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REVIEW

Chlorophyll fluorescence emission spectroscopy of oxygenic organisms at 77 K

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Abstract

Photosynthetic fluorescence emission spectra measurement at the temperature of 77 K (–196°C) is an often-used technique in photosynthesis research. At low temperature, biochemical and physiological processes that modulate fluorescence are mostly abolished, and the fluorescence emission of both PSI and PSII become easily distinguishable. Here we briefly review the history of low-temperature chlorophyll fluorescence methods and the characteristics of the acquired emission spectra in oxygen-producing organisms. We discuss the contribution of different photosynthetic complexes and physiological processes to fluorescence emission at 77 K in cyanobacteria, green algae, heterokont algae, and plants. Furthermore, we describe practical aspects for obtaining and presenting 77 K fluorescence spectra.

Additional key words: fluorescence; low temperature; photosynthesis.

Introduction

Historical background

"Chlorophyll fluorescence is red and beautiful" (Govindjee 1995) and has fascinated researchers for a long time (*see* reviews by Govindjee 1995, 2004). Chlorophyll (Chl) extracts prepared from leaves possess a very intense red fluorescence that caught the attention of Brewster in the 1830's (Brewster 1834). Correlations between the weaker *in vivo* Chl fluorescence emission and photosynthetic performance have been suggested (Müller 1887, as referenced by Govindjee 1995, 2004), but Kautsky and Hirsch were the first to unambiguously relate Chl fluorescence yield to the rate of photosynthesis (Kautsky and Hirsch 1931). The utility of Chl fluorescence for exploring photosynthesis was consequently demonstrated in many different photosynthetic organisms. Many breakthroughs in our understanding of photosynthesis are the result of researchers' creativity to develop techniques to resolve the spectral and temporal characteristics of Chl fluorescence in intact photosynthetic systems and by purifying individual components thereof.

One Chl fluorescence-based technique, which has been widely adopted, was developed by Seymour Steven Brody while working on his Ph. D. in the Rabinowitch laboratory at the University of Illinois at Urbana Champaign (Brody 1958, Rabinowitch and Govindjee 1969). It had been previously demonstrated that lowering the temperatures sharpens spectral characteristics, such as absorption and fluorescence, due to the loss of intramolecular vibrations. However, the improved spectral resolution was not the primary motivation for Brody to investigate fluorescence characteristics of the green algae *Chlorella* at liquid nitrogen temperatures. Brody wanted to eliminate biochemical and physiological processes (Hirsch and Rich

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Abbreviations: Chl – chlorophyll; LHC – light-harvesting complex; PSI – photosystem I; PSII – photosystem II.
This review is dedicated to Govindjee. In addition to Govindjee's original contributions to the field, we, the authors, are also very thankful to Govindjee for sharing historical context and personal connections, which contributed to researchers making their discoveries. In the case of 77 K fluorescence, the topic of this review, Govindjee was not only witness to its first implementation, but also went on to refine and extend the interpretation of this powerful measuring technique. The following short historical introduction into 77 K fluorescence often refers to information obtained from publications by Govindjee and coworkers.
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2010), and thereby gained direct insights into excitation and electron transfer processes involved in photosynthesis using Chl fluorescence as a reporter. Around the same time during which Brody investigated fluorescence emission by the green alga *Chlorella* (Brody 1958), Litvin and Krasnovsky (1957) investigated fluorescence emission of plant material at 77 K.

One immediate finding of these measurements was that in addition to the main Chl fluorescence band at 685 nm, which is readily observed at room temperature, a second emission band with peak intensity at 720 nm becomes more prominent at lower temperatures (Fig. 1) (Brody 1958). Frederick Cho and Govindjee demonstrated that at even lower temperatures - at liquid helium temperatures (4 K) (Rabinowitch and Govindjee 1969, Cho 1970a, b, Cho and Spencer 1966) a third emission band at 695 nm can be separated (Fig. 2).

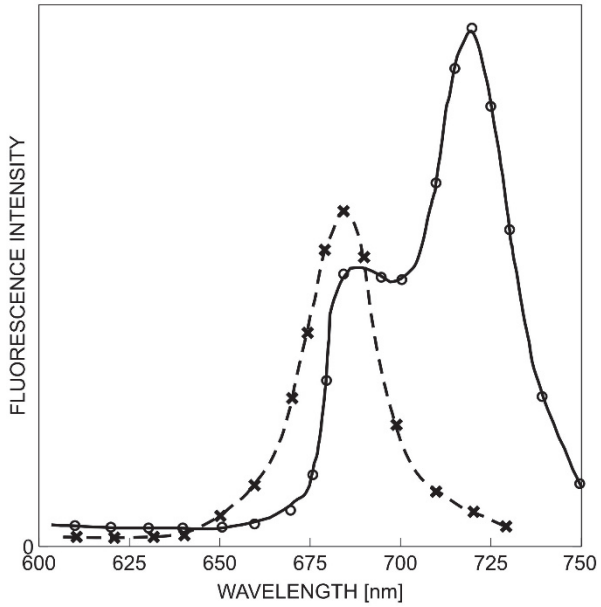

Fig. 1. Chlorophyll fluorescence emission by *Chlorella* cells at room temperature and –193°C. *The original figure legend*: „Fluorescence spectra of *Chlorella* at room temperature (*crosses*) and –193°C (*open circles*). The fluorescence intensities indicated are the same for both curves. The decrease in fluorescence yield at 690 mμ is probably due to the increased scattering of the exciting and fluorescent light".
Remarks: The fluorescence yield at 690 nm is expected to be about two times larger at –196°C compared to room temperature fluorescence yield, as suggested by Brody in the original figure legend. The term mμ is a historical notation that is equivalent with nanometer (nm).
This publication represents the first report of the increased long-wavelength chlorophyll fluorescence emission at 77 K, which has been established to be mainly associated with PSI.
Data was digitized from Brody (1958).

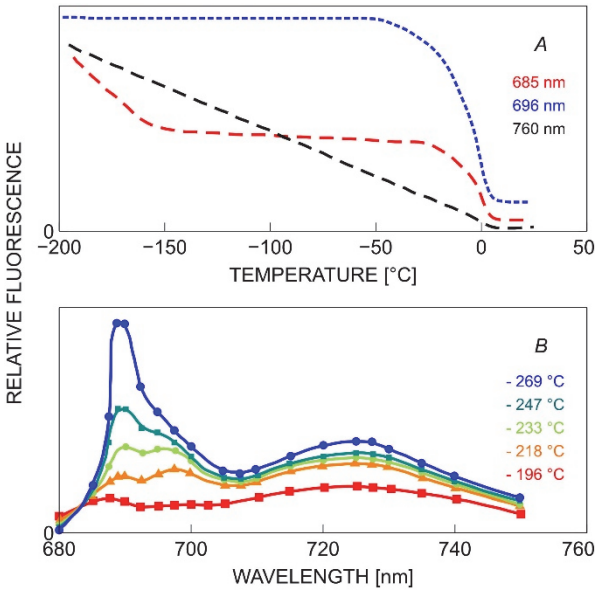

Fig. 2. Chlorophyll fluorescence emission of *Chlorella pyrenoidosa* as a function of temperature
(*A*): Chlorophyll fluorescence emission at specific wavelengths as a function of temperature in *Chlorella pyrenoidosa* as a function. The excitation light wavelength was 400 nm. *The original figure legend*: "Emission at 685 mμ (F685), at 696 mμ (F696), and at 760 mμ (F738; also referred to as F720 in the text) as a function of temperature (–196 to 20°C)." Panel *B* has been digitized from Cho *et al.* (Cho *et al.* 1966).
(*B*): Chlorophyll fluorescence emission spectra of *Chlorella pyrenoidosa* as a function of temperature (–269, –247, –233, –218, –196°C). Excitation light wavelength was 485 nm. *The original figure description*: "Fig. 1. shows the emission spectra measured in the 680–720 mμ range for several temperatures (–269, –247, –233, –218, and –196°C). Upon warming the sample, a shift from 695 mμ to 699 mμ in the location of the peak of the "F697.5" band is noticeable. As the temperature decreases from -196 to -269°C, the fluorescence increases steadily. The total intensity at –269°C is about 2 times that at –196°C. The profile of the fluorescence spectrum at –196°C shows clearly the F689, F697.5, and F725 bands; at –269°C, the F689 appears as a very sharp band and it dominates both F697.5 and 725, the F697.5 band shows only as a shoulder at –269°C" Panel *A* has been digitized from Govindjee and Yang (1966).
Remarks: The term mμ is a historical notation that is equivalent with nanometer (nm). This figure was used to demonstrate that the 738 nm (PSI) emission band is not a reabsorption artifact.

Associating these fluorescence emission bands with the concept of two types of photosystems (PSI and PSII) (Emerson 1957, Govindjee *et al*. 1960, Govindjee 1963) as part of the Z-scheme of photosynthesis, was achieved by the work of a community of researchers (reviewed by Govindjee and Björn 2017). So it was recognized that in red algae, which have a spectrally distinct light-harvesting system, preferentially exciting these light-harvesting systems, the phycobilisomes, increases the fluorescence emission at 685/695 nm (Krey 1966). Exciting Chl *b* in higher plants also increased the fluorescence at 685/695 nm, thus suggesting that Chl *b*-containing light-harvesting

systems are associated with PSII (Govindjee and Yang 1966, Rabinowitch and Govindjee 1969). Fractionation of spinach thylakoids into PSII-enriched and PSI-enriched fractions confirmed the assignments of Chl fluorescence emission bands at 685/695 nm to PSII and the emission band at 720 nm to PSI (Boardman *et al*. 1966).

That the number of light-absorbing pigments associated with PSI and PSII can be modulated in a physiologically relevant manner was shown by Murata (1969, 1970), in red alga using 77 K Chl fluorescence measurements. These "state transitions," are not unique to red algae, but have also been observed in other organism groups. Bonaventura and Myers (1969) showed dynamic adjustment of energy distribution between the photosystems in a green alga using room temperature fluorescence paired with oxygen measurements. However, 77 K Chl fluorescence emission measurements are by now the preferred method to establish changes in the association of light-harvesting systems between the two photosystems in green algae and plants (Goldschmidt-Clermont and Bassi 2015, Minagawa 2011).

