Changes in the in vivo absorption and fluorescence excitation spectra with growth irradiance in three species of phytoplankton

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The fluorescence excitation spectrum is sometimes used as a proxy for the action spectrum of photosynthesis in phytoplankton. The main assumption behind this approximation is that the shapes of absorption and fluorescence excitation spectra are similar except for the absorption by photoprotective pigments, which do not contribute to the fluorescence spectrum. In this study, we compare the shapes of the absorption and fluorescence spectra in three species of phytoplankton grown at different irradiances: two diatoms (Thalassiosira weissflogii and Chaetoceros sp.) and a cyanophyte (Synechococcus sp.). The contribution to absorption by photoprotective pigments was estimated for each experiment. Results showed that the differences between the shapes of absorption and fluorescence spectra were similar to the estimated absorption by photoprotective pigments only in the case of T. weissflogii. In Synechococcus sp., and to a lesser degree in Chaetoceros sp., the differences between the two types of spectra were larger than the absorption by photoprotective pigments. In the case of Synechococcus sp., the difference between these spectra was apparently due mainly to the extreme imbalance of chlorophyll a distribution between the two photosystems. Chaetoceros sp. seemed to be an intermediate case: a small part of the chlorophyll a of the cell appeared to be exclusively associated with photosystem I and therefore did not contribute to fluorescence. Fluorescence and absorption values were normalized to their values at 545 nm, and the ratio of normalized absorption to normalized fluorescence was computed for the blue (439 nm) and red (676 nm) peaks in the spectra. The results showed that these peak ratios can be used to distinguish between the effects of photoprotective pigments and the arrangement of the photosynthetic apparatus on differences between fluorescence and absorption spectra.

INTRODUCTION

In the visible part of the spectrum, which is important for photosynthesis, the absorption characteristics of phytoplankton are determined by their pigment composition, as well as by the manner in which they are packaged into the cell. Pigments are not uniformly distributed in solution inside cells, but are bound to specific proteins, forming pigment–protein complexes or chromoproteins (Prézelin, 1981; Larkum and Barrett, 1983; Porra et al., 1997). These chromoproteins are the main components of the photosynthetic units which are located in the thylakoid membranes. A photosynthetic unit (PSU) may be defined as the smallest unit able to carry out photosynthesis (Prézelin, 1981).

The absorption spectrum of a particular species of phytoplankton represents the sum of the individual absorptions of all of the pigment–protein complexes present in the cell. Light-harvesting complexes (LHCs) make up the bulk of the pigment content of the cell and thus they are the main agents responsible for absorption. The fact that pigments are enclosed within a cell, forming intricate structures, also affects their absorption characteristics. This is known as the ‘packaging effect’ (Duyvenga, 1956), according to which cell size and intracellular concentration of pigments influence phytoplankton
The fluorescence excitation spectrum of an alga at ambient temperature, by contrast, results from the re-emission of light reaching chlorophyll (Chl) a of photosystem II (PSII) (Goedheer, 1969; Kiefer and Reynolds, 1992; Govindjee, 1995). Each photosystem (PSI and PSII) is composed of a reaction centre (RCI and RCII) and a subantenna surrounding the RC, which contains mainly Chl a, together with small amounts of Chl b or Chl c, depending on the species and carotenoids (Larkum and Barrett, 1983; Przela and Boczar, 1986). The fluoresced light has a longer wavelength than the absorbed light, and has a peak in intensity at ~680 nm (Clarck, 1980; Govindjee, 1995). For a fixed wavelength of emission, the intensity of fluorescence varies with the wavelength of incident light. This wavelength dependence is caused by the differential absorption of light by the pigment–protein complexes associated with PSII. Since pigments associated with both PSI and PSII absorb, whereas only pigments of PSII contribute to fluorescence, some of the differences between fluorescence excitation spectra and absorption spectra may depend on the structures of the PSU, which may vary with species and their physiological condition. Another source of differences between absorption and fluorescence spectra is the photoprotective pigments (PP), which absorb light but do not transmit the energy to any of the photosystems, so that they do not contribute to fluorescence (Przela, 1981; Siegermann-Harnow, 1987).

The structure of the PSU in a species undergoes changes in response to changes in environmental conditions, e.g. light quantity and quality, nutrient concentration and temperature [reviewed in (Larkum and Barrett, 1983; Przela and Boczar, 1986; Falkowski and Raven, 1997)]. Photoacclimation to changes in light intensity works at two time scales. Short-term changes (in the order of seconds to minutes), such as state transitions (Bonaventura and Myers, 1969; Mullineaux and Allen, 1988; Kroon, 1994; Schubert et al., 1995) and the xanthophyll cycle (Demming-Adams, 1990; Oliandri and Yamamoto, 1994), were not considered in this study. Long-term acclimation (in the order of hours to days) involves increase in levels of PP at high irradiance (Biddle et al., 1993; Moore et al., 1995), and increase in levels of LHCs at low irradiance (Richardson et al., 1985; Falkowski and Lo Roche, 1991).

