for the existing conditions, the density of the cultures will not increase further.

Calculating the growth rates of cells are an important part of microalgal studies. Growth of microalgae can be measured in terms of cell counts in a known volume of water. Counting microalgae requires the most regular lab equipment-a microscope.

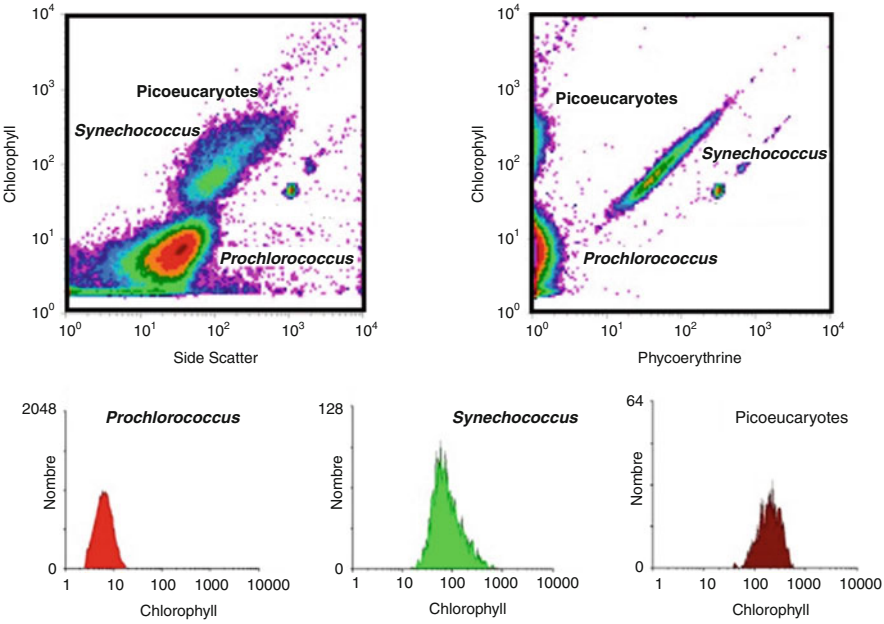

Fig. 23 Flow cytometry *dot plots* (from a sample of photosynthetic picoplankton) and activated cell sorting (FACS) (Image courtesy: Daniel Vaulot, CNRS, Station Biologique de Roscoff, CC-BY-SA 2.5, Wikimedia Commons)

It is performed with "counting chambers". Counting necessitates some knowledge of the algal taxonomy. Algal counts in mixed assemblages (i.e., in field samples or during competition experiments) are performed using Sedgewick-Rafter chamber, Palmer-Maloney Slide or inverted microscope method. Measuring cell density and counting algae in unialgal samples (i.e., during growth and bioassay experiments) requires the use of hemocytometer, coulter counter or flow cytometer. Microalgae contain pigments and increase in their number leads to an increase in the intensity of the colour of the culture which can be easily quantified using a spectrophotometer or fluorometer. Optical density and chlorophyll *a* measurement are generally used for quantification of algal biomass over a growth cycle.

Growth curves are prepared using data obtained from any of the above mentioned methods. Cultures are sampled at definite time intervals, depending on the algal growth rate. The growth curve is plotted using the number of cells or biomass against time (in days). From these curves, specific growth rate and division time can be calculated.

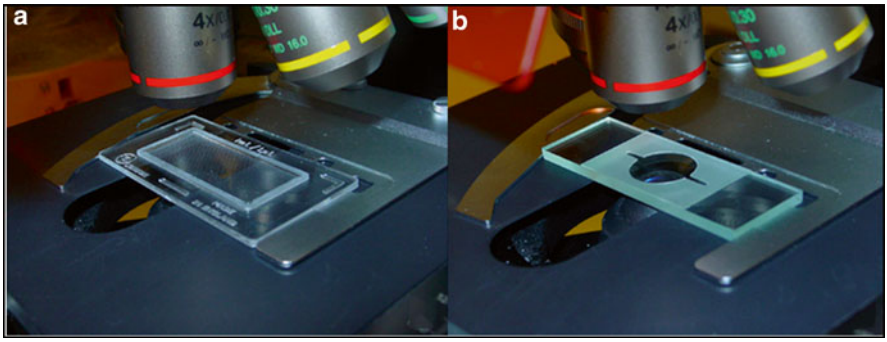

Fig. 24 (**a**) Sedgewick Rafter Chamber, (**b**) Palmer-Malony Cell (Picture courtesy: Agriculture, Fisheries and Conservation Department, HKSAR (Hong Kong Special Administrative Region))

4.1 Counting Cells in Mixed Assemblages

4.1.1 Sedgewick-Rafter Chamber Method

The Sedgewick Rafter counting Chamber is specifically used for the quantitative estimation of cells in a defined volume of fluid (Fig. 24a). The dimensions of this chamber are 50×20×1 mm. The chamber is made up of a grid of 100×1 mm squares. The chamber has an area of 1000 mm^2 and traps a volume of 1.0 ml when a cover glass is placed over it. The chamber is filled with the well mixed sample using a micropipette. A coverslip is properly positioned on the chamber. The sample is allowed to stand for at least 15 min to allow algae to settle to the bottom. Counts are performed using the lower power objectives (4× or 10×) of the compound microscope.

4.1.2 The Palmer-Maloney Slide

The Palmer-Maloney Slide comprises a glass slide onto which is epoxied a circular chamber, of volume 0.1 mL and has two narrow loading channels opposite to each other (Fig. 24b). This chamber is applicable for the enumeration of large algal cells (up to 150 mm) as well as very small nanoplanktons. However, for large algae present at low concentration, it may be appropriate to use a chamber that can hold a larger volume of sample (i.e., Sedgewick-Rafter Chamber).

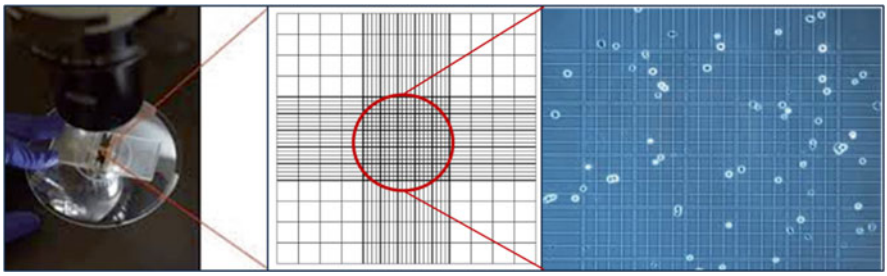

Fig. 25 A hemocytometer with its counting grids

4.1.3 Inverted Microscope Method

Inverted microscope is very advantageous for counting algae in mixed assemblages. In this microscope depth of the counting chamber does not prevent the usage of high power objective lenses. In the inverted microscope method, a known volume of algal sample is added to the chamber and allowed to stand for at least an hour to allow the algae to settle to the bottom. Utermőhl devised a standard settling and enumeration technique using inverted microscope and his own sedimentation chambers. The technique involves the gravitational sedimentation of a known volume of preserved algal samples into an Utermőhl chamber. Subsequently, counts are made on at least 20 random fields and a minimum of 200 cells are counted.