We use the term "77 K Chl fluorescence" in this review article, as the majority of researchers currently uses this expression. It is derived from the fact that the boiling point of liquid nitrogen under standard conditions is 77.355 K (195.795°C). However, several expressions have been used more frequently in the past, including "Chl fluorescence at liquid nitrogen temperatures" and "Chl fluorescence at –196ºC".

We have limited this review to steady-state fluorescence emission at 77 K and discuss the underlying physical processes, spectra, and its components. In addition to steady-state Chl fluorescence measurements at 77 K, many powerful techniques that use low-temperature Chl fluorescence emission have been developed, including time-resolved techniques (Strasser *et al*. 2004). Our focus here is on the physiological interpretation of information obtained from 77 K Chl fluorescence spectra. For this, we provide a visual overview of spectral features and physiological responses in different organism groups.

Physical background

Excitation of molecules

When molecules interact with light, the energy contained in the photons can be used to transfer an electron to an energetically higher orbital, thus generating a molecule in an excited state. Molecules with an extended conjugated system of bonds are likely to interact with photons in the visible spectrum. All Chls possess extended conjugated bond systems and interact with photons centered at two wavelengths that represent the first and second excited state of the Chl. The absorption band at higher energy is often termed "the blue absorption band", "B band" (consisting of several states) or "Soret band", whereas the lower energy absorption band can be referred to as the "red absorption band" or the "Q band" (consisting of two states, Q_x and Q_y) (Gouterman *et al*. 1963) (Fig. 3).

An excited Chl can return to the ground state through dissipating of the energy difference between excited state and ground state through different modes, which compete with one another. The second excited state of Chls is converted efficiently to the first excited state, and the energy difference is released as heat. The energy of the first excited state is either transferred to another pigment, or converted into: (*1*) heat, (*2*) chemical energy by driving charge separation, (*3*) a long-lived triplet state through a reversal of the spin of the electron, or (*4*) the emission of a photon. The energy of the emitted photon corresponds to the energy difference between the lower vibronic sublevels of the first excited state and ground state. This "Stokes-shifted" photon is the fluorescence that is the basis of many spectroscopic techniques, including 77 K fluorescence emission analysis.

The fluorescence characteristics of photosynthetic machinery is dependent on the emission characteristics of individual Chls and the cooperative excitation-coupling network these Chl form. A single Chl molecule possesses a fluorescence emission band, which reflects the transition from the first excited state to the ground state (Clayton 1980). This fluorescence band around 685 nm is broadened at room temperature as vibronic sublevels with higher energy are populated. Energy levels of excited states of individual Chls are also influenced by the protein and lipid environment that a Chl experiences. *In vivo*, the second broad fluorescence emission band around 735–740 nm is thought to be due to an increased emission wavelength through self-absorption at shorter wavelengths (Franck *et al*. 2002).

The overall emission of photosynthetic systems is a combination of main and vibronic sublevel emission, environment of individual Chls, and the energetic landscape of the Chl collective. For example, in plants, Chls associated with the PSII reaction center fluoresce at 685 and 695 nm – due to the main transition emission, and around 735–740 nm – due self-absorption enhanced fluorescence in this region. In intact PSII, Chls in the peripheral antennae, when isolated fluoresce at 680 nm, are coupled efficiently to the Chls within the PSII reaction center. Thus, only a small amount of 680 nm fluorescence emission is observed in intact systems, as the low energy Chls within the PSII reaction center (with a main emission at 685 and 695 nm) emit fluorescence. Furthermore, fluorescence emission from vibronic sublevels of Chls within the peripheral antennae and reaction center is emitted as a broad band centered at 730 nm. PSI has additional fluorescence emission that combines with that of PSII, light-harvesting complexes, and vibronic sublevel emission to generate the combined fluorescence emission pattern of an organism (Fig. 4).

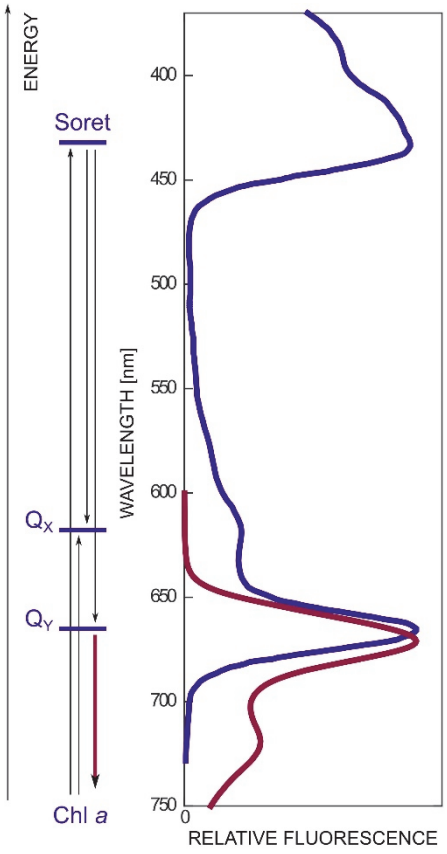

Fig. 3. Diagram relating the energy levels with the absorption spectrum and the fluorescence of chlorophyll (Chl) *a*. The data on energy levels in Chl *a* was obtained from Frank *et al*. (1994), absorption data for Chl *a* was obtained from Chen and Blankenship (2011), and the fluorescence data was retrieved from http://omlc.org/spectra/PhotochemCAD/html/122.html.

Table 1 gives an overview of major emission bands observed in various photosynthetic materials.

Excitation transfer

In whole, intact photosynthetic systems, Chl fluorescence represents a tiny fraction of all excitation energy captured by pigments in intact photosynthetic organisms, as energy is efficiently channeled into charge separation (Hillier and Babcock 2001). The bulk of the light-absorbing molecules in known photosynthetic organisms – except Heliobacteria – are not positioned within the photosynthetic reaction centers, but in separate peripheral light-harvesting systems. Light energy, absorbed by pigments in these light-harvesting systems is transferred *via* other pigments within the light-harvesting system (Förster 1965, Şener *et al*. 2011) to the reaction center antennae and finally to the reaction center core, where the excitation energy is used to accomplish a charge separation event.

Pigments

Oxygenic organisms employ three main classes of pigments for light harvesting: Chls, phycobilins, and carotenoids.

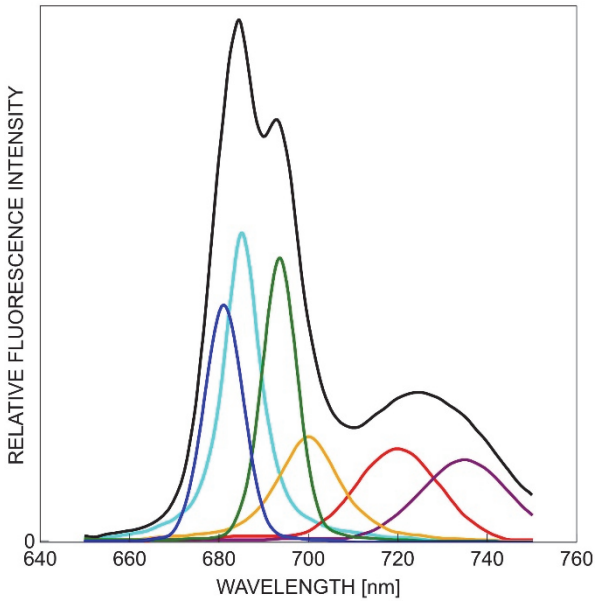

Fig. 4. Chlorophyll fluorescence emission spectra of spinach thylakoids at 77 K and decomposition in Gaussian components. A typical fluorescence emission spectrum with Gaussian decomposition representing known fluorescence emitters from spinach. The main components represent F_{680} (LHCII), F_{685} (CP47/CP43), F_{695} (CP47), F_{700} (aggregated LHCII trimer), F_{720} (PSI core complex), and F_{735} (LHCI). This figure has been digitized from Yamamoto *et al*. (2013), and a modified legend has been provided.

Chls and phycobilins possess a large absorption cross-section and are very fluorescent – features that make them well suited to participate in the Förster-type exchange of excitation. Indeed, the consortia of Chls (*i.e*., the light-harvesting systems of plants) and phycobilins (*i.e*., phycobilisomes, the light-harvesting system of cyanobacteria) are very efficient in channeling excitation to the Chl-containing reaction centers.

Carotenoids absorb light efficiently between 450–550 nm and display tiny fluorescence yields (Frank *et al*. 2006). Thus, the efficient excitation transfer between carotenoids and Chls cannot be understood by a Förster-type excitation transfer theory. Instead, energy transfer between carotenoids and Chls may be understood to be mediated by the singlet–singlet excitation energy transfer process (Owens 1992, Young and Frank 1996). Table 2 gives an overview of common pigments found in various organisms.

Fluorescence emission spectroscopy

A way to assess the interaction of light-harvesting systems with the reaction centers is to expose a sample to monochromatic light centered on a specific pigment absorption maximum and monitor the fluorescence emission. In cyanobacteria and red algae, phycobilins have an absorption spectrum that is distinct from the Chls of the reaction centers. For this reason, many early insights into

photosynthesis were obtained in phycobilin-containing organisms. Alga and plants expand the range of absorbed photons with Chls *b* and *c*, which are distinct from the Chl *a* of the reaction centers. The association of light-harvesting systems with the reaction centers can, therefore, be probed by exciting the light-harvesting system pigments and monitoring the fluorescence emission that is specific for PSII and PSI.