Based on the assumption of an equal distribution of energy between photosystems, it has been proposed that the fluorescence excitation spectrum should represent the ‘photosynthetic absorption spectrum’ i.e. phytoplankton absorption without the contribution of PP, and that it could serve as a proxy for the action spectrum of photosynthesis in studies of primary production in the ocean (Sakshaug et al., 1991; Bahin et al., 1995; Sosik and Mitchell, 1995). On the other hand, it is known that phycobilin-containing algae (rhodophytes, cryptophytes and cyanophytes) show an extreme imbalance in energy distribution between photosystems (Hash and Blanks, 1990; Neori et al., 1988; Warnock, 1990). Phycobilins, the main LHCs in these algae, transfer the energy they collect to PSII, while PSI receives energy from pigment–protein complexes which contain most of the Chl a of the cell (Goedheer, 1969; Myers et al., 1986; Bidigare et al., 1989). Therefore, cyanophytes show a striking difference between fluorescence and absorption spectra (Johnsen and Sakshaug, 1996). Other algae (e.g. dinoflagellates, diatoms, pynemoprotephytes) have also shown small imbalances in energy distribution between photosystems (Schofield et al., 1989, 1996; Johnsen et al., 1994, 1997). Lewis et al. observed a PSI-based action spectrum of Tri-chodesmium (ex. Oscillatoria) in the Sargasso Sea (Lewis et al., 1988). Furthermore, in a study conducted in the North Atlantic, we found that differences between the shapes of the absorption and fluorescence excitation spectra were not exclusively related to levels of PP in the samples (Lutz et al., 1998), especially in areas where cyanophytes dominated the phytoplankton community.

Two simple models proposed by Knox may be used to explain energy transfer inside the PSU: the so-called ‘lake’ and ‘puddle’ models (Knox, 1975). The models originally described energy transfer between photosystems of the same type, but this concept can easily be extended to explain energy transfer between photosystems of two different types (PSI and PSII). Basically, the ‘lake’ model is similar to the tripartite model proposed by Butler, where energy absorbed by all LHCs is shared by both photosystems, so that the shape of the fluorescence excitation spectrum (representing energy reaching PSII) will resemble that of the absorption spectrum (representing both PSI and PSII), once the effects of PP have been removed (Butler, 1978). In the ‘puddle’ model, each photosystem is served by a particular LHC (LHCl or LHCl), in the case, if LHCl and LHClII have different pigment compositions, the absorption of pigments exclusive to LHCl will not appear in the fluorescence excitation spectrum. This will lead to a difference in the shapes of the fluorescence and absorption spectra.

In this study, we conducted a series of laboratory experiments to examine the shapes of absorption and fluorescence excitation spectra for three different species of phytoplankton. We used the diatoms Thalassiosira ovario-floccus (which is frequently used in physiological experiments) and Chaetoceros sp. (which was previously found to be abundant in the Labrador Sea and was isolated from that source (Lutz et al., 1998)) and a cyanophyte Synechococcus sp. (cyanophytes were previously found to be
abundant close to the Canary Isles (Lutz et al., 1998). We chose these species because they are commonly occurring species in natural assemblages and because they were expected to have different types of photosynthetic apparatus. A simplified description of the distribution of pigments in different types of pigment–protein complex in diatoms and cyanophytes is shown in Table I. At the onset, we expected that the pathway of energy inside the PSUs would correspond to the lake model in diatoms and to an extreme case of a puddle model in cyanophytes. In this paper, we examined the variations in the shape of absorption and fluorescence spectra, and their potential relationship to changes in the structure of the PSU, as reflected in the pigment composition of the cells. We also investigated the contributions of varying concentrations of PP (Table II), to the difference between absorption and fluorescence spectra, by growing the cultures at different irradiances.

**METHOD**

**Phytoplankton cultures**

Cultures of *Chaetoceros* sp. (a strain isolated from the Labrador Sea by D. V. Subba Rao), *T. weissflogii* (clone CCMP 1336; Provasoli–Guillard National Center for Culture of Marine Phytoplankton) and *Synechococcus* sp. (clone CCMP 837; Provasoli–Guillard National Center for Culture of Marine Phytoplankton) were grown in f/2 medium under continuous illumination provided by a rack of cool-white fluorescent tubes. *Chaetoceros* sp. and *T. weissflogii* were grown in semicontinuous cultures, maintained at exponential growth by frequent addition of fresh medium, at 10 and 20°C, respectively. *Synechococcus* sp. was grown in batch cultures at 20°C. *Chaetoceros* sp. was grown at two irradiances [photosynthetically available radiation (PAR) 400–700 nm]: 30 and 100 μmol quanta m⁻² s⁻¹; *T. weissflogii* was grown at five irradiances [PAR]: 20, 50, 100, 250 and 450 μmol quanta m⁻² s⁻¹; and *Synechococcus* sp. at three irradiances [PAR]: 20, 50 and 100 μmol quanta m⁻² s⁻¹. All cultures were initiated from small inocula and grown for at least 10 days at the designated irradiance before performing the measurements, to ensure complete acclimation to the growth conditions. Note that the highest irradiance at which *Chaetoceros* sp. was grown was lower than that used for *T. weissflogii*. *Chaetoceros* sp. was isolated from the cold waters of the Labrador Sea and grown at 10°C; thus, the cells were probably predisposed to a photosynthetic apparatus acclimated to low temperatures, which is somewhat equivalent to acclimation to high light (Maxwell et al., 1995), which reduces their ability to grow at higher irradiances. In the case of *Synechococcus* sp., we moved cells growing at very low light (<20 μmol quanta m⁻² s⁻¹) directly to the higher irradiances without allowing them to gradually adjust to increasing irradiances as suggested by Kana and Gilbert (Kana and Gilbert, 1987). Consequently, the maximum irradiance at which the cells were able to grow was 100 μmol quanta m⁻² s⁻¹.