4.2 Counting Unialgal Samples

4.2.1 Hemocytometer Method

In case of unialgal samples, a chamber such as hemocytometer is commonly used to estimate the culture density. The modern-day hemocytometer has a double-chamber layout and counting grids developed by O. Neubauer (Fig. 25). This configuration permits the user to do two cell counts for every sample without cleaning the hemocytometer. The two counting areas or chambers are etched up with grids. Each chamber is composed of nine squares, each square 1×1 mm ($1\ mm^2$). The chamber is 0.1 mm deep. Hence the total area of each chamber is $9\ mm^2$ and the total volume is $0.9\ mm^3$. These nine squares are further subdivided into small areas. The squares at the four corners are subdivided into 16 intermediate squares, whereas the central square is subdivided into 25 intermediate squares. Generally the total number of cells in the central large square is counted. To obtain the total number of cells in this

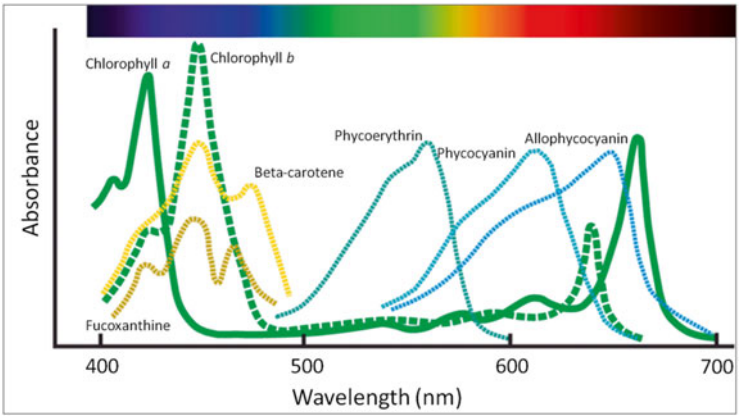

Fig. 26 Absorption spectra for various algal pigments

large square, the number of cells in each of the 25 intermediate squares are counted, recorded then added. The volume of central large square is $1 \times 1 \times 0.1$ mm $= 0.1$ mm^3. Since 1000 mm^3 (0.1 mm$^3 \times 10^4$) $= 1$ ml, multiplication of average cell counts in the central large square by 10^4 will give the cell density per ml. Hence 10^4 is the chamber conversion factor (for improved Neubauer standard hemocytometer).

4.2.2 Electronic Particle Counter (e.g. Coulter Counter) and Flow Cytometer

A Coulter counter is a device for counting particles suspended in electrolytes. In this method an algal suspension is placed inside an electronic particle counter, within which algae are passed through a tiny orifice. This orifice connects the two compartments of the counter which contain an electrically conductive solution. As each alga passes through the orifice, the electrical resistance between the two compartments increases. This generates an electrical signal which is automatically counted. In addition, flow cytometry can also be used to count unialgal samples.

5 Techniques for the Study of Algal Pigments

The major pigments in algae comprise chlorophylls, carotenoids and phycobiliproteins. These pigment molecules absorb light only in the wavelength range of 400–700 nm, this range is referred as photosynthetically active radiation (PAR). Each of these pigments has evolved to absorb only certain wavelengths of visible light and reflect the wavelengths they cannot absorb. Each type of pigment can be identified by the specific pattern of wavelengths it absorbs from visible light, which is the absorption spectrum (Fig. 26). Chlorophyll absorbs light in blue and red region.

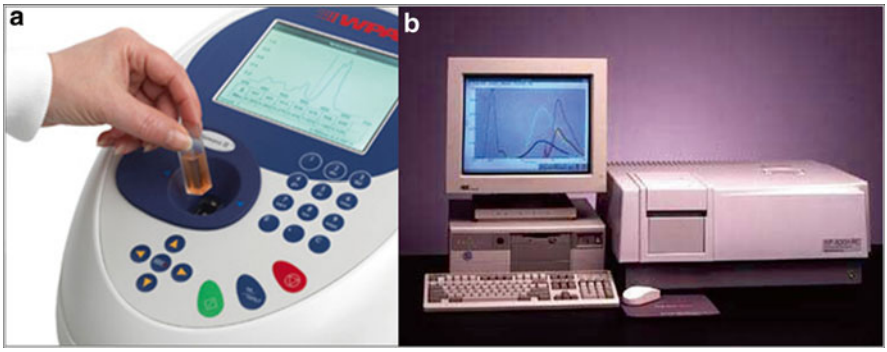

Fig. 27 (**a**) Spectrophotometer, (**b**) Fluorometer

Chlorophyll does not absorb green light; it reflects green light, hence appears green. Carotenoids absorb light energy in the blue-green and violet region. They reflect yellow, red, and orange light, hence appear yellow red or orange to us.

5.1 Spectrophotometric Determination

The types of pigments present in an alga can be determined by using a spectrophotometer (Fig. 27a). A spectrophotometer can differentiate the wavelengths of light a pigment can absorb. Spectrophotometer measures the intensity of transmitted light to determine the absorbance of the sample at that particular wavelength. By extracting pigments from algae and placing these samples into a spectrophotometer, we can identify which wavelengths of light an alga can absorb. Chlorophyll *a* is the major photosynthetic pigment of algae. It has been commonly used as an indicator of biomass or primary productivity. The spectrophotometric methods are routinely used to study the pigment composition in various algal species. However, the spectrophotometric method is not very sensitive and needs a large sample volume. In addition, an appropriate empirical formula for chlorophyll must be chosen (Jeffrey and Humphery 1975; Porra et al. 1989).

5.2 Fluorometric Method

Fluorometry is the measurement of fluorescence. A fluorometer excites the solution and can measure the emission. No two pigments can have the same fluorescence signature (excitation and emission spectra). This principle makes fluorometry an extremely specific analytical technique. The fluorometric method (Fig. 27b) is extensively used for the quantitative analysis of chlorophyll *a* and phaeopigments (non-photosynthetic pigments which are the degradation product of algal

chlorophyll pigments). Phaeopigments are usually formed during and after the formation of marine phytoplankton blooms). In vivo fluorescence measurements allow real-time monitoring of phytoplankton distribution (Fig. 1c, d).

High precision liquid chromatography (HPLC) can be used to discriminate and quantify pigments directly from water samples. HPLC provides detailed information about the composition of phytoplankton communities based on indicative carotenoids (i.e., peridinin for Dinophytes and alloxanthin for Cryptophytes) and some other marker pigments. It is worthwhile to consider the most appropriate technique. At present HPLC is the method of choice for the analysis of marker pigments and for achieving accurate measurements of Chl *a* and a variety of accessory pigments in the extracts of algae.

5.3 High-Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography (HPLC) is a technique used to separate, identify and quantify the algal pigments in a mixture. High performance liquid chromatography (HPLC) pigment method provides accurate chlorophyll *a* data along with extensive information about the composition of algal communities (Mantoura and Llewellyn 1983). This method is based on the assumption that different algal classes include specific marker pigments. For instance, fucoxanthin is the marker pigment for bacillariophyta (diatoms), zeaxanthin for cyanobacteria (blue-green algae), and chlorophyll *b* for chlorophyta (green algae) (Stauber and Jeffrey 1988; Millie et al. 1993; Jeffrey and Vest 1997).

HPLC apparatus consists of high pressure solvent pumps, sample injector, chromatography column, detectors and a data recording and processing unit (computer) (Fig. 28a, b). In this technique solvent pumps are used to pass a highly pressurized solvent (mobile phase) containing the sample through a specially designed column filled with a solid adsorbent material (stationary phase). All components in the sample interact somewhat differently with the adsorbent material, resulting into different flow rates for various components. This leads to the separation of different components as they get eluted from the column. The time taken for a specific compound to pass through the system (starting from the column inlet up to the detector) is known as its retention time. The detector provides the output to a computer equipped with data acquisition software where the data is recorded and analyzed (Fig. 28c). The resulting chromatogram is used for the identification and quantification of a particular compound (Fig. 28d). The presence or absence of certain pigments helps in differentiating the major algal groups present in natural waters (Bidigare et al. 2005). Therefore HPLC has proved to be a powerful tool to study the processes that have an effect on phytoplankton pigment pool.