Room temperature fluorescence emission of plants

In whole, intact photosynthetic systems, only 1–2% of excitation energy captured is lost as fluorescence (Maxwell and Johnson 2000). At room temperature, PSII emits 90% of fluorescence while PSI contributes the remaining 10% (Govindjee 1995). Due to the low room temperature fluorescence emission of PSI compared to PSII, the overlapping fluorescence spectra of the main fluorescence emission band of Chls associated with PSI, and vibronic emission of Chls associated with PSII around 720–730 nm (Franck *et al*. 2002), it is difficult to assess the magnitude of PSI fluorescence emission. Another factor that makes the assignment of fluorescence to PSI and PSII difficult at room temperature is the modulation of fluorescence yield due to the reduction state of the electron acceptors within the photosystems. The prime modulator of overall Chl fluorescence at room temperature is the reduction state of the first stable electron acceptor of PSII, a quinone named Q_A (Duysens and Sweers 1963). In contrast, there is almost no modulation of PSI fluorescence yield by the reduction state of the electron acceptors of PSI at room temperature, which is due to the efficient quenching of excitation by $P700^+$ (Dau 1994a, b).

Fluorescence at low temperatures

Fluorescence emission spectra at 77 K offer a key advantage over room temperature measurements, as modulation caused by physiological acclimations and biochemical reactions are eliminated. Compared to room temperature, the 77 K fluorescence yield of the PSII core complex is about two times higher, and fluorescence yield of PSI increases by a factor of around 20 (Mukerji and Sauer 1988, Dekker *et al*. 1995), thus PSII and PSI fluorescence signatures become discernable from each other.

Electron transport reactions, apart from those involved in primary charge separation and charge stabilization within the photosystems, are inhibited at 77 K. Electrons accumulate on the acceptor side of PSII, and on the acceptor side of a fraction of PSI (Sétif *et al*. 1984, Schlodder *et al*. 1998) after brief illumination, and remain at the electron acceptors, even during low-intensity illumination, such as applied during the collection of fluorescence emission spectra. However, when samples are dark-adapted and remain unexposed to light after being frozen freezing at 77 K, fluorescence yield changes upon illumination at 77 K reflecting the electron transport within the reaction centers (Ley and Butler 1980). Lowering the temperature to 77 K also leads to a decrease in energy and thus electrons occupy lower vibrational levels, reflected by a sharpening of the fluorescence emission band compared to room temperature. Excitations are more likely trapped on the longer wavelength Chls, as the energy of the vibrational ground state is higher in the shorter-wavelength Chls. Thus, at temperatures lower than 77 K, more fluorescence within PSII is emitted at 695 nm (in addition to 685 nm), PSII and PSI fluorescence emission shifts to longer wavelengths, and emission of additional low energy pigments increase. For example, in plants fluorescence of Chls in LHCII complexes (Rabinowitch and Govindjee 1969, Cho and Spencer 1966) are thought to trap excitation below liquid nitrogen temperature increasingly, thereby contributing to fluorescence emission at 680 nm (Rijgersberg *et al*. 1979).

Having a discernible PSI-specific fluorescence signal enables the investigation of the association of light-harvesting systems with PSI or PSII, by exciting pigments (such as Chl *b*, *c*, carotenoids, and phycobilins) that are preferentially located in the peripheral light-harvesting systems. Furthermore, direct excitation of Chl *a* and the resulting fluorescence emission patterns can be used to determine the stoichiometries of the photosystems (Murakami 1997), and has provided major insights into physiological adaptations of photosynthetic organisms.

Technical and practical aspects for 77 K fluorescence measurements

Several methodologies have been developed to obtain 77 K fluorescence spectra with a variety of instruments and sample preparation procedures. The following section contains an overview of these techniques and procedures.

Fluorometer and measurements

In a fluorometer that is capable of quantifying fluorescence emission, monochromatic light is used to excite a sample at the desired wavelength. The fluorescence is detected at an angle of 90° to the incident excitation beam. The illumination and detection wavelength can be modulated by optical filters in combination with monochromators. For obtaining 77 K fluorescence emission and excitation spectra, instruments that use monochromators in combination with full spectrum excitation light sources and photomultiplier tubes have traditionally been used (Hipkins and Baker 1985). However, for obtaining fluorescence emission measurements, a less costly instrument that uses LEDs as an illumination source and a CCD-array spectrometers can also be employed (Lamb *et al*. 2015; Fig. 5).

To obtain useful spectral data that lacks artifacts, care must be taken to avoid spectral distortions by self-shading (*see* Fig. 6) (Govindjee and Yang 1966, Weis 1985). A dilution series of the sample can be used to find the concentrations where spectral distortions occur. Comparing these dilution spectra reveals that self-shading

Table 1. Assignment of fluorescence emission signals at 77 K.

Band assignment	Origin of 77 K fluorescence	Key reference
F_{640}	phycoerythrin	Sobiechowska-Sasim *et al*. 2014
F_{645}	phycocyanin	Sobiechowska-Sasim *et al*. 2014
F_{660}	allophycocyanin	Sobiechowska-Sasim *et al*. 2014
F_{680}	LCM, Lhca, Lhcb, Lhcf (Lhcv), Lhxr, RedCLH	Rijgersberg *et al*. 1979
F_{685}	PSII core: CP43, IsiA	Andrizhiyevskaya *et al*. 2005
F_{695}	PSII core: CP47	Andrizhiyevskaya *et al*. 2005
$F_{710–720}$	Lhc aggregates	Yamamoto *et al*. 2013
$F_{720–760}$	PSI reaction center antenna (cyanobaceria) PSI peripheral antenna (plants) chlorophyll vibronic sublevels	Karapetyan *et al*. 2014

Table 2. Distribution of photosynthetic pigments. [1]Many cyanobacterial species contain Chl *a* as their only chlorophyll-type pigment. [2]Heterokont algae.

	Bilins	Chl *a*	Chl *b*	Chl *c*	Chl *d,f*
Cyanobacteria	√	√	√[1]	√[1]	√[1]
Red algae	√	√			
Diatoms[2]		√		√	
Eustigmatophyta[2]		√			
Brown algae[2]		√		√	
Dinoflagellates[2]		√		√	
Cryptomonads	√	√		√	
Green algae		√	√		
Plants (Viridiplantae)		√	√		

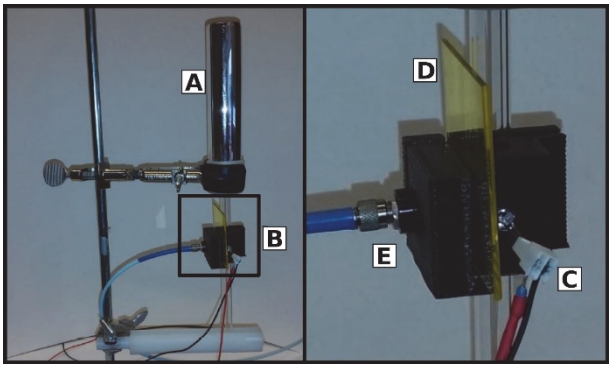

Fig. 5. Custom-built fluorometer. Images of a custom-built instrument setup published by Lamb *et al.* (2015). During data acquisition the instrument is covered by a black cloth, which has been omitted to show the setup. A: Dewar that holds the samples immersed in liquid nitrogen. The dewar part that orientates the sample in regards to the LED and detection fiber is supported by a white piece of plastic at the bottom, B: a 3D-printed housing, C: an excitation LED, D: a long-pass filter, and E: an optical fiber detector (at right angle to excitation LED). (Lamb *et al.* 2015).

leads to decrease in fluorescence yield of the bands associated with PSII (685/695 nm) compared to the PSI band, and a red shift of fluorescence emission spectra. With the sensitivity of modern instruments, compromises between spectra quality and signal-to-noise levels are unlikely to occur, but strategies and models for compensating for unavoidable self-absorption artifacts in tissues have been developed (Cordón and Lagorio 2006).

Optical filters can be employed to avoid spectral artifacts that are inherent to the optical components of the instruments and the characteristics of the sample. A (narrow) band pass filter can be used to eliminate stray light that has been transmitted by the excitation monochromator. Another artifact that can be removed by inserting a (narrow) band pass filter is the elimination of photons due to harmonic transmission by the excitation monochromators (*i.e.*, photons with 1.5-times the excitation wavelength and 2-times the excitation wavelength may also pass the monochromators). This 1.5-times wavelength harmonic is of relevance when Chl is exited by 435-nm light (resulting in an artefactual peak at 652.5 nm), whereas the 2-times wavelength transmission artifact is of relevance if Chl is excited in the UV part of the spectrum.

Furthermore, a long-pass filter can be used to avoid excitation light, which is scattered by the sample, from entering the detector (Lakowicz 1983a, b).

Data processing of fluorescence spectra

Before measurements are reported, it is necessary to correct the spectra, and explicitly state the measuring configuration used. Corrections to spectra are necessary due to spectral features of the illuminating light source, as well as the spectral response of the detector used. Useful instructions and background for obtaining corrected spectra have been provided before (Lakowicz 1983a,b; Hofstraat *et al*. 1992).