Cultures were acclimated to dim light, <10 μmol quanta m⁻² s⁻¹, for at least 30 min before measurements were made, to eliminate fast changes in the energy flow within the PSU, such as the xanthophyll cycle or state transitions. Samples of 10 ml of culture were filtered through GF/F glass fibre filters for pigment determination and kept in liquid nitrogen until analysis. Another aliquot of 10 ml was inoculated with 3-(3,4-dichlorophenyl) 1-1 dimethylurea to a final concentration of 18 µM (to block electron transfer and enhance the fluorescence emission by PSII, filtered through GF/F filters after ~10 min, and the filters were then immediately used to measure the fluorescence and absorption spectra. All measurements were made in triplicate for *Chaetoceros* sp. and *T. weissflogii*, and in duplicate for *Synechococcus* sp. Results shown are the averages of these replicates.

In the case of *Chaetoceros* sp. and *T. weissflogii*, cells were counted on the day of each experiment, using a haemocytometer. Cell size was measured using a calibrated ocular eyepiece in the microscope. Two dimensions were

<table>
<thead>
<tr>
<th>Complex</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diatoms</td>
<td></td>
</tr>
<tr>
<td>LHCs</td>
<td>Chlorophyll a–chlorophyll ε–fucoxanthin–protein</td>
</tr>
<tr>
<td></td>
<td>Chlorophyll a–chlorophyll ε–protein</td>
</tr>
<tr>
<td></td>
<td>Chlorophyll a–fucoxanthin–protein</td>
</tr>
<tr>
<td>PSI</td>
<td>RCI: chlorophyll a–protein (P680)</td>
</tr>
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<td></td>
<td>Sub antennae II: chlorophyll a–(<em>T</em>)–protein</td>
</tr>
<tr>
<td>PSI</td>
<td>RCI: chlorophyll a–protein (P700)</td>
</tr>
<tr>
<td></td>
<td>Sub antennae II: chlorophyll a–(<em>T</em>)–protein</td>
</tr>
<tr>
<td></td>
<td>PSI: chlorophyll a–protein</td>
</tr>
<tr>
<td>Cyanobacteria</td>
<td></td>
</tr>
<tr>
<td>LHCs</td>
<td>Phycocyanin</td>
</tr>
<tr>
<td></td>
<td>Chlorophyll a–protein</td>
</tr>
<tr>
<td>PSI</td>
<td>RCI: chlorophyll a–protein (P680)</td>
</tr>
<tr>
<td></td>
<td>Sub antennae II: chlorophyll a–(<em>T</em>)–protein</td>
</tr>
<tr>
<td>PSI</td>
<td>RCI: chlorophyll a–protein (P700)</td>
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<tr>
<td></td>
<td>Sub antennae II: chlorophyll a–(<em>T</em>)–protein</td>
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</tbody>
</table>

LHCs, light harvesting complexes; PSI and PSII, photosystem I and II; RCI and RCI, reaction centre I and II. * Probably minor amounts of other pigments.
measured for the diatoms and the volumes were calculated assuming a cylindrical shape. From these volume estimates, the cell radius was calculated for an equivalent spherical volume. For \textit{Synechococcus} sp., samples were fixed with 2% glutaraldehyde and stored in cryovials at –70°C until analysis by flow cytometry. Cell numbers were counted using a flow cytometer following the method described by Li (Li, 1994). Flow cytometric measurements of forward scattered light were used to estimate cell size (Olson \textit{et al.}, 1989) by transforming them into equivalent spherical diameter using a coefficient obtained for calibrated plastic beads. This estimation of cell volume is affected by differences in the shape and in the refractive indexes of the plastic beads used in the calibration, and those of the cells measured. Hence, cell volumes calculated by flow cytometry should not be expected to be the same as those estimated by microscopy.