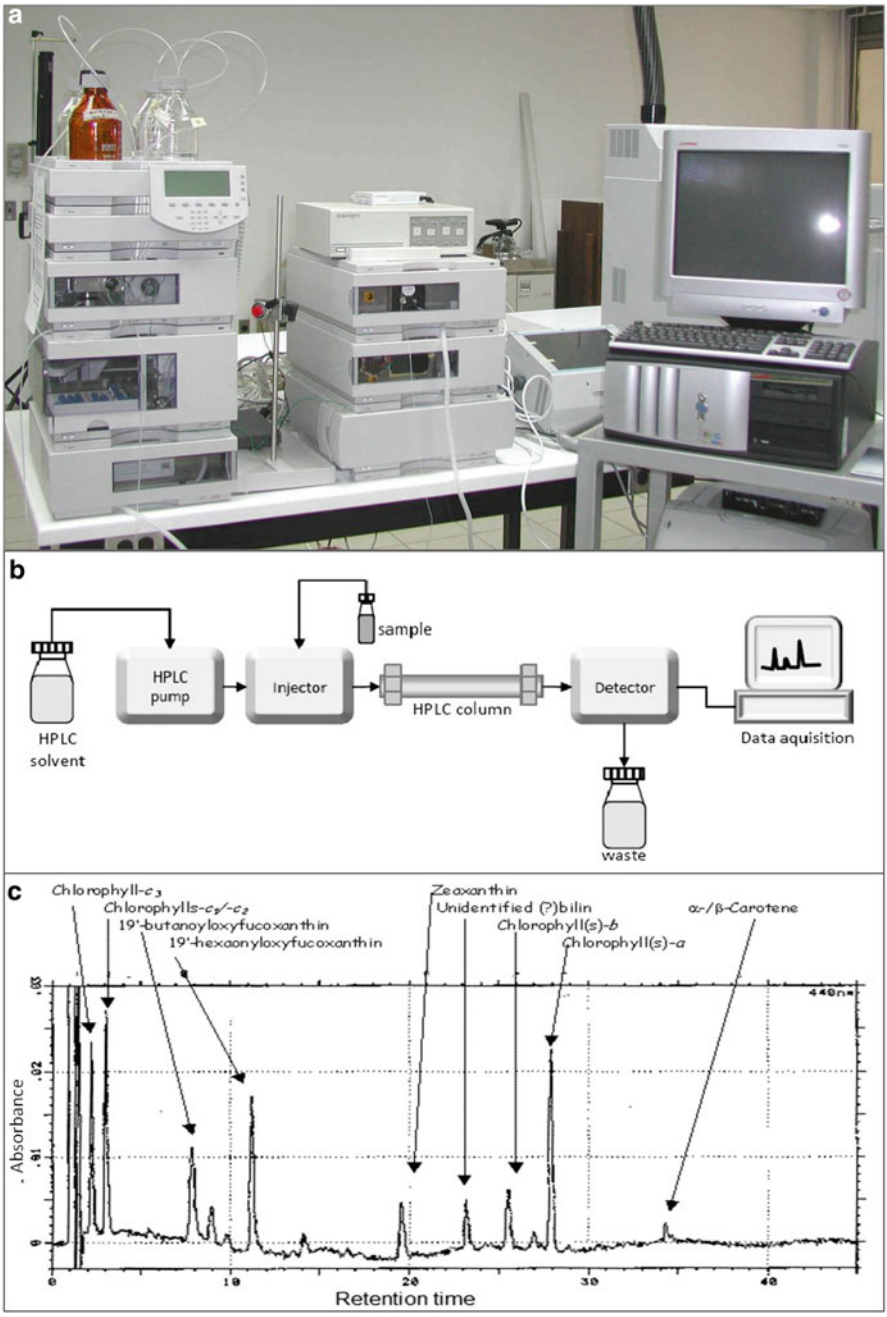

Fig. 28 Pigment analysis using high-performance liquid chromatography (HPLC)

Acknowledgement The authors are grateful to Dr. Ravidas Naik for his valuable suggestions towards the preparation of this manuscript.

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Culturing Algae

Salam Sonia Devi and Dinabandhu Sahoo

1 Introduction

The artificial medium where the alga grows is defined as culture. Artificial culture medium is supplemented with various chemicals so that it resembles with the natural environment. The isolation of axenic culture of algae is the first and foremost step in any aquaculture process. A proper management and precautions is required in each and every step of unialgal cultures so that axenic culture can be preserved for longer period of time. It is crucial to maintain the algal reservoir free of various contaminants and bacteria. Various culture media were developed for various algae as the habitat of algae vary from one species to another. Algal culture media are divided into two broad classes viz Fresh water culture media and marine culture media. The samples which are collected are usually not pure. Therefore various purification steps should be followed so that large impurities and unwanted materials are removed. The generalized scheme of possible isolation and purification methods for microalgae are outlined below (Guillard and Morton 2003).

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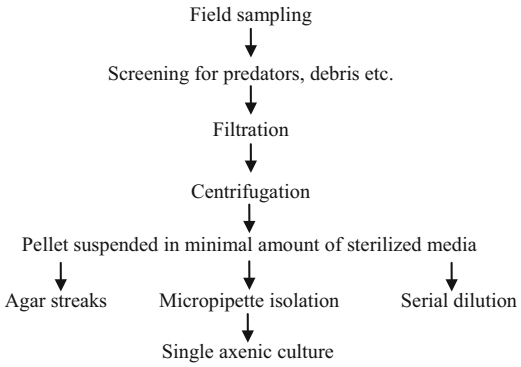
D. Sahoo, J. Seckbach (eds.), *The Algae World*, Cellular Origin, Life in Extreme Habitats and Astrobiology 26, DOI 10.1007/978-94-017-7321-8_22



2 Sterilization and Sterile Technique

Before the start of any culture whether fresh water algae or marine algae sterilization of all the glasswares are very much essential. Sterilization is usually done to develop an aseptic culture environment by killing the microorganisms. This process is one of the most important step in any culture work so that pure culture can be maintained (Masanobu and Mary Héléne 2005). There are various types of sterilization methods such as heat sterilization, electromagnetic, chemical sterilization etc. Out of which autoclaving which is a method of heat sterilization is commonly practiced for algal cultures. All the glasswares, pipettes, plasticwares, utensils and medium (both solid and liquid) should be autoclaved before used. Autoclave is a specialized closed chamber where high steam pressure is maintained. Autoclaving is usually done at 15 lb/in. at 121 °C for 15 min (Chen and Yue 2005). Autoclaving kills all the microbes giving infection free product.

Following precautions should be taken into consideration before culturing algae.

- Laminar flow should be turned on before the start of the culture work.
- The working surface should be cleaned with 70 % ethanol.
- The Bunsen burner should be used.
- After organizing all requirements under the laminar flow, hands should be cleaned with 70 % ethanol.
- All the sterile pipette, loop or any material which are going to be used in culture should be flamed and it should be cooled before used.
- All the rim of the test tubes, petriplates should be flamed and cooled down before use.
- The loop or pipette should be cleaned after every use.

3 Preparation of Stock Solutions

Media are generally composed of three components macronutrients, trace elements and vitamins. All the three components are termed as stock solutions. Stock solutions for each and every component are made separately. The preparation of stock solutions is important because it reduces the number of repetitive chemical measurements involved in media preparation. Moreover direct weighing of media components that are required only in milligram or microgram (micronutrients or vitamins) in final formulation cannot be measured accurately. Therefore concentrated stock solutions for such components are prepared and are subsequently diluted to the final media concentration. In addition, concentrated solutions of some chemicals are more stable and can be stored for longer periods than the dilute solutions. Stock solutions are generally prepared in quantities of 100 mL to 1 liter depending on the amount of uses in final medium.

To prepare stock solutions clean flasks of various sizes are taken. Desire amount of chemicals are weighed and dissolved it in the flask containing required volume of distilled or deionised water. The flask is stirred continuously till the component is completely dissolved. If more than one component has to be added in a flask in case of trace metal solution then make sure that the first component is completely dissolved before adding the second one and so on. Some chemicals are easily dissolved while stirring but those chemicals which are not dissolved easily should be given a warm heat. Once the chemicals are fully dissolved the final volume of the stock solutions are made up to the desired volume with distilled or deionized water. The sock solutions are stored at 4 °C in a tightly sealed glass bottle or plastic bottle so that the final concentration of the solution does not change.

The sock solution for macronutrient is always prepared separately for each chemical. But for trace metal some chemicals are added together in one solution. Some of the common media used for microalgae cultures is given below.

3.1 Medium for Bold Basal (Bold 1949; Bischoff and Bold 1963)

Bold Basal medium (BBM) are used for the culture of fresh water algae. This medium can be prepared using the following components given in Table 1 in distilled water. Ten milliliters each from macronutrients, 1 mL each from EDTA, Iron, Boron and Trace Metal Solution are taken and made the volume up to 1 liter by distilled water. The pH of the culture is maintained at 6.5 for the optimal growth of the culture. Solid agar based medium are prepared by using 1.5 % agar in the liquid medium. The medium is autoclaved before use (Chen and Yue 2005).