A feature of most Chl fluorescence data is that they are reported in "relative fluorescence units." The reason that most of the time non-quantified spectra, which report the fluorescence yield per absorbed photon, are reported, is the impracticality imposed by the lack of suitable equipment, as well as differences in the optical properties of samples, including scattering. Other factors that limit quantitative reporting of Chl fluorescence are changes in optical properties of the samples due to freezing, and difficulty in attaining reproducible orientation of the sample within the

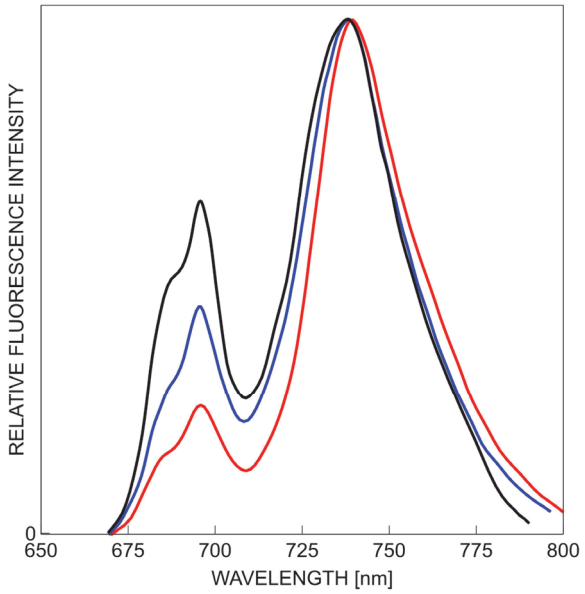

Fig. 6. Chlorophyll fluorescence emission of chloroplast fragments as a function of chlorophyll concentration. *The original figure legend*: "Emission spectra at –196°C of chloroplast fragments of spinach showing the effect of using different concentrations of chloroplasts. Exciting wavelength is 430 mμ. Further information from the text explaining the data: "The concentration is expressed on the graphs in per cent absorption at 680 mμ, ranging from about 100°-~ to 2%. These experiments show that reabsorption of the 696 mμ and 685 mμ emission bands is insignificant at less than 5%~ absorption at 680 mμ, but becomes significant at the higher concentrations. Thus, the 738 mμ band must be a "real" band (*i. e.* not due to reabsorption of the main band). (Further quantitative investigation is, however, required because our present data have not been corrected for reabsorption within a single chloroplast fragment.)"
This figure has been digitized from Govindjee and Yang (1966). *Remarks*: The term mμ is a historical notation that is equivalent with nanometer (nm). This figure was used to demonstrate that the 738 nm (PSI) emission band is not a reabsorption artifact.

used dewar. One way of standardizing the fluorescence measurements is the inclusion of a known quantity of fluorescent dye. The emission spectra of the included dye can then be used to normalize spectra against each other (*see* section on fluorescein below).

Dewars and cryostats

The dewar is at the heart of 77 K fluorescence measurements as it maintains the sample at low temperatures. Named after its inventor Sir James Dewar, a dewar is a specialized vacuum flask that houses low-temperature liquids, such as nitrogen or helium. For 77 K fluorescence measurements, the dewar takes the form of an open vessel, where a vacuum separates two glass walls. The dewar consists of a larger reservoir that houses the majority of liquid nitrogen and a thinner extension, which orientates the sample within the fluorometer. Samples that have already been frozen in liquid nitrogen are transferred to the dewar and maintained at a temperature of 77 K for the duration of the measurement. Dewars that have been designed to hold tubes for EPR experiments and other spectrophotometric measurements are commercially available. However, researchers who are lucky enough to have access to a good glassblower may find custom-designed dewars to be a cheaper alternative.

In addition to the relatively simple dewar-based setup, which can be adapted to many fluorometers, highly sophisticated instruments that interface with a defined suite of devices is also available. These instruments use cryostats, which allow precise temperature control down to liquid helium temperatures (4°C) and have optical interfaces for specific instruments or fiber optics.

Sample tubes

Unlike the situation for spectrophotometers, no standardized optical path length is used for 77 K experiments. In some laboratories, dewars made for EPR experiments are used, and thus matching EPR tubes with an inner diameter of 3–5 mm are used for measurements. An alternative to these commercial, closed-end tubes is an open-ended tube design, which provides a very cost-effective and practical alternative. The liquid samples can enter the tube by simply lowering the tube into the sample liquid. Once the tube is filled with the desired amount, the user seals the tube using their thumb and then freezes the sample in liquid nitrogen. The frozen liquid may escape from an open tube during measurements, and it is good practice to measure Chl fluorescence without a sample to assess contamination. In our experience, contamination does not occur even after measuring up to one hundred tubes. The real advantage of the open tube design is the ease of cleaning the tube so they can be reused continuously. When tubes are stored in a liquid nitrogen dewar for a long time, a film of ice can form, which should be removed with tissue paper, before inserting the sample into the dewar for measurements.

Fluorescein

An approach to compare the amplitude of fluorescence spectra is to introduce a fluorescent molecule to the sample at a known concentration. This allows the spectra from different samples to be normalized irrespective of their sample preparation properties, thus providing a quantitative insight into the changes between samples. One such molecule that can be included in the sample is fluorescein (Sjöback *et al*. 1995). When light is used to excite Chl *a*, it has an excitation maximum between 435–460 nm, and this light also excites fluorescein. The emission maximum of fluorescein is then observed at 508 nm (El Bissati *et al*. 2000), 545 nm (Walters and Horton 1991), or 535 nm (Krause *et al*. 1983, Krause and Weis 1984).

Glycerol

Glycerol has frequently been added to samples before freezing to 77 K as a cryoprotectant. This treatment reduces the formation of ice crystals during the freezing

procedure, therefore reducing damage to the sample and preventing light scattering. Unfortunately, glycerol treatment has been found to alter 77 K spectra in cyanobacteria (Mullineaux 1994) by reducing the affinity of phycobilisomes to PSII, resulting in increased fluorescence emission by the phycobilisomes (Mullineaux 1994). A detailed study on the effect of glycerol on the photosynthetic machinery of the cyanobacterium *Spirulina platensis* (Li *et al*. 2007) revealed that glycerol weakens energy transfer of the terminal phycobilisome emitter (L_{CM}) to the reaction center of PSII as well as interfering with energy transfer between phycobilins.

Sample preparation

Samples for measurement of fluorescence at 77 K can either be suspensions, such as algae, extracted thylakoids, and chloroplasts, or photosynthetic tissues, such as whole leaves. Dependent on the sample type, different sample preparation methods for measurements at 77 K have been developed. For standardization, samples are diluted or concentrated to the same Chl concentration. This concentration must be low enough to avoid self-shading of excitation and re-absorption of the emission light, yet high enough to obtain a high signal to noise ratio.

Cell suspensions and thylakoids

The fluorescence emission of many single-celled organisms can be measured in their respective growth medium. However, some cyanobacteria and alga accumulate fluorescent molecules within their growth medium, and thus washing with fresh culture medium may be required. When cells are spun down for washing with the fresh medium, it is crucial to develop a rapid procedure to do so, as prolonged exposure to new conditions, such as the absence of light within the centrifuge or changes in pH may elicit a physiological response. A physiological response that has severe consequences for Chl fluorescence within a short period is anaerobic incubation (Hohmann-Marriott *et al*. 2010) that can rapidly occur within cell pellets.

Thylakoids of cyanobacteria, algae, and plants contain the photosynthetically active protein complexes. For some experiments, it is desirable to remove the thylakoids from the organisms to control physical conditions, such as pH and the concentrations of ions, and to administer artificial electron donors and acceptors. Isolated chloroplasts and thylakoid membranes can be stored (Farkas and Malkin 1979) in the freezer. Once isolated and resuspended in their buffered media, samples can be inserted directly into sample tubes and frozen using liquid nitrogen, and are ready for 77 K fluorescence analysis. An alternative to freezing cells and thylakoids within the medium is to soak these samples up within filter paper and then rapidly freeze the sample by plunging it in liquid nitrogen. The resulting samples can then be treated like leaf tissue.

Photosynthetic tissues

For the photosynthetic tissue of multicellular organisms, such as plants and macroalgae, sample preparation differs from that of unicellular organisms. Leaf disks can be prepared readily and inserted into a dewar. However, the orientation of the tissue is a crucial parameter, as is the case for plant leaves, where the upper and lower side of the leaves have different spectroscopic properties (Björkman and Demmig 1987). Therefore, a tissue sample must have a known orientation that is stable during measurements. Due to the high Chl concentration, there is substantial self-shading in most intact photosynthetic tissues. To overcome self-shading, Egelbert Weis developed a method that dilutes the Chl concentration within tissues to a lower concentration (Weis 1985). For this, the tissue sample is ground up in a liquid nitrogen-cooled mortar, and water (Pfundel and Pfeffer 1997) or quartz (Weis 1985) is added to dilute the sample. The ground sample is then inserted into a closed, cooled glass tube with a small diameter. The original description of the method for "diluted leaf powder" (Weis 1985) also demonstrates the spectral shifts of fluorescence emission spectra that occur due to self-shading.

Processing of 77 K fluorescence emission data

Recorded raw 77 K fluorescence spectra are usually processed, before presenting them in publications, in order to remove artifacts. After compensating spectra for light source and instrument response curves (*see* "Data processing section"), remaining artifacts arise from the fluorescence of the medium the samples are suspended in, and scattering. Recording of media fluorescence emission spectra can be used to compensate for these artifacts. Spectral features of the medium that fall outside the fluorescence spectra of the sample can be used to scale the medium fluorescence and subtract it from the sample spectrum. Some bacteria and algae produce fluorescent molecules that accumulate in the medium. The fluorescence of these molecules together with scattering artifacts can form a broad spectral band that extends into the Chl fluorescence spectra. This spectral band can often be effectively subtracted from the Chl fluorescence spectrum by an exponential or Gaussian function. These functions can be anchored at a wavelength were no, or very little Chl fluorescence is expected, which is usually at 800 nm or 850 nm in oxygenic organisms. Adjusting the fluorescence level at 850 nm to zero, even without the subtraction of a logarithmic function for artifact removal, is often performed to display data in figures.

77 K fluorescence emission of isolated complexes and organisms

Photosystems

The dominant room temperature fluorescence emitted by PSII, together with its variability, has been an early focus of spectral analysis in photosynthesis research. The insight that two photosystems are working in concert in oxygenic photosynthesis (Rabinowitch and Govindjee 1969) lead to a hunt for the fluorescence signal that is emitted by the second type of reaction center. Fluorescence measurement at 77 K played a crucial part in establishing the identity of the second type of photosystem we now know as PSI. The discovery and miss-assignments of Chl fluorescence signals have been reviewed by Govindjee (2004) and Strasser *et al*. (2004).

Photosystem II

In this section, we focus on the fluorescence emitted by PSII. As PSII is associated with different light-harvesting systems in different organisms, we only discuss the fluorescence characteristics of the PSII reaction center antennae that contain Chl *a,* which are well established. There remains uncertainty about a functional assignment within PSII in Chl *d*-containing *Acaryochloris* species, as well as Chl *d*- and *f*- containing cyanobacteria capable of red light photoacclimation (FaRLiP) (Gan *et al*. 2015). The 77 K spectra of Chl *d*- (Miyashita *et al*. 1996) and Chl *f*- (Chen *et al*. 2012) containing PSII are discussed in details in the original literature.