**Pigment determination**

The efficiency of pigment extraction by different solvents on \textit{Chaetoceros} sp. was examined. A modification of the solvent mixture proposed by Wright \textit{et al.} (Wright \textit{et al.}, 1991) and Kraay \textit{et al.} (Kraay \textit{et al.}, 1992), using 98% ethanol:2% 0.5 M ammonium acetate (instead of 98% methanol), proved to be the best for this species. It is known that the extraction of pigments from cyanophytes is more difficult than from diatoms, but because of technical problems it was impossible to check the efficiency of extraction of different solvents from \textit{Synechococcus} sp. Nevertheless, according to Wright \textit{et al.} (Wright \textit{et al.}, 1997), the commonly used method of soaking the samples in 90% acetone can cause a severe underestimation of pigments, particularly in cyanophytes. Therefore, all filters were extracted in a mixture of 98% ethanol:2% 0.5 M ammonium acetate by grinding using an electrical homogenizer. Filters were taken from the liquid nitrogen and immediately ground, keeping the temperature low (~5°C) by maintaining the grinding container in an ice bath, to minimize the conversion of Chl \textit{a} into chlorophyllide \textit{a} by the action of chlorophyllase (Barrett and Jeffrey, 1964; Owens and Falkowski, 1982). Samples were then analysed by HPLC according to the method of Head and Horne (1993).

**In vivo fluorescence**

\textit{In vivo} fluorescence excitation spectra were measured on particulate material retained on a filter rather than in suspension, as is commonly done [e.g. (Sosik and Mitchell, 1995; Johnsen \textit{et al.}, 1997)], to relate these experimental results to previous field observations, where the low concentrations of phytoplankton, especially in oligotrophic waters, would have precluded measurements in suspension (Lutz \textit{et al.}, 1998). The instrument used was a Photon Technology International QM-1 spectrofluorometer equipped with a red-sensitive photomultiplier and a reference photodetector with a photodiode array for the correction of the excitation spectrum. To avoid any interference by stray light, two interference filters were used: a cut-off filter located immediately after the excitation monochromator to eliminate light of wavelengths >700 nm, and another cut-off filter located immediately before the emission monochromator to eliminate light of wavelengths <720 nm. The efficacy of the correction procedure was tested by comparing the shapes of the fluorescence excitation and absorption spectra of a 90% acetone solution of pure Chl \textit{a}, which gave a good match. Fluorescence excitation spectra were recorded between 380 and 700 nm at an emission wavelength of 730 nm. The excitation slits were

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**Table II:** Intracellular concentration of photosynthetic (PS) and photoprotective (PP) carotenoids (ng m$^{-3}$ 10$^{-5}$) in the three species studied (T. weissflogii, Chaetoceros sp. and \textit{Synechococcus} sp.) at the lowest (LL) and highest (HL) irradiances

<table>
<thead>
<tr>
<th>Species</th>
<th>Irradiance</th>
<th>PS</th>
<th>PP</th>
<th>m</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>fu</td>
<td>d+t</td>
<td>β-c</td>
</tr>
<tr>
<td>T. weissflogii</td>
<td>LL</td>
<td>18.95</td>
<td>2.22</td>
<td>1.26</td>
</tr>
<tr>
<td></td>
<td>HL</td>
<td>16.88</td>
<td>10.51</td>
<td>1.79</td>
</tr>
<tr>
<td>Chaetoceros</td>
<td>LL</td>
<td>95.20</td>
<td>6.65</td>
<td>4.27</td>
</tr>
<tr>
<td></td>
<td>HL</td>
<td>82.90</td>
<td>12.81</td>
<td>4.55</td>
</tr>
<tr>
<td>\textit{Synechococcus}</td>
<td>LL</td>
<td>–</td>
<td>–</td>
<td>24.21</td>
</tr>
<tr>
<td></td>
<td>HL</td>
<td>–</td>
<td>–</td>
<td>5.31</td>
</tr>
</tbody>
</table>

Triplicate measurements of pigment concentration showed a CV of <5% (except in some cases for β-carotene, which was found in minor concentrations).

fu, fucoxanthin; d+t, diadinoxanthin + diatoxanthin; β-c, β-carotene; ze, zeaxanthin.
set at a bandpass width of 2 nm and the emission slit at 4 nm. The blank was obtained by filtering the filtrate of the corresponding sample through a GF/F filter. Filters were wetted with filtered sea water before reading. The shapes of the spectra were also corrected for differences between measurements in suspension and on a filter as described in Lutz et al. (1990). The ratio of the fluorescence excitation spectrum on a filter to that in suspension was calculated from triplicate measurements for each species (data not shown). All measurements of the fluorescence excitation spectrum were then divided by their corresponding ratio (filter/suspension). Spectra were then smoothed using a running average of 5 nm and normalized to 1 at 345 nm (Lutz et al., 1998). This wavelength was chosen because the two spectra are expected to be equal here. By contrast, if the spectra (absorption and fluorescence) had been normalized at a wavelength of maximum absorption of Chl a (e.g. 676 nm), an artificial overshooting of the fluorescence over absorption would have occurred, in species where a significant amount of the Chl a was in PSI (not contributing to fluorescence), at wavelengths where photosynthetic pigments serving both photosystems were absorbing Johnsen and Sakshaug (Johnsen and Sakshaug, 1996) proposed that the spectra (absorption and fluorescence) be normalized in diatoms to the region between 520 and 535 nm, where fucoxanthin absorbs light. We decided to use 545 nm for the normalization because this wavelength includes not only photosynthetic carotenoids, but also absorption by phycoerythrin, which is a common LHC in cyanophytes. These spectra are designated as $f'_\text{a}(\lambda)$. The average from triplicate samples was calculated in each case. The mean coefficient of variation (CV), from the CVs calculated from the replicate values of $f'_\text{a}(\lambda)$ in each case, was 3.0 ± 2.3% for all experiments ($n = 10$).