Table 1 Bold basal medium composition

Component	Stock solution (gL^{-1} dH_2O)	Quantity to be used (mL)	Final concentrations (M)
Macronutrients			
$NaNO_3$	25.00	10	2.94×10^{-3}
$CaCl_2$ $2H_2O$	2.50	10	1.70×10^{-4}
$MgSO_4$	7.50	10	3.04×10^{-4}
K_2HPO_4	7.50	10	4.31×10^{-4}
KH_2PO_4	17.50	10	1.29×10^{-3}
NaCl	2.50	10	4.28×10^{-4}
EDTA solution		**1**	
EDTA	50.00		1.71×10^{-4}
KOH	31.00		5.53×10^{-4}
Iron solution		**1**	
$FeSO_4.7H_2O$	4.98		1.79×10^{-5}
H_2SO_4		**1**	
Boron solution		**1**	
H_3BO_3	11.42		1.85×10^{-4}
Trace metals solution		**1**	
$ZnSO_4.7H_2O$	8.82		3.07×10^{-5}
$MnCl_24H_2O$	1.44		7.28×10^{-6}
MoO_3	0.71		4.93×10^{-6}
Cu $SO_4.5H_2O$	1.57		6.29×10^{-6}
Co $(NO_3)_2.6H_2O$	0.49		1.68×10^{-6}

3.2 Chu 10 (Chu 1942)

Chu 10 is another medium used for the culture of various algae such as green algae, diatoms and cyanobacteria. It is an antificial medium where no chelators, vitamins and trace metals are added (Table 2). One milliliter each from component can be taken and make the volume up to 1 l by distilled water. The pH of the culture is maintained at 6.5.

3.3 Medium for Diatom (Cohn and Pickett-Heaps 1988; Cohn et al. 2003)

This is mainly for the culture of various diatom species. The medium uses soil extract. The following components are dissolved in 900 mL of dH_2O except vitamins. The final volume is brought up to 1 liter using dH_2O and sterilized. After cooling the solution vitamins are added and the pH is adjusted to 6.7. For the preparation of vitamin solution thiamine HCl is added into 950 mL of dH_2O. From all the stock solution 1 mL each is used and is brought to 1 liter. The solution is store frozen. The components of diatom medium are given in Table 3.

Table 2 Chu 10 medium compositions

Component	Stock solution ($g.L^{-1}$ dH_2O)	Quantity to be used (mL)	Final concentration (M)
$Ca(NO_3)_2$	40	1	2.44×10^{-4}
K_2HPO_4	5	1	2.87×10^{-5}
$MgSO_4.7H_2O$	25	1	1.01×10^{-4}
Na_2CO_3	20	1	1.89×10^{-4}
Na_2SiO_3	25	1	2.05×10^{-4}
$FeCl_3$	0.8	1	4.93×10^{-6}

Table 3 Compositions of diatom medium

Component	Stock solution ($g.L^{-1}$ dH_2O)	Quantity to be used (mL)	Final concentration (M)
$Ca(NO_3)_2.4H_2O$	70.85	1	3.00×10^{-4}
K_2HPO_4	54.44	1	4.00×10^{-4}
$MgSO_4.7H_2O$	24.65	1	1.00×10^{-4}
Na_2SiO_3 (27 % aq. sat. soln.)	20 mL pH 8.5	5	$\sim 3.00 \times 10^{-4}$
$FeSO_4.7H_2O$	0.278	1	1.00×10^{-6}
$MnCl_2.4H_2O$	0.02	1	1.00×10^{-7}
Soil extract	–	50	–
Vitamins solution		**1 mL**	
Thiamine HCl (Vitamin B_1)	–	1 g	2.97×10^{-6}
Biotin (Vitamin H)	–	1 g	4.09×10^{-6}
Nicotinic acid (niacin)	–	1 g	8.12×10^{-6}
Cyanocobalamin (Vitamin B_{12})	1	1 mL	7.38×10^{-10}

3.4 Medium for Volvox *(Provasoli and Pintner 1960)*

This medium is mainly for the culture of *Volvox* species. *Volvox* medium is also used for the culture of some strains of *Eudorina*, *Pandorina* and *Gonium*. In 900 mL of dH_2O calcium nitrate and glycylglycine are dissolved followed by other stock solutions. Vitamins are later added. The details components of *Volvox* medium are given below in Table 4.

3.5 Medium for BG-11 (Allen 1968; Allen and Stanier 1968; Rippka et al. 1979)

BG-11 (Blue Green Algae Medium) is mainly for the culture of Cyanobacteria. It is primarily used for freshwater, soil and marine organisms which do not require high ionic strength (Table 5). One milliliter each of the components and the trace metal was taken. The volume was made up to 1 liter by distilled water. The pH of the culture was maintained at 7.5 for the optimal growth of the culture and autoclaved.

Table 4 *Volvox* medium

Component	Stock solution ($g.L^{-1}dH_2O$)	Quantity to be used	Final concentration (M)
Glycylglycine	–	500.0 mg	2.46×10^{-3}
$Ca(NO_3)_2.4H_2O$	–	117.8 mg	5.00×10^{-4}
Na_2 ß-glycerophosphate $5H_2O$	50.0	1 mL	1.63×10^{-4}
$MgSO_4\ 7H_2O$	40.0	1 mL	1.62×10^{-4}
KCl	50.0	1 mL	6.71×10^{-4}
Biotin (Vitamin H)	0.0001	1 mL	4.09×10^{-10}
Cyanocobalamin (Vitamin B_{12})	0.0001	1 mL	7.38×10^{-11}
PIV trace metals solution		**3 mL**	
HOEDTA	–	1.398 g	1.22×10^{-5}
$FeCl_3.6H_2O$	–	0.194 g	2.15×10^{-6}
$MnCl_2.4H_2O$	–	0.036 g	5.46×10^{-7}
$ZnCl_2$	–	0.104 g	2.29×10^{-7}
$Na_2MoO_4.2H_2O$	–	0.013 g	1.56×10^{-7}
$CoCl_2.6H_2O$	4.04	1 mL	5.09×10^{-8}

HOEDTA Hydroxyethyl ethylenediamine triacetic acid

Table 5 BG-11 compositions

Component	Stock solution ($g.\ L^{-1}\ dH_2O$)	Quantity used (mL)	Final concentration (M)
Citric acid	6.0	1.0	3.12×10^{-5}
Ferric ammonium citrate	6.0	1.0	3.00×10^{-5}
$NaNO_3$		1.5 g	1.76×10^{-2}
$K_2HPO_4.3H_2O$	40.0	1.0	1.75×10^{-4}
$MgSO_4.7H_2O$	75.0	1.0	3.04×10^{-4}
$CaCl_2.2H_2O$	36.0	1.0	2.45×10^{-4}
Na_2CO_3	20.0	1.0	1.89×10^{-4}
$MgNa_2EDTA.H_2O$	1.0	1.0	2.79×10^{-6}
Trace metals solution			
H_3BO_4		2.86 g/L	4.63×10^{-5}
$MnCl_2.4H_2O$		1.81 g/L	9.15×10^{-6}
$ZnSO_4.7H_2O$		0.22 g/L	7.65×10^{-7}
$Na_2MoO_4.2H_2O$		0.39 g/L	1.61×10^{-6}
$CuSO_4.5H_2O$	79.0	1.0	3.16×10^{-7}
$Co(NO_3)_2.6H_2O$	49.4	1.0	1.70×10^{-7}

3.6 *Medium for* Spirulina *(Aiba and Ogawa 1977; Schlösser 1994)*

The main components of *Spirulina* medium are given below in Table 6. Solutions I and Solutions II are prepared separately. In case of solution II 1 mL of trace metals solution are added. The two solutions are autoclaved separately and allow them to cool. The two solutions are combined aseptically and after that 1 mL of cyanocobalamin (B_{12}) is added.