PSII is composed of the reaction center core and the reaction center antenna. The PSII reaction center core houses 6 molecules of Chl *a*, and 2 *β*-carotenes, 2 phaeophytins, and 2 quinones are associated with D1 (PsbA) and D2 (PsbD). Four of these Chls are part of the special set of Chls that mediates charge separation, while the remaining 2 Chls energetically couple the special set of Chls to the reaction center core antenna. The 32 Chl *a* molecules, and 6 *β*-carotenes, which make up the core antennae pigments, are housed in the proteins CP43 (PsbC) and CP47 (PsbD). The absorption and fluorescence spectra of isolated CP43 and CP47 (Fig. 7*A*,*B*) and isolated PSII (Fig 7*C*,*B*) reaction centers of plants and cyanobacteria are very similar indicating a very stringent conservation through evolution.

At room temperature, PSII emission originates predominantly from Chls fluorescing at 695 nm. Upon further cooling to 77 K, a distinct fluorescence at 685 nm also gains prominence. The emission at 695 nm was early on correctly assigned to be emitted by Chls within PSII (reviewed by Govindjee 2004). The fluorescence emission at 685 nm, however, was first thought to emanate from the antenna complexes of plants (reviewed by Strasser *et al*. 2004). This fluorescence emission was later also assigned to the PSII reaction center antenna proteins CP43 (Rijgersberg *et al*. 1979) and CP47 (Nakatani *et al*. 1984). More recent studies on isolated plant PSII particles (Andrizhiyevskaya *et al*. 2005) confirm the association of

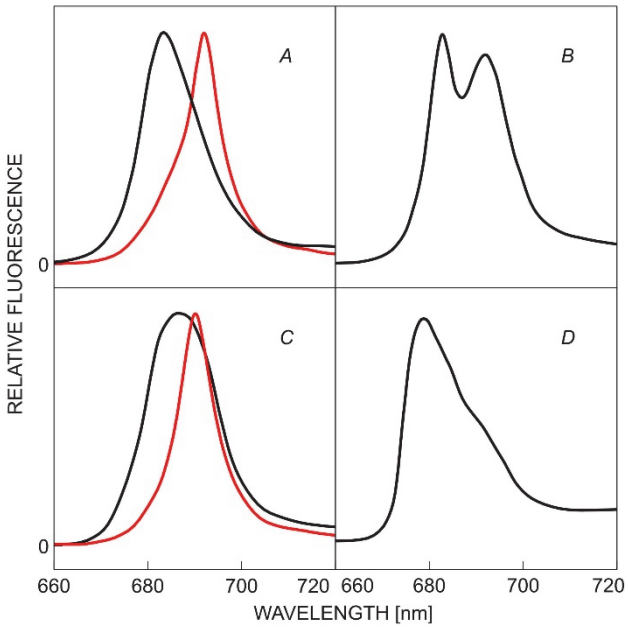

Fig. 7. Fluorescence emission from PSII, isolated PSII and CP43 and CP47 from cyanobacteria and plants. Fluorescence emission spectra of CP43 (*black*) and CP47 (*red*) (*A*) excited at 488 nm, isolated PSII that contains a histidine-tag, (*B*) excited at 435 nm from the cyanobacterium *Synechocystis* sp. PCC 6803. Fluorescence emission spectra CP43 (*black*) and CP47 (*red*) (*B*) excited at 488 nm, and isolated PSII (*D*) excited at 435 nm from plant *Arabidopsis thaliana*. The fluorescence spectra have been digitized from Boehm *et al*. (2011) (*A*, *C*), Liu *et al*. (2011), and Irrgang *et al*. (1988) (*D*).

695 nm emission with CP47, but indicate that the 685 nm emission arises from fluorescence emitted by both CP47 and CP43 at 77 K. Compared to room temperature, the fluorescence emission by PSII increases by a factor of about two when cooled to temperature of 77 K. At this temperature, PSII still contributes to long wavelength fluorescence, which overlaps with PSI fluorescence emission at 720/740 nm (Butler 1977).

PSII assembly and repair

The *de novo* assembly of PSII subunits is a fascinating topic (Eaton-Rye and Sobotka 2017) that also informs our understanding of the evolution of photosynthesis (Cardona 2016). In both cyanobacteria and plants, the assembly of PSII begins with binding of cytochrome b_{559} to the D2 subunit in the thylakoid membrane, forming the D2 pre-complex (Komenda *et al*. 2012, Nickelsen and Rengstl 2013). The D1 pre-complex is then bound, resulting in the heterodimeric reaction center pre-complex. Addition of the CP47 pre-complex to the heterodimeric reaction center pre-complex forms the RC47 complex (Boehm *et al*. 2012). The CP43 core antenna also forms a pre-complex with other subunits that bind to the RC47 complex.

Repair of damaged PSII shares common features with the assembly of PSII (Järvi *et al*. 2015). Damage to PSII reaction centers by light often affects D1 and results in the removal and consequent degradation of the damaged D1 protein (Komenda *et al*. 2012, Mulo *et al*. 2012). The repair of the damaged PSII involves the synthesis and insertion of a new D1 polypeptide into the PSII complex (Kyle *et al*. 1984, Järvi *et al*. 2015). This repair mechanism allows minimal energy expenditure during this process (Nixon *et al*. 2005, Takahashi and Badger 2011). Subcomplexes can be characterized by the presence or absence of fluorescence signal specific for the CP43 and CP47. In addition, increased fluorescence yield of peripheral light-harvesting complexes indicates that the assembly is not well coordinated or that PSII is damaged. Fluorescence spectroscopy at 77 K has provided valuable insights into the assembly and repair of the photosynthetic machinery (McCormac *et al*. 1996, Mysliwa-Kurdziel *et al*. 1997, Komenda *et al*. 2012, van Wijk *et al*. 1995).

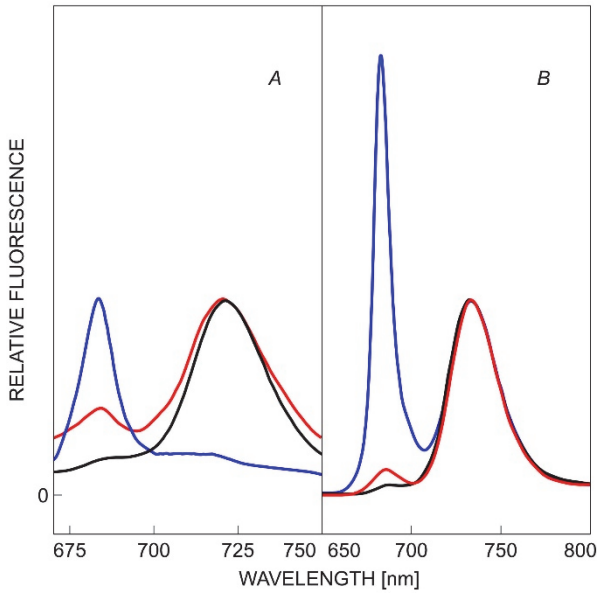

Fig. 8. Fluorescence emission from isolated PSI and PSI super complexes from cyanobacteria and plants. The fluorescence emission spectra (excited at 440 nm) of isolated PSI (*black*), isolated CP43' and (*blue*) isolated CP43'-PSI supercomplex (*red*) of the cyanobacterium *Synechocystis* PCC 6803 are shown in panel *A*. The fluorescence emission spectra (excited at 440 nm) of isolated PSI-Lhca-super complex (*black*), Lhcb-trimers (*blue*) PSI-Lhca-(Lhcb-trimer) super complex (*green*) of the plant *Arabidopsis thaliana* is shown in panel *B*. The emissions in both panels are normalized to the maximum of the Soret band.
The fluorescence spectra in panel *A* have been digitized from Bibby *et al*. (2001a).
The fluorescence spectra in panel *B* have been digitized from Galka *et al*. (2012).

Photosystem I

The discovery and assignment of the fluorescence emission at 720/730 nm to PSI is closely linked to the establishment of the two photosystem model for oxygenic photosynthesis (reviewed by Govindjee 2004). PSI complexes show a wide variety of emission spectra depending on the species and physiological conditions, but are located between 720–730 nm in oxygenic phototrophs, with some cyanobacteria having emission bands centered at wavelengths as long as 760 nm (Karapetyan *et al*. 2014).

The PSI reaction center of cyanobacteria (Fig. 8*A*), green algae, and plants (Fig. 8*B*) consists of two proteins (PsaA and PsaB) which together house around 85 Chls (Jordan *et al*. 2001). In addition, several smaller protein complexes contain about 10 Chl *a* molecules in cyanobacteria and plants. Cyanobacteria – which can form PSI trimers – are known to expand the trimeric PSI in stress conditions by circular antenna system that contributes an additional 180 (Boekema *et al*. 2001) to 218 (Bibby *et al*. 2001a) Chl *a* molecules.

The absorption cross-sections of PSI of green algae, red algae, heterokont algae, and plants are further expanded by light-harvesting systems that belong to the three trans-membrane helix family of light-harvesting complexes (Busch *et al*. 2010). In plants, four of these light-harvesting complexes (Lhca) are associated with PSI, adding 52 Chl *a* and 9 Chl *b* molecules. An additional 20 Chl *a* molecules interface the Lhca with the reaction center (Mazor *et al*. 2015). An unknown number of light-harvesting systems are associated with the PSI of red algae and heterokont algae.

In cyanobacteria and plants, fluorescence around 720 nm is emitted by Chls within the reaction center antennae (Karapetyan *et al*. 2014). Plants also possess an additional pool of long wavelength emitters located in the Lhca antenna (Morosinotto *et al*. 2003), specifically within Lhca3 and Lhca4 (Wientjes *et al*. 2011). This assignment is in agreement with previous greening studies, which indicated that the fluorescence emission at 736 nm is emitted by peripheral antennae complexes (containing Chl *b*), while the emission around 724 nm is emitted by the PSI reaction center antennae (Mullet *et al*. 1980a,b).