**In vivo absorption**

In vivo absorption spectra were measured using the filter technique (Yentsch, 1962; Mitchell and Kiefer, 1984), with a Shimadzu UV-2101 PC double-beam spectrophotometer equipped with an integrating sphere. Sample and blank filters were wetted in filtered sea water and placed in the spectrophotometer immediately after the fluorescence measurements. The optical density at 750 nm was subtracted from the spectrum (Johnsen and Sakshaug, 1996; Bricaud and Stramski, 1990). Optical densities $|D(\lambda)|$ were transformed into absorption $|a(\lambda)|$ by converting decimolar to natural logarithm (factor = 2.3); and taking into account the volume filtered and the area of the filter ($A = \text{volume filtered} \times \text{area of the filter}$). Spectra were corrected for the pathlength amplification factor using a quadratic equation (Mitchell, 1990; Hoepfner and Sathyendranath, 1992):

$$s_4(\lambda) = \frac{2}{3 \times (\pi \times |D(\lambda)| + 3 \times |D(\lambda)'|)}$$

where the coefficients $A$ and $B$ were determined individually for each species (for *Chaetoceros* sp., $A = 0.47$ and $B = 0.19$; for *T. weissflogii*, $A = 0.35$ and $B = 0.20$; for *Synechococcus* sp., $A = 0.41$ and $B = 0.32$). Absorption by detrital material was corrected for, to obtain $s_4(\lambda)$, using the method proposed by Kishino et al. (Kishino et al., 1985) as modified by Hoepfner and Sathyendranath (Hoepfner and Sathyendranath, 1992) for *Chaetoceros* sp. and *T. weissflogii* and the theoretical approach of Hoepfner and Sathyendranath (Hoepfner and Sathyendranath, 1995) for *Synechococcus* sp. Finally, spectra were normalized to 1 at 345 nm and the normalized spectra, denoted here as $a'_\text{a}(\lambda)$, were calculated as the average from replicate samples in each case. The mean CV for all the CVs calculated from the replicate values of $a'_\text{a}(\lambda)$ in each case, was 3.6 ± 2.6% for all experiments ($n = 10$).

**Estimation of the absorption by photoprotective pigments**

The contribution of PP to the normalized absorption spectrum was estimated using the method proposed by Bidigare et al. (Bidigare et al., 1990) as modified by Babin et al. (Babin et al., 1996). Briefly, the absorption spectrum was reconstructed $[a_{\text{pp}}(\lambda)]$ using pigment concentrations $[\phi]$ and the corresponding specific in vivo absorption coefficients $[a(\lambda)]$ given by Bidigare et al. (Bidigare et al., 1990):

$$a_{\text{pp}}(\lambda) = \sum_i \phi_i a_i(\lambda)$$

An initial estimate of absorption by PP $[a_{\text{pp}}(\lambda)]$ was computed similarly. This method uses the shapes of absorption by pure pigments in vitro (slightly shifted; see Bidigare et al., 1990), which are not the best representation of the absorption spectra of pigments which are present in pigment–protein complexes in vivo [see Johnsen et al., 1994]). Furthermore, this reconstruction procedure does not account for packaging of pigments within the cells. This last factor can be significant, especially in large cells grown at low irradiance, when both cell diameter and intracellular concentrations of pigments are high (Morel and Bricaud, 1981). Figure 1 shows an example of the discrepancies between reconstructed and measured absorption spectra for *T. weissflogii* grown at the lowest irradiance (20 μmol quanta m⁻² s⁻¹). Because of this discrepancy, we used a procedure similar to that described in Babin et al. (Babin et al., 1996) to obtain a corrected estimate of the normalized absorption due to PP. The bias resulting from the use of fixed in vitro shapes of absorption by individual pigments to represent the in vivo ones still persists in this case. This bias could only be assessed by measuring...
absorption from isolated pigment-protein complexes from different species of phytoplankton. Allowing that this method [Bidigare et al., 1996], as modified by Babin et al., 1996] is not perfect, it does nevertheless represent a first approximation of the contribution of absorption by photoprotective pigments to total phytoplankton absorption (Figure 1). The normalized absorption by PP [aΨ(λ)/a] was estimated as follows:

\[ \frac{a_{pp}(\lambda)}{a_{ph}(\lambda)} = \frac{\int \frac{1}{H_{100}} A_{pp}(\lambda) d\lambda}{\int \frac{1}{H_{100}} A_{ph}(\lambda) d\lambda} \]

In other words, the normalized phytoplankton absorption spectrum was multiplied by the ratio of the initial estimate of absorption by PP to the total reconstructed absorption at the same wavelength.