4 Isolation Methods of Algal Samples

The techniques for the isolation of microalgae first started by Beijerinck (1890). Water samples are collected in clean bottles and kept at lower temperature so that the cells remain viable. Techniques such as filtration, differential centrifugation,

Table 6 Compositions of *Spirulina* medium

Component	Stock solution (g. L^{-1} dH_2O)	Quantity used (mL)	Final concentration (M)
Solution I	**500 mL**	–	–
$NaHCO_3$	–	13.61 g	1.62×10^{-1}
Na_2CO_3	–	4.03 g	3.80×10^{-2}
K_2HPO_4	–	0.50 g	2.87×10^{-3}
Solution II	**500 mL**	–	-
$NaNO_3$	–	2.5 g	2.94×10^{-2}
K_2SO_4	–	1.0 g	5.74×10^{-3}
NaCl	–	1.0 g	1.71×10^{-2}
$MgSO_4.\ 7H_2O$	–	0.2 g	8.11×10^{-4}
$CaCl_2.2H_2O$	–	0.04 g	2.72×10^{-4}
$FeSO_4.\ 7H_2O$	–	0.01 g	3.60×10^{-5}
$Na_2EDTA.\ 2H_2O$	–	0.08 g	2.15×10^{-4}
$MgNa_2EDTA.H_2O$	1.0	1.0	2.79×10^{-6}
Trace metals solution		**1 mL**	–
$Na_2EDTA.\ 2H_2O$	–	0.8 g	2.15×10^{-6}
$FeSO_4.\ 7H_2O$	–	0.7 g	2.52×10^{-6}
$ZnSO_4.7H_2O$	1.0	1 mL	3.48×10^{-9}
$MnSO_4.\ 7H_2O$	2.0	1 mL	8.97×10^{-9}
H_3BO_3	10.0	1 mL	1.62×10^{-7}
$Co(NO_3)_2.6H_2O$	1.0	1 mL	3.44×10^{-9}
$Na_2MoO_4.2H_2O$	1.0	1 mL	4.13×10^{-9}
$CuSO_4.5H_2O$	0.005	1 mL	2.00×10^{-11}
Vitamin solution		1 mL	
Cyanocobalamin (Vitamin B_{12})	–	5 mg	3.69×10^{-9}

micropipetting, serial dilution and agar streaking are used for isolation and purification of the algal samples (Richmond 2003).

4.1 Filtration

The water samples are filtered through two layered muslin cloth or in a sieve, strainer, net of different size. In this separation method large particles and debris are discarded (Clark and Sigler 1963) to make the sample free from unwanted particles.

4.2 Differential Centrifugation

Centrifugation is the separation of larger and heavier cells from smaller cells such as algae and bacteria. But mild centrifugation for a short duration helps large dinoflagellates and diatoms to settle at the bottom forming a loose pellet. The process can be repeated several times if necessary. The centrifugation speed and time vary from species to species (Watanabe et al. 1998). This method allows the larger algae to settle at the bottom of the tube but is very difficult to obtain a unialgal culture; therefore further techniques for purifications are required.

4.3 Micropipette Isolation

This is the most commonly practiced method for the isolation of microalgae. The micropipette isolation is usually carried out with glass capillary. A few drops of water sample containing algal mixtures are placed in the cavity slide and observed under a microscope. The desired algal species is focused under the microscope and the algal cells are picked up from the side carefully into the capillary. This isolated algal cell is transferred to a drop of sterile medium. This process can be repeated several times with sterilized capillary to obtain reasonable concentration of algal cells to obtain proper growth on agar plate (Andersen and Kawachi 2005).

4.4 Serial Dilution Technique

Serial dilution technique is another method for the isolation of unialgal cells. In this technique a known volume of sample is mixed with distilled water. This technique reduces the concentration of the sample. In this process ten test tubes are taken and 9 mL of media are added in all the ten test tubes. The test tubes are labelled as 10^{-1}–10^{-10} indicating dilution factor. 1 mL of the sample is added to the first test

tube labelled as10^{-1} and mixed gently. From this test tube 1 mL of the sample is taken and added to the second test tube represented as 10^{-2} and mixed gently. The above procedure is repeated for the remaining tubes (10^{-3}–10^{-10}). The test tubes are maintained under normal temperature and light conditions. The cultures are examined periodically under the microscope.

4.5 Streaking Cells Across Agar Plates

A loop is loaded with a small amount of sample and then the sample is spread with the loop across the medium supplemented with 1.5 % agar (Heaney and Jaworski 1977). The origin of the streak typically has a mixture of algae that are not separated, but as the distance from the origin increases, single cell begins to separate. When the colonies started appearing, the cells are again taken from the agar plates with a loop and then restreaked on a new agar plate (Richmond 2003). This process can be repeated several times until a pure culture is obtained.

4.6 Raising of Mass Culture

Once the pure cultures are obtained, then the cultures are transferred to both into the liquid and solid media to develop small scale culture. After inoculation the culture experience an adaptation phase called the lag phase where the algae trys to stabilize the culture condition. At this stage the cells could not able to divide. When the cultures are allowed to grow continuously, a slight colour change can be noted. The cultures are light green indicating that the algal cells enter another stage where the algae started dividing. This stage is called the exponential stage (Fig. 1a–d). once the culture attained full growth, it is necessary to subculture so that algae maintain in its healthy state. Aliquot of algal cells can be aseptically transferred into a flask of freshly prepared medium and is cultivated under the same culture condition (Devi 2008).

5 Antibiotic Treatment

In order to obtain pure unialgal cells antibiotic treatment is sometimes necessary. Some of the common antibiotics used in the microalgal culture are penicillin, streptomycin and gentamycin. It diminishes the bacterial growth without affecting the algal growth. The concentration of antibiotics used in the medium differ from one species to another as the capability to tolerate the level of antibiotic concentration vary. A range of 50–500 mg/l concentrations is generally used. Krauss (1962) also presented data showing that streptomycin is inhibitory to a wide range of algae

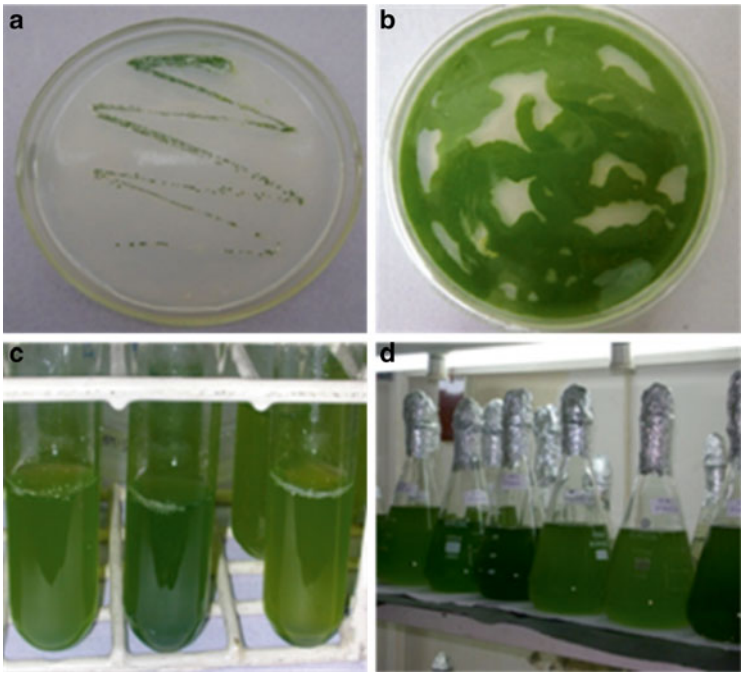


Fig. 1 (**a**–**d**) Various steps of Microalgal culture. (**a**) Streaking (**b**) Unialgal culture in solid medium (**c**) Unialgal culture in liquid medium (**d**) Raising of mass culture in the laboratory

between 1 and 40/μg/mL. Various combinations of broad-spectrum antibiotics such as chloramphenicol, tetracycline, streptomycin and neomycin are used for short exposure periods at various low concentrations, together with benzyl penicillin which is effective only against Gram-positive bacteria (Jones et al. 1973). The common antibiotics used in the algal culture are chloramphenicol, tetracycline, and bacitracin. The antibiotics prepared for the used in the algal culture are preserved in frozen condition until use.