In plants, the fluorescence emission of PSI at 77 K is not modulated by the reduction state of P700. However, in red alga (Ley and Butler 1977) and cyanobacteria, (Karapeyan *et al*. 2014), modulation of the fluorescence yield dependent on the reduction state of P700 have been reported, but during steady-state fluorescence emission measurements, this modulation can be neglected.

Interpretation of 77 K fluorescence in different organism groups

Cyanobacteria and red algae

Cyanobacteria are a diverse group of oxygen-producing organisms, which share many features of their photosynthetic machinery with their ancestors, which gave rise to the chloroplasts of photosynthetic eukaryotes (Hohmann-Marriott and Blankenship 2011). This evolutionary relationship is reflected in the photosynthetic machinery of red algae, which uses similar pigments and protein structures as cyanobacteria. Several cyanobacteria have achieved model status, including unicellular *Synechocystis* and *Synechococcus* species, as well as multicellular, nitrogen-fixing *Anabaena* and *Nostoc* species, while the unicellular *Cyanidioschyzon* and multicellular *Porphyridium* species are red algal model systems. In the following section, we mainly discuss the well-characterized photosynthetic machinery of cyanobacteria with the inference that the photosynthetic machinery of red algae is similar.

Peripheral light-harvesting systems and supercomplexes

What makes cyanobacteria a rewarding species for spectroscopic studies are their light-harvesting pigments, the phycobilins, which are covalently linked within phycobiliproteins. The phycobiliproteins can be assembled into large structures, the phycobilisomes (de Marsac 2003, Marx *et al*. 2014). Phycobilins absorb light within the "green gap" between the two main absorption bands of Chl *a*. Phycobilisomes are in many conditions primarily associated with PSII, but as discussed in detail later, can dynamically (Mullineaux 2014) adjust this association and can form supercomplexes with both photosystems (Liu *et al*. 2013). There are several types of phycobilins; including phycoerythrobilin, phycocyanobilin, and allophycocyanobilin, but not all species of cyanobacteria contain all of these phycobilins. For example, *Synechocystis* sp. PCC 6803 contains phycocyanin, allophycocyanin, and phycoerythrin, while the latter is absent in *Synechococcus* sp. PCC 7002. Phycoerythrin absorbs excitation between 475–575 nm (maximum 565 nm), and fluoresces maximally at 640 nm; phycocyanin absorbs excitation between 525–635 nm (maximum 620 nm), and fluoresces at maximally at 644 nm; allocyanophycin absorbs between 550–665 nm (maximum 650 nm), and fluoresces maximally around 660 nm (Sobiechowska-Sasim *et al*. 2014). Excitation of phycobilins in cyanobacteria and red algae at 77 K induces fluorescence emission by phycobilins, which are pronounced when the interactions of phycobilisomes with the reaction centers are disturbed (Kaňa *et al*. 2014). The terminal phycobilisome emitter, *i.e*., the protein containing the pigments that energetically couple the phycobilisomes to the core reaction center antenna, is different for PSI and PSII. An allophycocyanin (ApcD) interfaces with PSI (Dong *et al*. 2009), while PSII interfaces with a multidomain protein named L_{CM} (encoded by *apcE*) (Tang *et al*. 2015), which contains a phycocyanobilin with a geometry usually found in phytochromes. This pigment within L_{CM} absorbs maximally around 655 nm and fluoresces maximally around 670 nm. As the number of Chl per reaction center is higher in PSI (96 Chl *a* molecules) (Jordan *et al*. 2001) than that in PSII (35 Chl *a* molecules) (Umena *et al*. 2011), exciting Chls at 435 nm preferentially excites PSI, while photons with a wavelength of 590/635 nm preferably excite PSII in cyanobacteria. The PSI/PSII ratio in cyanobacteria is much higher than 1, and consequently more than 90% of Chls can be associated with PSI.

Some cyanobacteria can utilize other Chls, in addition to Chl *a,* to perform oxygenic photosynthesis. A polyphyletic group of organisms, the prochlorophytes, possess a membrane-embedded antenna system that houses Chl *b* along with Chl *a,* which is related to the stress-induced antenna protein isiA and the PSII-subunit CP43 (La Roche *et al*. 1996), with similar fluorescence emission spectra. Chl *d* is used by the *Acaryochloris marinus*, and Chl *d* and Chl *f* (Li and Chen 2015) are used by a diverse group of cyanobacteria that take advantage of environments rich in far-red photons.

Photosystem I

Cyanobacterial PSI has species-specific long wavelength-emitting Chls ranging from 727–760 nm with 2-7 Chls estimated to contribute to the emission (Karapetyan *et al*. 2014). There is also a spectral range of emission in red algal PSI, ranging from 708 nm for *P. cruentum* to 728 nm for *C. caldarum*. Trimeric PSI complexes are commonly found in cyanobacteria under a variety of conditions (Fig. 8*A*) (Boekema *et al*. 1987, 2001; Garczarek *et al*. 1998) and *Synechocystis* sp. PCC 6803 (Bibby *et al*. 2001a,b). Some cyanobacteria can form monomeric, trimeric, and tetrameric PSI, with each possessing different optical characteristics including Chl fluorescence emission bands at 77 K (monomer at 725 nm, trimer at 730 nm, and tetramer at 715 nm) (Li *et al*. 2014). In red algae, however, PSI appears to be monomeric (Gardian *et al*. 2007).

Red algae have light-harvesting complexes related to the Lhca and Lhcb of alga and plants, which are absent in cyanobacteria (Busch *et al*. 2010). These three-transmembrane helix proteins, named Lhcr, are exclusively associated with the PSI of red algae. As in cyanobacteria (Kondo *et al*. 2007, Watanabe *et al*. 2014), there is good evidence that a specific pool of phycobilisome proteins is associated with PSI in red algae also (Busch *et al*. 2010).

State transition

State transitions are dynamic adjustments of the

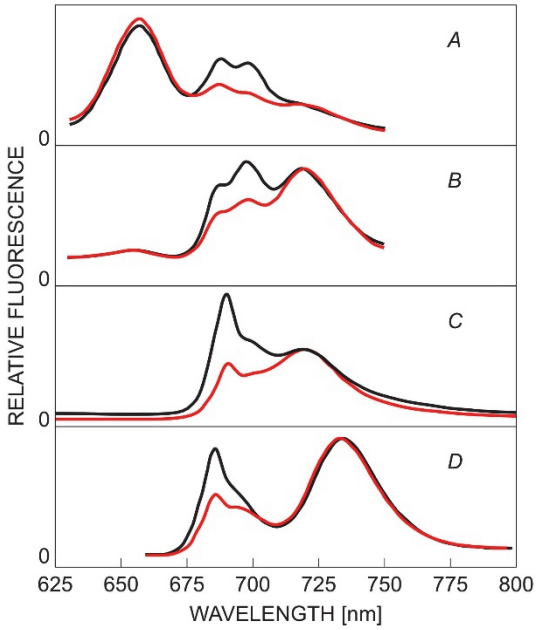

Fig. 9. Fluorescence emission characteristics of state transition in cyanobacteria, green algae, and plant during state transition. State 1 is indicated by the black trace while state 2 is indicated by the red trace. The cyanobacterium *Synechococcus* 6301 was excited at 600 nm (*A*) and 430 nm (*B*). S*ynechococcus* cells were locked in the state 1 by treating the cells with far-red light, which preferentially excites PSI, while the cells investigated in the state 2 were dark-acclimated. The green algae *Chlamydomonas reinhardtii* was excited at 455 nm (*C*). *Chlamydomonas* cells were locked in the state 1 by application of the PSII inhibitor DCMU under the light, and in the state 2 by treatment with the uncoupler FCCP. The plant *Arabidopsis thaliana was* excited at 435 nm (*D*). To lock the *Arabidopsis* cells in different states, excitation light favoring PSII and PSI, respectively, was utilized. Spectra were and normalized to the Soret emission band for all figures.
Data has been digitized from Mullineaux and Allen (1990) for *Synechococcus* 6301, Iwai *et al*. (2008) for *Chlamydomonas reinhardtii*, and Dietzel *et al*. (2011) for *Arabidopsis thaliana*. State 1 is shown in black and state 2 is shown in red.
Remarks: Excitation light at 600 nm preferentially excites phycobilins over chlorophyll *a*, thus panel *A* indicates that phycobilisomes donate more energy to PSII in state 1. *Synechococcus* 6301 can synthesize phycocyanin and allophycocyanin, while genes for phycoerythrin synthesis are missing, and therefore no fluorescence emission characteristic of phycoerythrin is observed.

photosynthetic machinery that modulate the distribution of absorbed light energy between PSI and PSII (Fig. 9*A*) (Mullineaux 2014). 77 K fluorescence studies in cyanobacteria and red algae (Murata *et al*. 1966), played a crucial role in establishing the concept of state transition, as changes in energy distribution could be easily investigated by preferentially illuminating the phycobilins while monitoring the fluorescence emission bands specific for PSI and PSII (Murata 1969). These studies indicate that both PSI and PSII receive excitation that was absorbed by phycobilins (Mullineaux 1992). Whether state transitions are based on a physical movement of phycobilisomes between PSI and PSII, or energy transfer occurs between Chl *a* in PSII and PSI, in a process called "spillover" (Biggins and Bruce 1989), was debated for a long time (McConnell *et al*. 2002, Li *et al*. 2004). However, there is now good evidence that the movement of phycobilisomes is a critical part of state transitions (Joshua and Mullineaux 2004), while spill-over appears not to be the major route for transferring energy from the phycobilisomes to PSI (Mullineaux 2008). A recent detailed study on the movement of phycobilisomes in different red algae (Kaňa *et al*. 2014) found that phycobilisomes have very limited mobility in the thermophilic red algae *C. caldarium*, while the mesophilic red algae *P. cruentum* exhibited phycobilisome movement in analogy to state transitions observed in cyanobacteria.