RESULTS

Changes in pigment composition with irradiance

We investigated variations in pigment ratios in the three species when exposed to the two extreme growth irradiances, hereafter referred to as the lowest light (LL) and the highest light (HL). For *T. weissflogii*, LL = 20 µmol quanta m⁻² s⁻¹ and HL = 450 µmol quanta m⁻² s⁻¹; for Chaetoceros sp., LL = 30 µmol quanta m⁻² s⁻¹ and HL = 100 µmol quanta m⁻² s⁻¹; and for Synechococcus sp., LL = 20 µmol quanta m⁻² s⁻¹ and HL = 100 µmol quanta m⁻² s⁻¹. These pigment ratios at different irradiances are later used to infer changes in the distribution of pigments in different parts of the PSU. We also estimated cell size in the three species at LL and HL. Because cell size changed with irradiance, we preferred to look at the intracellular concentration of pigments rather than the concentration of pigments per cell. Cell volumes in diatoms may be affected by the presence of vacuoles, but this effect was not considered in this study so that effective intracellular concentrations of pigments may have been underestimated.

The intracellular concentrations of the different photosynthetic (PS) and photoprotective (PP) carotenoids are shows in Table II. For the diatoms, concentrations of PS were higher at LL, and those of PP were higher at HL. In *Synechococcus*, concentrations of PP changed in a different manner: the intracellular concentration of β-carotene was higher at LL, whereas that of zeaxanthin remained almost constant at both light intensities.

In *T. weissflogii*, the ratios of Chl a to Chl c (cc/ca) and PS/ca were higher at LL than at HL, while the ratio PP/ca was higher at HL (Table III). This species seemed to have compensated for a general increase in pigment content per cell at LL by increasing its volume by ~64%, compared with that at HL. The concentration of Chl a increased proportionally with cell size, so that intracellular concentration was ~12% more than at HL, while intracellular concentrations of PS were ~70% higher at HL than at LL (Table III). These results are consistent with changes in cell volume and intracellular concentrations of Chl a found in the same species by Sosik et al. (Sosik et al., 1989), although they are in contrast to the findings of Falkowski et al., who found that cell volume decreased with a decrease in irradiance (Falkowski et al., 1985). There is a wide variation in reported cell sizes for *T. weissflogii* from 500 µm³ (Berge et al., 1996) to 2800 µm³ (Costillo and Chisholm, 1981). These variations in cell size can be partly explained by the large genetic variation observed in isolates of *T. weissflogii* (Armbrust and Chisholm, 1992), which can undergo different cycles of auxospore formation.

In Chaetoceros sp., in contrast to the situation in *T. weissflogii*, the ratios cc/ca and PS/ca were higher at HL than at LL, as was the ratio of PP/ca (Table III). In *Chlamydomonas* sp., which is smaller than *T. weissflogii*, cell volume actually increased with irradiance (Table III). Consequently, the total intracellular concentration of Chl a was ~30% higher at LL than at HL. The intracellular concentrations of Chl c and PS were also higher at LL than at HL (~10%), while intracellular concentrations of PP were ~36% higher at HL (Table III).

In *Synechococcus*, as in the other species, the ratios of PP/PS also increased at HL (Table III). Since we were not able to measure the concentration of phycobilins, the
main LHCs in this species, we do not know how their concentrations changed with irradiance. As in Chaetoceros sp., cell volume increased with irradiance in Synechococcus sp. These results are consistent with those of Stramski et al. (Stramski et al., 1995), but contrast with those of Kana and Glibert (Kana and Glibert, 1987), who found that cell volume increased with a decrease in irradiance. This might reflect differences in the cell sizes of different strains of Synechococcus and in their physiological responses to growth conditions. The intracellular concentration of Chl a was >90% higher at LL than at HL. The concentration of PP was ~20% higher at LL (Table III). Hence, the increase in PP/Chl a at HL was due to a decrease in the concentration of Chl a rather than to an increase in the concentration of PP [see (Kana et al., 1988)].