6 Modern Microalgal Isolation Method

Some automated isolation method for microalgae has been developed as the earlier method needs skill and time consuming. Not only this it is very difficult to isolate manually a very small algal cells (Li 2002). Flow cytometry is another automatic isolation method for cell sorting (Fig. 2). It is a laser based technology mainly developed for cell counting, single cell isolation by suspending cells in a stream of fluid. The main principle involved in Flow cytometry is light scattering, excitation and emission of fluorochrome molecules to generate specific multi-parameter data from particles and cells in the size range of 0.5–40 μm in diameter.

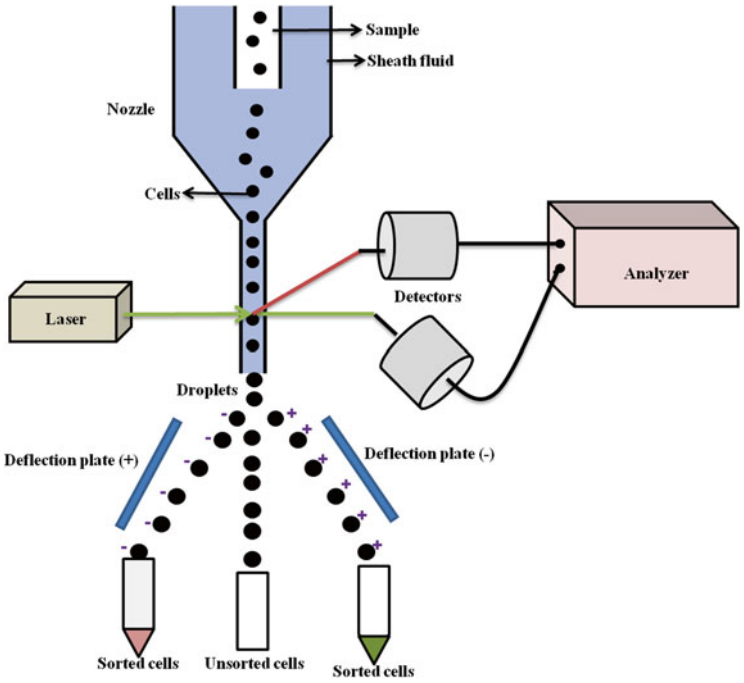


Fig. 2 Schematic diagram of flow cytometry

The light source used in flow cytometry are mainly lasers as lasers produce a high intensity of monochromatic light. Lasers have a very small size hole to focus light so that large single cells are excited thereby reducing the chance of more than one cells.

When the cells intercept a light source they scatter light and fluorochromes are excited to a higher energy state. The light which are scattered and emitted from cells are converted to electrical pulses with the help of optical detectors. Confocal lenses picked up all the collimated light and are passed to different detectors. Photomultiplier tube (PMT) is the most common type of detector used in flow cytometry. Logarithmic amplification is most often used to measure fluorescence in cells which help in expansion of weak signals and compression of strong signals. The data of flow cytometry are stored in computer files. Haugen et al. (1987) tested four fragile algae (*Chroomonas*, *Micromonas*, *Tetraselmis*, and *Gyrodinium*) for possible damaging effects by flow cytometry and neither flow cytometric fluidics nor laser exposure caused cell loss or inability to grow. Several authors have reported successful isolation of microalgae by flow cytometric sorting. Sensen et al. (1993) started unialgal cultures of *Cyanophora*, *Haematococcus*, *Monomastix*, *Scherffelia*, and *Spermatozopsis* (Andersen 2005).

7 Outdoor Cultivation of Microalgae

For the mass cultivation of microalgae, they can be grown either in the open or closed systems. In early and late 1970s, the production of algae was initiated in East Europe, Israel and Japan and the algae were mainly cultured in the open ponds for food. Open ponds are of two types namely natural lakes, lagoons or ponds and artificial system. Of these the open ponds which include raceway tanks or circular ponds etc. are very common because they are easier to handle. The major benefit of open system is that they are easy to handle than the other systems. But the raceway pond faces some disadvantages such as requirement of large land areas, evaporation looses contamination etc. At the same time mixing of the culture is very difficult in open systems thereby producing less biomass.

7.1 Raceway Ponds

Raceways are oval shaped, closed loop which are filled with water up to certain level so that the culture can be mixed and circulated properly. In raceway ponds, paddlewheels are used to maintain constant mixing of the algae. This wheel works continuously to avoid any settlement below the pond. They are built in concrete and covered with white plastic. Raceway ponds for mass culture of microalgae have been used since the 1950s. The largest raceway-based biomass production facility occupies an area of 440,000 m^2 (Spolaore et al. 2006) (Fig. 3).

Fig. 3 Microalgae cultivation in the field (image courtesy © D.Sahoo)

7.1.1 Advantages of Open Culture

Compare to closed system, open system has the following advantages such as

- Open culture system is cheaper.
- The open system does not require existing agricultural land, since they can be established in wasteland.
- Energy requirement is low.
- Regular maintenance and cleaning is much easier in open culture system.

7.2 Closed Culture System

The closed culture system for microalgae is usually carried out by using photobioreactors. Photobioreactors are of various types such as tubular, flat panel or column reactors (Janssen et al. 2002; Carvalho et al. 2006). They used external light supplies for their growth.

7.2.1 Tubular Photobioreactor

Tubular photobioreactors used long, transparent tubes which can be horizontal (Carlozzi et al. 2006), vertical (Converti et al. 2006; Perner-Nochta et al. 2007), inclined (Vunjak-Novakovic et al. 2005), or as a helix (Hall et al. 2003). Mechanical pumps or airlifts create the pumping force which allow CO_2 and O_2 to be exchanged between the liquid medium and the aeration gas (Hall et al. 2003; Converti et al. 2006), while almost no gas-exchange takes place in the tubes (Fig. 4).

Fig. 4 A tubular photobioreactor used for growing microalgae in a power plant (image courtesy © D. Sahoo)

7.2.2 Flat Panel Photobioreactors

In case of flat panel photobioreactors the algal culture is mixed across the flat panel system (Richmond et al. 2003). The light is absorbed by the algae which are above the culture. They are good for immobilization of algae. It has been reported that with flat-panel photobioreactors, high photosynthetic efficiencies can be achieved.

7.2.3 Column Photobioreactors

Column photobioreactors are reactors which can be in vertical position or can be bubbled from below. The light are passed from the transparent walls through the culture. This source of light can be given internally (Suh and Lee 2001). This type of photobioreactors give a very good mixing of the culture, the rate of gas transfer are very high.

8 Seaweed (Macro algae) Culture in the Laboratory

Seaweeds are macroscopic, multicellular benthic marine algae mainly found growing in the sea and brackish water. They comprise of green, brown and red algae. Seaweeds collected from the field are brought to the laboratory. They are cleaned manually by removing attached epiphytes and epifauna. To maintain a culture of seaweeds vegetative tissue or the spores are used. The cultures are kept in such chambers where the temperature, light can be manipulated. Direct use of natural sea water for macroalgal culture is seldom acceptable as the growth of the macroalgae is very slow. But for large scale cultivation the macroalgae are usually cultured in natural seawater (Fig. 5). For laboratory culture it is necessary to supply nutrients and other trace metal in the media for the growth. Thus for the culture of seaweeds in the laboratory artificial sea water is used along with various other nutrients required for the proper growth of the desired species (Fig. 6). To observe the development of the thallus it is very important to culture the algae of interest in the artificial conditions. But it is always difficult to culture the desire macroalgae in natural seawater. By improving the culture media composition algal culture of macroalgae became widely studied in 1960s (Bold 1942; Tatewaki 1966).