Stress-induced adaptations

Iron limitation induces changes in the composition of the photosynthetic machinery in cyanobacteria. Under iron limitation, proteins encoded by the *isiAB* operon are expressed (Pakrasi *et al*. 1985a,b, Laudenbach *et al*. 1988, Riethman and Sherman 1988, Burnap *et al*. 1993). While *isiB* codes for a thioredoxin that can functionally replace ferredoxin, IsiA possesses homology to the Chl *a*-binding PSII protein CP43 (Burnap *et al*. 1993, Falk *et al*. 1995), and can function as a PSI antenna system under stress conditions. During iron limitation, 18 IsiA from an antenna ring around trimeric PSI in *Synechococcus* sp. PCC 7942 (Boekema *et al*. 2001) and *Synechocystis* sp. PCC 6803 (Fig. 8*A*) (Bibby *et al*. 2001a,b). It was also suggested that IsiA could replace CP43, thus acting as an alternative antenna complex for PSII (Pakrasi *et al*. 1985b), or as an excitation energy dissipater, with the ability to protect PSII from photoinhibitory damage during iron starvation (Park *et al*. 1999). The induction of the IsiA protein under strong light, even in the presence of iron, confirmed its photoprotective role (Havaux *et al*. 2005). The presence of IsiA can be discerned by an increase in 685 nm emission (Burnap *et al*. 1993, Falk *et al*. 1995, Park *et al*. 1999), and an increase in energy partitioning to PSI. The latter is reflected by a more prominent fluorescence emission by the red Chls of the PSI reaction center antenna Chls around at 720 nm.

Green algae and plants

Green alga and plants share a common ancestor and consequently share features of their photosynthetic machinery (Keeling 2013). The green algae *Chlamydomonas reinhradtii* has many features that make it a valuable

organism for photosynthesis research, such as toolboxes for genetic manipulation, and the ability to grow non-photosynthetically. However, *Chlamydomonas* possesses many features that are altered or unique compared to plants (Erickson *et al*. 2015).

Peripheral light-harvesting systems and super-complexes

In plants and green algae, the absorption cross-section of the reaction center is extended by membrane-embedded light-harvesting systems. In addition to Chl *a* that is also found in the reaction center core and reaction center antennae, plants and green algae also synthesize Chl *b* that is associated with peripheral membrane-bound antenna complexes (LHCs). These antenna complexes and non-pigment-containing linker complexes form different supercomplexes with PSI and PSII, some of which can be isolated and structurally and spectroscopically characterized (Fig. 10) (Tokutsu and Minagawa 2013, Wei *et al*. 2016).

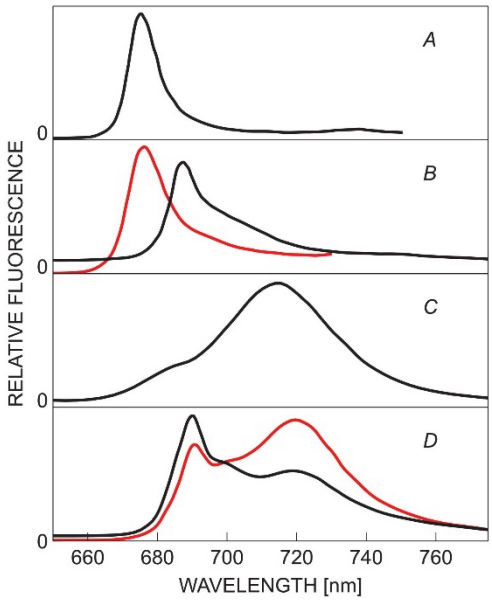

Fig. 10. Fluorescence emission spectra of photosynthetic components of the green algae *Chlamydomonas reinhardtii*. (*A*) The 77 K fluorescence emission spectrum (excitation wavelength 440 nm) of trimeric LHCII complexes isolated from *C. reinhardtii* (Natali and Croce 2015). (*B*) The 77 K fluorescence emission spectra of an isolated His-tagged PSII core complex (*black*) (excitation wavelength 435 nm) (Sugiura and Inoue 1999), and the 77 K fluorescence emission (excitation wavelength 440 nm) of PSII-LHCII super complex (*red*) (Drop *et al*. 2014). (*C*) The 77 K fluorescence emission spectra of LHCI-PSI super complex (excitation wavelength is 435 nm) (Kargul *et al*. 2003). (*D*) The 77 K fluorescence emission spectrum of a whole cell in state 1 (*black*) and state 2 (*red*) (excitation wavelength 440nm) (Iwai *et al*. 2008).
Panel *A* was digitized form Natali and Croce (2015). Panel *B* was digitized from Sugiura and Inoue (1999) and Drop *et al*. (2014). Panel *C* was digitized from Kargul *et al*. 2003 (2003). Panel *D* was digitized from Iwai *et al*. 2008 (2008).

Major and minor light-harvesting complexes

The protein complexes that house the pigments of the peripheral antennae can be grouped into monomeric Chl-binding complexes often called minor antennae complexes and trimer-forming LHCs (Busch *et al*. 2010) that are predominantly associated with PSII (Lhcb) or PSI (Lhca). Some Lhcb proteins in plants and algae can change the association between PSII and PSI during state transitions. Lhcb can form homo- and hetero-trimers that house 24 Chl *a* molecules, 18 Chl *b* molecules, and 12 carotenoids, with isolated trimers emitting fluorescence maximally between 678–680 nm at 77 K (Standfuss and Kühlbrandt 2004). Modeling in combination with mutational deletion of Chl ligands (Novoderezhkin *et al*. 2005) identified a spatially clustered group of three Chls as the final emitters, *i.e*., these Chls are likely to donate excitation to the PSII reaction center. These Chls are likely responsible for fluorescence emission at 680 nm (F_{680}). Lhcb can also form aggregated states that may be involved in photoprotection through excitation quenching, and exhibit a red-shifted fluorescence emission at 77 K (700–715 nm) (Ruban *et al*. 1997, 2012).

Photosystem II

The PSII of green alga and plants is organized in a dimer and Lhcb trimers can interface with both reaction centers of this dimer. Excitation transfer from the trimer to the reaction center is accomplished *via* three pigment-containing protein subunits (CP29, CP26, and CP24) associated with each reaction center monomer. Structural studies show that the subunit CP29 contains 10 Chl *a* molecules, 3 Chl *b* molecules, 3 carotenoids, and CP26 contains 9 Chl *a* molecules, 3 Chl *b* molecules, 3 carotenoids (Wei *et al*. 2016). Mutational analysis and modeling suggest that CP24 contains 5 Chl *a* molecules, 5 Chl *b* molecules, and two carotenoids (Passarini *et al*. 2009). These minor Chl-containing complexes are not known to contribute to 77 K fluorescence in intact systems largely.

Photosystem I

In contrast to PSI of cyanobacteria, the green algal and plant PSI possess tightly associated Lhca (Ben-Shem *et al*. 2003). The PSI supercomplex of *Pisum sativum* contains the reaction center proteins (PsaA and PsaB) as well as four Lhca (Lhca1–4) that form the peripheral antennae as a "dimer of dimers" (Mazor *et al*. 2015). Around 20 additional Chls energetically link the PSI reaction center with the peripheral antennae (Ben-Shem *et al*. 2003). The fluorescence emission of plant PSI is mainly due to Chl *a* in the Lhca antenna (Croce *et al*. 1998). Specifically, it is thought that one Chl *a* dimer within each Lhca (Qin *et al*. 2015) is the emitter of long wavelength fluorescence, whereas the reaction center core is only a minor contributor to this emission. Interestingly, no long wavelength emission was observed in PSI isolated from a prasinophyte green algae (Swingley *et al*. 2010).

State transition

The term "state transition" and its historical development has been introduced in the section on cyanobacteria. It became apparent that algae and plants also perform state transitions (Bonaventura and Myers 1969). As in cyanobacteria, the physiological basis for state transitions in green algae and plants appears to be the imbalance of electrons produced by PSII and utilized by carbon fixation (Minagawa 2011). However, in green algae and plants, the excitation coupling of Lhcb, not the excitation coupling of phycobilisomes to the two photosystems, is modulated. In the presence of a (partly) oxidized PQ pool, Lhcb are tightly excitationally coupled to PSII. Upon reduction of the PQ pool, some Lhcb are phosphorylated (Allen 1992) and consequently more Lhcb donate excitation to PSI. Whether much physical movement of the Lhcb is required for state transition is still unclear. It seems unlikely that a substantial mass migration of Lhcb between PSII-rich grana and PSI-rich stroma occurs, but instead, Lhcb may make additional contact with PSI. State transitions can be readily observed (Fig. 9*B*) by exiting samples at either the Chl *a* (430/440 nm) or Chl *b* (450/455 nm) Soret absorption band, while observing the emission bands of PSII (685/695 nm) and PSI (720/740 nm), with Chl *b* excitation providing a more specific signal for changes in excitation distribution.

Heterokont algae

Heterokonts comprise many photosynthetic algae including diatoms (*Bacillariophyceae*), brown algae (*Phaeophyceae*), and *Eustigmatophyceae*. Four membranes surround the chloroplast of these algae, indicating a secondary (Cavalier-Smith 1999) or even more complex endosymbiotic history (Keeling 2013).

Phaeodactylum

Major and minor light-harvesting complexes

Phaedactylum tricornutum is a pennate diatom that, despite several clade-unspecific features regarding its live cycle and morphology, has achieved model status. The photosynthetic machinery of *Phaeodactylum,* however, shows features that are consistent and typical for most diatoms. In addition to light absorbed by Chl *a,* diatoms use alternative pigments, in particular, *c*-type Chls ($c_{1–3}$) and carotenoids, with fucoxanthin being the most common. This composition of pigments was used to name the light-harvesting systems that are typical of many heterokont algae, the fucoxanthin-Chl-binding proteins (FCP) (Gundermann and Büchel 2014). The peripheral light-harvesting systems of most heterokont algae can be preferentially excited using the Soret band of Chl *c* (465 nm) and fucoxanthin (530 nm), while Chl *a*-specific photons can be used to excite the reaction center antenna preferentially.