**Variation in the packaging effect**

To evaluate the differences in the packaging effect in these three species at the different light intensities, we calculated the specific absorption coefficients (absorption per unit concentration of Chl a; Figure 2). As expected, *T. weissflogii* (which has the largest cell size) showed the lowest specific coefficients, followed by *Chaetoceros* sp. and *Synechococcus* sp. In the two diatoms, there was a marked increase in the packaging effect at LL, concomitant with the increase in the intracellular concentration of PS (Table III). In *Synechococcus*, the differences between the specific absorption coefficients at LL and HL were not as large as those in the diatoms, and there was an apparent increase in the specific absorption coefficients at LL at the red and the green regions of the spectrum. It is known that *Synechococcus*, because of its small size, is less affected by the packaging effect than larger cells (Morel et al., 1993), so that differences between packaging at different irradiances were also expected to be small. On the other hand, the specific absorption coefficients measured in this study were lower ([a·ph] > 0.020) than those previously reported by Morel et al. (Morel et al., 1993; [a·ph] > 0.020). This difference may partly be due to the use of a more efficient extraction procedure for pigments in the present study: we ground the filters in 98% ethanol:2% 0.5 M ammonium acetate, instead of soaking them in 100% acetone (Morel et al., 1993). It should also be mentioned that since this species was grown in batch cultures, we cannot disregard some self-shading effect among the cells.

**Variation in the absorption and fluorescence excitation spectra at low and high light**

We examined the variation in the normalized absorption and fluorescence excitation spectra at LL and HL. If each species is considered separately, differences between absorption spectra at LL and HL were always

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**Table III: Cellular volume (µm3), intracellular concentration of pigments (mg m–3 10–5) and ratios among them in the three species studied.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Irradiance</th>
<th>Cell volume</th>
<th>Intracellular concentrations of pigments (mg m–3 10–5)</th>
<th>Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chl a</td>
<td>Chl c</td>
</tr>
<tr>
<td><em>T. weissflogii</em></td>
<td>LL</td>
<td>2004 ± 346</td>
<td>41.98</td>
<td>4.07</td>
</tr>
<tr>
<td></td>
<td>HL</td>
<td>1407 ± 367</td>
<td>3.48</td>
<td>0.13</td>
</tr>
<tr>
<td><em>Chaetoceros</em></td>
<td>LL</td>
<td>49 ± 16</td>
<td>139.56</td>
<td>32.04</td>
</tr>
<tr>
<td></td>
<td>HL</td>
<td>65 ± 30</td>
<td>105.56</td>
<td>29.97</td>
</tr>
<tr>
<td><em>Synechococcus</em></td>
<td>LL</td>
<td>0.30 ± 0.04</td>
<td>171.99</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>HL</td>
<td>0.45 ± 0.05</td>
<td>87.70</td>
<td>–</td>
</tr>
</tbody>
</table>

The percentage difference of the values at low light (LL) from those at high light (HL) are indicated.
larger than the corresponding differences in the fluorescence excitation spectra (Figure 3). The CV between the normalized (545 nm) absorption spectra at the blue peak (439 nm) between LL and HL was 34.6% for Chaetoceros sp., 41.6% for T. weissflogii and 35.8% for Synechococcus sp. The CV between the normalized (545 nm) fluorescence spectra at the blue peak was 26.3% for Chaetoceros sp., 24.9% for T. weissflogii and 22.7% for Synechococcus sp. On the other hand, if results from all the experiments on the three species were considered together, it is obvious that variations in the shape of fluorescence spectra would be larger than those of the absorption spectra.

Comparison between absorption and fluorescence spectra at each irradiance

We examined the shapes of the normalized absorption and fluorescence spectra at each irradiance, and the corresponding difference spectrum (absorption – fluorescence, $a_f^\text{ph} - a_c^\text{ph}$) in relation to the absorption by PP. For T. weissflogii, the shape of the fluorescence excitation spectrum was similar to that of the absorption spectrum at LL, where the contribution of PP to absorption was very low (Figure 4). At HL, the normalized fluorescence spectrum was lower than the absorption spectrum in the blue region, and $a_f^\text{ph} - a_c^\text{ph}$ matched the shape of the PP absorption in that region. In Chaetoceros sp., the spectra for LL showed the same trend as in T. weissflogii, although $a_f^\text{ph} - a_c^\text{ph}$ was slightly larger than the absorption by PP. At HL, the difference spectrum was much larger than absorption by PP. In the two diatoms, both at LL and HL, the difference spectrum showed a conspicuous peak at the far red region (~690 nm). In the case of Synechococcus sp., for both LL and HL, the shape of the fluorescence spectrum resembled the shape of phycoerythrin absorption (maximum at ~567 nm) and almost completely missed the peaks at the blue and red regions of the spectrum, where Chl a absorbs. Therefore, the difference spectrum ($a_f^\text{ph} - a_c^\text{ph}$) showed absorption by Chl a and PP.