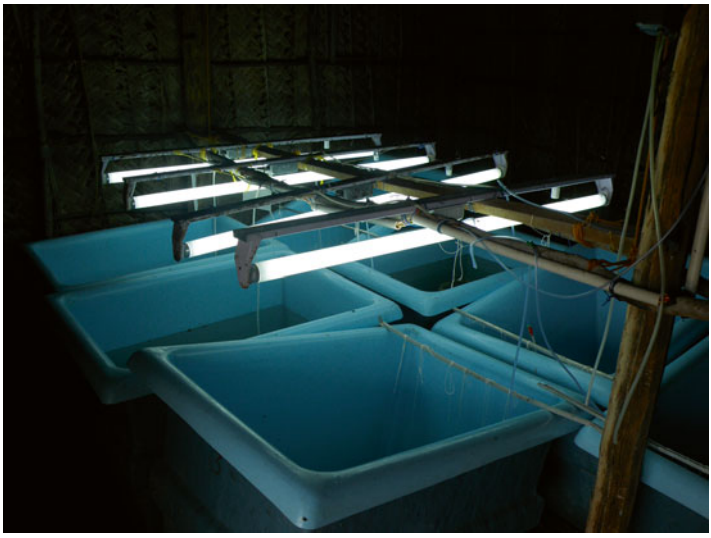


Fig. 5 Tank culture of seaweed for pilot cultivation

Fig. 6 Laboratory culture of seaweed

9 Isolation and Culture

Macroalgae have high potential for regeneration. Therefore the unialgal culture of macroalgae is generally practiced by cutting off the vegetative cells. Algal cultures can also be developed from zoospores and planogametes, zygotes, carpospores, tetraspores, or aplanospores (Kawai et al. 2005). Macroalgae are cultured in a sterilized glass or petridishes filled with enriched medium which are covered with parafilm. After every 2–4 weeks or depending on the material and temperature culture media are changed. If the culture was still contaminated the isolation processes are repeated several times.

But before the start of any culture work, the thalli are allowed to acclimatize for 4–5 days in sterilized seawater with desired salinity. The thalli are weighed properly and are cut into small pieces of 2–3 cm. They are transferred into 500 mL of cleaned conical flask containing 200 mL of sterilized liquid media. The most commonly used culture media for macroalgae is F/2 medium and PES medium.

Direct use of natural sea water is not acceptable for the culture of macroalgae as natural seawater contains various elements and lots of organic compounds which are not always favored for the growth of macroalgae. So it is very much necessary to add extra nutrients and trace metals to make better growth of the algae. Therefore artificial sea water is enriched by adding macronutrients, trace elements and vitamins for the laboratory culture of macroalgae. Compositions of some of the common marine media are given below.

9.1 Aquil or Artificial Seawater Media (Morel et al. 1979; Price et al. 1989)

Aquil is an artificial seawater medium that has been widely used with various modifications and supports the growth of many marine taxa. The details compositions of Aquil modified medium is given in Table 7. To prepare the media in 600 mL of distilled water each of the anhydrous salts is dissolved individually. In another 300 mL of distilled water each of the hydrous salts are dissolved individually. The two solutions are combined and 1 mL of trace metal solution and 1 mL of vitamins solution are added. The final salinity is maintained at 35 psu.

9.2 Medium for Von Stosch (Grund) (Guiry and Cunningham 1984)

Von Stosch (1963) modified enriched seawater medium from Grund medium. This medium is used by Guiry and Cunningham (1984) in the culture of *Gigartina* and various other red seaweeds. Nine hundred fifty milliliters of filtered natural seawater is taken and 10 mL of each of the following stock solutions given in Table 8 are added aseptically.

Table 7 Composition of aquil medium

Component	Stock solution (gL^{-1} dH_2O)	Quantity to be used	Final concentration (M)
Anhydrous salts			
NaCl	—	24.540 g	4.20×10^{-1}
Na_2SO_4	—	4.090 g	2.88×10^{-2}
KCl	—	0.700 g	9.39×10^{-3}
$NaHCO_3$	—	0.200 g	2.38×10^{-3}
KBr	—	0.100 g	8.40×10^{-4}
H_3BO_3	—	0.003 g	4.85×10^{-5}
NaF	—	0.003 g	7.15×10^{-5}
Hydrous salts			
$MgCl_2.\ 6H_2O$	—	11.100 g	5.46×10^{-2}
$CaCl_2.\ 2H_2O$	—	1.540 g	1.05×10^{-2}
$SrCl_2.\ 6H_2O$	—	0.017 g	6.38×10^{-5}
Major nutrients (In 900 mL of distilled water 1 mL each stock solutions are added and final volume is brought to 1 liter)			
$NaH_2PO_4.\ H_2O$	1.38	1 mL	1.00×10^{-5}
$NaNO_3$	85.00	1 mL	1.00×10^{-4}
$Na_2SiO_3.\ 9H_2O$	28.40	1 mL	1.00×10^{-4}
Trace metal stock solution (In 900 mL of distilled water, EDTA is dissolved and 1 mL each stock solutions are added and final volume is brought to 1 liter)			
EDTA (anhydrous)	—	29.200 g	1.00×10^{-5}
$FeCl_3.\ 6H_2O$	—	0.270 g	1.00×10^{-6}
$ZnSO_4.\ 7H_2O$	—	0.230 g	7.97×10^{-8}
$MnCl_2.\ 4H_2O$	—	0.0240 g	1.21×10^{-7}
$CoCl_2.\ 6H_2O$	—	0.0120 g	5.03×10^{-8}
$Na_2MoO_4.\ 2H_2O$	—	0.0242 g	1.00×10^{-7}
$CuSO_4.\ 5H_2O$	4.9	1 mL	1.96×10^{-8}
Na_2SeO_3	1.9	1 mL	1.00×10^{-8}
Vitamin stock solution (In 900 mL of distilled water, thiamine HCl is dissolved and 1 mL each stock solutions are added and final volume is brought to 1 liter)			
Thiamine. HCl (vitamin B1)	–	100 mg	2.97×10^{-7}
Biotin (vitamin H)	5.0	1 mL	2.25×10^{-9}
Cyanocobalamin (vitamin B12)	5.5	1 mL	3.70×10^{-10}

Table 8 Components of Von Stosch (grund) medium

Component	Stock solution (gL^{-1} dH_2O)	Quantity used	Concentration in final medium (M)
Na_2 ß-glycerophosphate	5.36	10 mL	2.48×10^{-4}
$NaNO_3$	42.52	10 mL	5.00×10^{-3}
$FeSO_4.\ 7H_2O$	0.28	10 mL	1.00×10^{-5}
$MnCl_2.\ 4H_2O$	1.96	10 mL	1.00×10^{-4}
$Na_2EDTA.\ 2H_2O$	3.72	10 mL	1.00×10^{-4}
Vitamin stock solution (In 950 mL of distilled water, thiamine HCl is dissolved and 1 mL each stock solutions are added and final volume is brought to 1 liter)			
Thiamine. HCl (vitamin B1)	–	200 mg	5.93×10^{-6}
Biotin (vitamin H)	0.1	1 mL	4.09×10^{-9}
Cyanocobalamin (vitamin B12)	0.2	1 mL	1.48×10^{-9}

Table 9 Composition of f/2 medium

Component	Stock solution (g. L^{-1} dH_2O)	Quantity used (mL)	Final concentration (M)
$NaNO_3$	75	1	8.82×10^{-4}
NaH_2PO_4. H_2O	5	1	3.62×10^{-5}
Na_2SiO_3. $9H_2O$	30	1	1.06×10^{-4}
Trace metal solution		1	
Vitamin solution		0.5	
f/2 trace metal solution			
950 mL of dH_2O is taken and EDTA and other components are added making the final volume to 1 liter with distilled water			
$FeCl_3$. $6H_2O$		3.15 g	1.17×10^{-5}
Na_2EDTA. $2H_2O$		4.36 g	1.17×10^{-5}
$CuSO_4$. $5H_2O$	9.8	1	3.93×10^{-8}
Na_2MoO_4 $2H_2O$	6.3	1	2.60×10^{-8}
$ZnSO_4$. $7H_2O$	22.0	1	7.65×10^{-8}
$CoCl_2$. $6H_2O$	10.0	1	4.20×10^{-8}
$MnCl_2$. $4H_2O$	180.0	1	9.10×10^{-7}
f/2 vitamin solution			
950 mL of dH_2O is taken and dissolved thiamine. HCl add 1 mL each of the stocks and bring final volume to 1 liter with dH_2O. The final solutions are stored in the freezer			
Thiamine HCl (vitamin B1)		200 mg	2.96×10^{-7}
Biotin (vit. H)	1.0	1	2.05×10^{-9}
Cyanocobalamin (vitamin B12)	1.0	1	3.69×10^{-10}

9.3 F/2 Medium (Guillard and Ryther 1962; Guillard 1975)

f/2 medium is widely used enriched seawater medium for the culture of marine algae (Table 9).