FCP is related to the three transmembrane-helix LHCs of plants and algae. The FCP can be grouped into three fractions: (1) FCPs, which are unique to heterokont algae (Lhcf), (2) FCPs, which are related to red algal Lhca (Lhcr), and (3) FCPs, which related to LhcSR, a protein complex characterized in the green algae *Chlamydomonas* and the moss *Physcomitrella* (Lhcx) (Gundermann and Büchel 2014). Homology modeling in combination with spectroscopic and biochemical characterization suggest that each Lhcf can bind 6 Chl *a* molecules, 4 Chl *c* molecules, and 5–6 carotenoids, which in addition to fucoxanthin may also include lutein, diadinoxanthin, and diatoxanthin (Gundermann and Büchel 2014). In addition to Lhcf, another distantly related three-transmembrane helix protein belonging to the "red lineage Chl *a*/-binding-like proteins" (RedCAP) is also present in heterokont algae.

Lhcf can form trimers (Lepetit *et al*. 2007, Nagao *et al*. 2013) analogous to the Lhcb trimers in plants and algae. While some uncertainty about the association of Lhcf to PSII to heterokont algae remains, there is good evidence that Lhcf associates predominantly with PSI (Juhas and Büchel 2012).

The fluorescence emitted by PSI-associated FCP has two prominent bands at 685 nm and 697 nm (Fig. 11*A*). These fluorescence bands, however, are emitted by a group of Chl *a*, which are unlikely to be at homologous locations to the terminal emitter in the Lhca and Lhcb of plants (Gundermann and Büchel 2014). The absence of emission bands associated with Chl *c* species indicates that all Chl *c* is efficiently coupled to Chl *a* within the FCPs.

Photosystem II

PSII core complexes form dimers in diatoms, such as *Phaeodactylum*, and exhibit a fluorescence emission band with a maximum at 692 nm (Fig 10*B*) (Yokono *et al*. 2015). This emission band appears to be a combination of a 692 nm and a 684 nm emitter located in CP47 (Yokono *et al*. 2015), while CP43 fluorescence emission is absent at 77 K. A different 77 K fluorescence emission pattern is observed in monomeric PSII, where the maximum fluorescence is emitted around 687 nm (Yokono *et al*. 2015). There is little biochemical evidence that any FCPs are associated with PSII to form stable super complexes in heterokont algae (Grouneva *et al*. 2011) or at least not into very stable super complexes (Nagao *et al*. 2010). However, spectroscopy-based studies reveal that the functional antenna size of PSII can be dynamically adjusted, thus suggesting a functional association between PSII and some FCPs (Miloslavina *et al*. 2009).

Photosystem I

Isolated PSI of diatoms has a long wavelength emission at

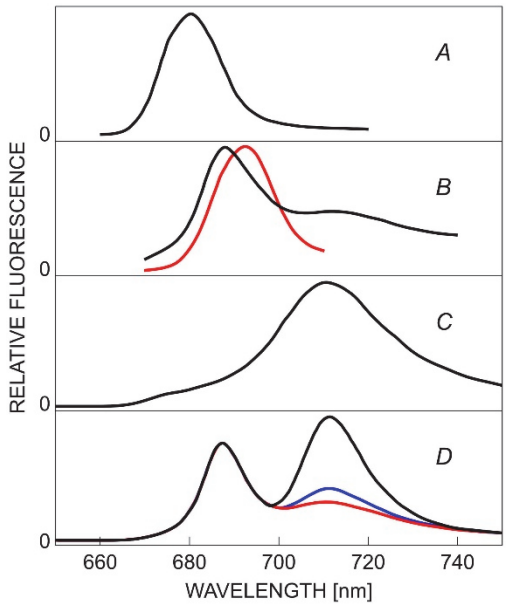

Fig. 11. Fluorescence emission spectra of photosynthetic components of heterokont diatom algae (*Phaeodactylum and Chaetoceros gracilis*). (*A*) The 77 K fluorescence emission spectrum (excitation wavelength 435 nm) of FCP from *Phaeodactylum tricornutum* (Litvín *et al*. 2016). (*B*) The 77 K fluorescence emission spectrum (excitation wavelength 425 nm) of PSII monomer (*red*) and dimer (*black*) from *Phaeodactylum tricornutum*, excited at 425 nm (Yokono *et al*. 2015). (*C*) The 77 K fluorescence emission spectrum (excitation wavelength 425 nm) of FCP-PSI super complexes from *Chaetoceros gracilis*, excited at 450 nm (Ikeda *et al*. 2008). (*D*) The 77 K fluorescence emission spectra (excitation wavelength 425 nm) of whole cells of *Phaedactylum tricornutum* with different levels of non-photochemical quenching (Lavaud and Lepetit 2013).
Panel *A* was digitized form Litvín *et al*. (2016).
Panel *B* was digitized from Yokono *et al*. (2015)
Panel *C* was digitized from Ikeda *et al*. (2008)
Panel *D* was digitized from Lavaud and Lepetit (2013).

715–720 nm (Fig. 11*C*) (Berkaloff *et al*. 1990, Veith and Büchel 2007, Ikeda *et al*. 2008). In addition, there is also a broad emission centered at 740–750 nm, which is a combination of vibronic sub-bands of all Chls within the sample.

Nannochloropsis

Nannochloropsisis species are heterokont algae belonging to the Eustigmatophyceae family. *Nannochloropsis oceanica* and *gaditana* have received increased attention as they can accumulate high amounts of lipids. The architecture of the photosynthetic machinery of *Nannochloropsis* species has recently been investigated in detail (Herbstová *et al*. 2015, Litvín *et al*. 2016).

Eustigmatophyceae are unique among the heterokont algae in lacking Chl *c* and fucoxanthin in their LHCs. Thus, Chl *a* serves as the only Chl of both PSI and PSII, as well as in the peripheral LHCs. *Nannochloropsis* species

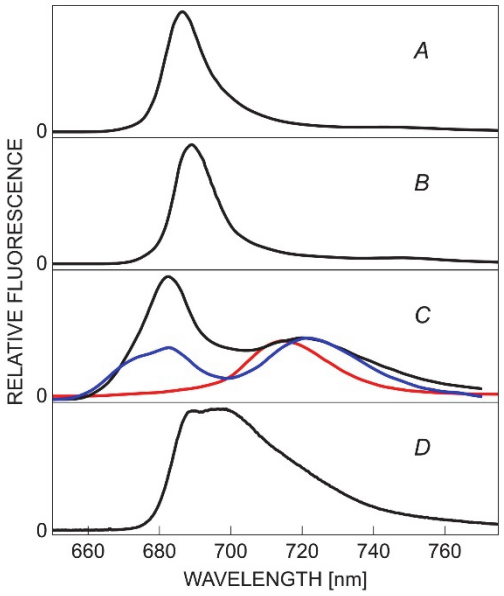

Fig. 12. Fluorescence emission spectra of photosynthetic components of heterokont algae *Eustigmataphyceaen* alga *Nannochloropsis*. (*A*) The 77 K fluorescence emission spectrum (excitation wavelength 440 nm) of isolated VCP from *Nannochloropsis oceanica* (excited at 435 nm) (Litvín *et al*. 2016). (*B*) The 77 K fluorescence emission spectrum of isolated PSII from *Nannochloropsis oceanica* (excitation wavelength 435 nm) (Litvín *et al*. 2016). (*C*) The 77 K fluorescence emission spectrum of isolated PSI-LHC complexes isolated from *Nannochloropsis gaditana* (mainly dimeric: *black*, mainly monomeric: *blue*) (Alboresi *et al*. 2017) and PSI-supercomplex (*red*) (Litvín *et al*. 2016) (excitation wavelength 440 nm). (*D*) The fluorescence emission spectrum of whole cell *Nannochloropsis oceanica* (excitation wavelength 435 nm). Panels *A* and *B* were digitized from Litvín *et al*. (2016). (*C*) was digitized from Alboresi *et al*. (2017).

use predominantly the carotenoids violaxanthin and vaucheroaxanthin as light-harvesting pigments and for photoprotection. Chl *a* and the carotenoids are housed in LHCs, called VCP for viola-/vaucheriaxanthin Chl protein (Fig. 12*A*) (Litvín *et al*. 2016).

Light-harvesting complexes

Nannochloropsis species possess LHCs that can be clustered according to their evolutionary origin as diatoms including Lhcx and Lhcv – the *Eustigmatophycean* version of the diatom Lhcf, and Lhcr. Furthermore, RedCLH, a light-harvesting protein first identified in *Chromera velia,* is also present (Litvín *et al*. 2016). Biochemical isolation suggests that VCP can form trimers (Litvín *et al*. 2016). VCP seems to be very rich in carotenoids, with about one carotenoid per two Chls, with violaxanthin and vaucheriaxanthin being the dominant carotenoids (Litvín *et al*. 2016). Until high-resolution structural data of Lchv is available, it may be assumed that VCPs bind pigments analogous to Lhca, Lhcb (in green algae and plants), and Lhcf (in diatoms).

Photosystem II

Biochemical separation procedures suggest that the PSII complexes can contain about 4–5 VCPs per PSII core (Litvín *et al*. 2016) that belong to the Lhcr family. Interestingly, 77 K fluorescence spectra of isolated PSII particles show no fluorescence emission band at 685 nm and PSII that lack CP43 are present when PSII is isolated (Fig 11*B*) (Litvín *et al*. 2016). Whether this 77 K fluorescence emission pattern in combination with the CP43-lacking PSII complexes indicate that PSII has structural features, which are different from green algae and plants, needs to be resolved in the future.

Photosystem I

The PSI reaction center core complex of *Nannochloropsis* has very similar fluorescence emission characteristics (Fig. 11*C*) to those observed in diatoms (Herbstová *et al*. 2015)**,** with a single emission band around 715 nm. However, *Nanochloropsis* PSI supercomplexes with associated complexes can be isolated, which contain Lhcr, Lhcx, and RedCAP (Bína *et al*. 2017, Litvín *et al*. 2016), the latter being a Chl-binding protein also present in other heterokont algae.

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