9.4 Provasoli Enriched Seawater (PES) Medium (Provasoli 1968)

To prepare the Provasoli Enriched Seawater (PES) medium 900 mL of distilled water is taken and to this the compositions are added. The final volume is made up to 1 liter with distilled water (Table 10).

Table 10 PES stock solution

Component	Stock solution (gL^{-1} dH_2O)	Quantity used	Concentration in final medium (M)
Tris base	—	5.0 g	8.26×10^{-4}
$NaNO_3$	—	3.5 g	8.24×10^{-4}
Na_2 b-glycerophosphate·H_2O	—	0.5 g	4.63×10^{-5}
Iron–EDTA solution	(See following recipe)	250 mL	—
Trace metals solution	(See following recipe)	25 mL	—
Thiamine · HCl (vitamin B1)	—	0.500 mg	2.96×10^{-8}
Biotin (vitamin H)	0.005	1 mL	4.09×10^{-10}
Cyanocobalamin (vitamin B12)	0.010	1 mL	1.48×10^{-10}
Iron–EDTA solution			
Into 900 mL of dH_2O, EDTA is dissolved followed by iron sulphate and the final volume is brought to 1 liter			
$Na_2EDTA·2H_2O$	—	0.841 g	1.13×10^{-5}
$Fe(NH_4)_2(SO_4)_2·6H_2O$	—	0.702 g	8.95×10^{-6}
Trace metals solution			
Into 900 mL of dH_2O, EDTA is dissolved and then individually dissolved the following components. The final volume is brought to 1 liter			
$Na_2EDTA·2H_2O$	—	12.74 g	1.71×10^{-4}
FeCl3·6H2O	—	0.484 g	8.95×10^{-6}
H_3BO_3	—	11.439 g	9.25×10^{-5}
$MnSO_4·4H_2O$	—	1.624 g	3.64×10^{-5}
$ZnSO_4·7H_2O$	—	0.220 g	3.82×10^{-6}
$CoSO_4·7H_2O$	—	0.048 g	8.48×10^{-7}

10 Treatment of Thalli in Disinfectant Series

Before the start of culture, the thalli are treated with various disinfectant series so that the culture is free from contamination. For disinfectant series the thalli are treated with 1 % IKI solution (Markham and Hasmeier 1982) for 1 min to eliminate surface microbes. Sterilization is done by using broad spectrum antibiotic mixture (Polne-Fuller and Gibor 1984) to prevent the growth of bacteria. The sterilized healthy thalli are cultured in 300 mL of autoclaved seawater in a flask. To this 1 % of GeO_2 (Germinium dioxide) is added (Markham and Hasmeier 1982) to prevent the growth of diatoms.

10.1 Preparation of 1 % GeO_2 (Markham and Hasmeier 1982)

Four grams of sodium hydroxide (NaOH) is boiled and 250 mg of GeO_2 is added all at one time. The mixture is stirred properly with glass rod so that the compounds are dissolved properly and the solution is kept for cooling. pH is adjusted to 7.8–8.0 by 1 N HCl. The final volume is brought up to 250 mL with distilled water. From this, 1 mL is used in 100 mL of culture medium.

10.2 Preparation of 1 % IKI Solution (Markham and Hasmeier 1982)

In 300 mL of distilled water 2 g of potassium iodide (KI) and 1 g of iodine is dissolved. 1 mL of IKI solution is taken and added in 250 mL of sterilized seawater.

11 Preparation of Broad Spectrum Antibiotics (Polne-Fuller and Gibor 1984)

The following components are dissolved in 100 mL of distilled water and filter sterilized. Dissolve Nystatin in DMSO separately and add after filter sterilization.

Component	Quantity used
Penicillin G	1 g
Streptomycin sulphated	2 g
Kannamycin	1 g
Nystatin	25 mg
Neomycin	200 mg

There are different microalgal culture collection centres located all over the world from where the pure microalgal culture can be obtained. Some of the important microalgal culture collection centres are given below in Table 11.

Table 11 List of microalgal culture collections around the world

Collection centres	Name	Country
Academy of Sciences of the Czech Republic, Institute of Botany (CCALA)	Czech Collection of Algae and Cyanobacteria (http://ccala.butbn.cas.cz/)	Praque
Centre for Ecology and Hydrology (CCAP)	Culture Collection of Algae and Protozoa (http://www.ccap.ac.uk/)	Scotland
Chinese Academy of Sciences, Culture Collection Committee (CTCCCAS)	Freshwater Algae Culture Collection (http://www.ctcccas.ac.cn/typecc/danshui/en.html)	China
CSIRO, Marine research (CS)	CSIRO Collection of Living Microalgae (http://www.marine.csiro.au/algaedb/search.htm)	Hobart, Tasmania
Charles University Prague, Department of Botany (CAUP)	Collection of Algae of Charles University Prague (http://botany.natur.cuni.cz/algo/caup-list.html)	Praque
Duke University, Chlamydomonas Genetics Center (CGC)	Chlamydomonas Genetics Center Collection (http://www.chlamy.org/strains.html)	USA
Institut Pasteur, Paris (PCC)	Pasteur Culture Collection of Cyanobacteria (http://www.pasteur.fr/ip/easysite/pasteur/en/research/collections/crbip/general-informations-concerning-the-collections/iv-the-open-collections/iv-iii-pasteur-culture-collection-of-cyanobacteria)	Paris
Loras College, Iowa, USA	The Loras College Freshwater Diatom Culture Collection (http://www2.bgsu.edu/departments/biology/facilities/algae/html/DiatomCulture.html)	Lowa, USA
Thailand Network on Culture Collections (TNCC)	BIOTEC Culture Collection (http://www.1a.biotec.or.th/TNCC/dbstore/BCC_search.asp)	Thailand
Philipps-University Marburg, Department of Cell Biology and Applied Botany (CCAM)	Culture Collection of Algae Marburg (marburg.de/~cellbio/welcomeframe.html)	Germany
Provasoli-Guillard National Center for Culture of Marine Phytoplankton, ME (CCMP)	Provasoli-Guillard National Center for Culture of Marine Phytoplankton (https://ncma.bigelow.org/)	Maine
Station Biologique Roscoff, Phytoplancton (RCC)	Roscoff Culture Collection of Marine Phytoplancton (http://www.sb-roscoff.fr/Phyto/RCC/index.php)	France
University of Caen Basse, Laboratoire de Biologie et Biotechnologies Marines (ALGOBANK)	Algobank (http://www.unicaen.fr/algobank/accueil/)	

(continued)

Table 11 (continued)

Collection centres	Name	Country
University of Coimbra, Department of Botany (ACOI)	Coimbra Collection of Algae (http://www.uc.pt/botanica/ACOI.htm)	Portugal
University of Cologne, Institute of Botany (CCAC)	Culture Collection of Algae at the University of Cologne (http://www.ccac.uni-koeln.de/recherche.shtml)	Germany
University of Göttingen, Sammlung von Algenkulturen (SAG)	Culture Collection of Algae at the University of Göttingen (http://www.uni-goettingen.de/en/184982.htmlcgi-bin/epsag/website/cgi/show_page.cgi?kuerzel=start)	Germany
University of Texas at Austin, School of Biological Sciences (UTEX)	Culture Collection of Algae at the University of Texas at Austin (http://web.biosci.utexas.edu/utex/)	Austin, USA
University of Toronto, Department of Botany (UTCC)	University of Toronto Culture Collection of Algae and Cyanobacteria (http://www.botany.utoronto.ca/utcc/List%20of%20Cultures.html)	Toronto

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D. Sahoo, J. Seckbach (eds.), *The Algae World*, Cellular Origin, Life in Extreme Habitats and Astrobiology 26, DOI 10.1007/978-94-017-7321-8

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