Relationship of differences between absorption and fluorescence spectra and the ratio of PP to Chl a

Differences between the normalized (545 nm) absorption and fluorescence spectra were quantified as the ratios of absorption to fluorescence at 439 nm [$S(439)$] and at 676 nm [$S(676)$] for all the growth irradiances. The relationship between $S(439)$ and the ratio PP to Chl a (PP/CA) for T. weissflogii showed a good correlation ($r^2 \approx 0.95$; Figure 5a). If we plotted on the same graph the values for Chaetoceros sp. and for Synechococcus sp., points for the former species lay close to the regression line for T. weissflogii, but those for Synechococcus sp. lay well above this regression line. Since PP absorb only in the blue–green region of the spectrum, while Chl a absorbs in both blue and red regions of the spectrum, a better way to single out the effect of PP should be to examine the ratio $S(439)/S(676)$, in which the effect of Chl a of PSI is cancelled out. Consistent with this argument, the relationship between $S(439)/S(676)$ and PP/CA showed a good correlation for T. weissflogii ($r^2 \approx 0.99$) and the values for Synechococcus sp. were closer to this regression line (Figure 5b).
DISCUSSION

Changes in the PSU

Studies at high resolution of the molecular structure of LHCs [e.g. peridinin–chlorophyll–protein in dinoflagellates (Hofmann et al., 1996); Chl a–Chl b–protein complex in terrestrial plants (Kühlbrandt et al., 1994)] have shown that these are very organized and elaborate structures. It seems likely that the structure of any individual LHC should be conservative (i.e. it should maintain the same ratios of its constituent molecules), whereas the cell may change the concentrations of...
different types of LHCs according to physiological growth conditions (Johnsen et al., 1997). We believe that examining changes in the components of the PSU (i.e. concentrations of different LHCs and the ratio of PSI:PSII) in different species, or under different physiological conditions, should provide a better way of understanding changes in the fluorescence excitation spectra than looking at changes, for example, in the total concentrations of isolated pigments.

**Fig. 4.** Comparison between absorption and fluorescence excitation spectra, both normalized to 1 at 545 nm, for the three species studied at low (LL) and high light (HL). The correspondent absorption by PP, the difference total phytoplankton absorption minus PP absorption ($a^{PP}_{n}$) and the difference total phytoplankton absorption minus fluorescence ($a^{n}_{f}$) spectra are also shown.
Fluorescence emission originates mainly from the subantenna of PSII (Govindjee, 1995), i.e., from a small fraction of very specialized pigment–protein complexes. Hence, knowing how the total cellular amount of Chl \( \text{a} \) changes under different conditions does not tell us much about expected variations in the fluorescence excitation spectrum, unless we know how the proportion of those particular subantenna–II complexes changes within the PSU, as well as how the proportion of LHGs serving subantenna II changes (i.e., how energy is distributed between the photosystems). To our knowledge, the structure of LHGs in diatoms has not been described to a sufficiently high level of resolution to identify unequivocally the composition of different types of LHGs [see (Préaladin and Boczar, 1986; Boczar and Préaladin, 1989)]. Therefore, we cannot carry out any detailed quantitative assignment of pigments to the different complexes.

Despite the absence of quantification, however, changes in the ratios amongst the pigments should provide some information as to how the different components of the PSU change with irradiance, if we take into account the basic pigment distribution within the PSU (Table I). In \( T. \) weissflogii, both \( \text{cc}/\text{ca} \) and \( \text{PS}/\text{ca} \) ratios were higher at LL, while the intracellular concentration of Chl \( \text{a} \) did not change (Table III). We can infer from this that this species had relatively more Chl \( \text{a} \) in subantennae complexes at HL. Thus, at HL, there were fewer LHGs per photosystem, as expected in photoacclimation to HL (Richardson et al., 1983; Falkowski and LaRoche, 1991). Chaetoceros sp., on the other hand, seemed to have more Chl \( \text{a} \) in subantennae complexes at LL, since both the \( \text{cc}/\text{ca} \) and \( \text{PS}/\text{ca} \) ratios were lower at LL, concomitant with a higher intracellular concentration of Chl \( \text{a} \) (Table III). This is consistent with there being an increase in the proportion of PSII at LL, as has been demonstrated by Falkowski et al. (Falkowski et al., 1981) for Skeletonema costatum (PSI:PSII ratio changed from \( \sim 1.0 \) at HL to \( \sim 0.5 \) at LL). This might also influence the way in which the packaging will affect the fluorescence spectrum (coming only from PSII) and the absorption spectrum (representing all parts of the PSU).

In the case of Synechococcus sp., we could not quantify the concentrations of phycobilins (the main LHG for PSII), although there are reports that they increase at LL (Kana and Gilber, 1986). The ratio of the normalized (at 545 nm) absorption value at 567 to that at 676 nm (Figure 3), however, suggests that the amount of phycoerythrin (the main phycobilin in the clone used here) increased proportionally with the amount of Chl \( \text{a} \), an observation which is consistent with the constant ratio of Chl \( \text{a} \) to phycoerythrin observed by Bidigare et al. for Synechococcus sp. grown under different light conditions (Bidigare et al., 1989). The increased intracellular concentration of Chl \( \text{a} \) at LL was probably located in the subantennae of the photosystems (mainly in PSI), perhaps forming more specialized LHGs.

On the other hand, the two PP in cyanophytes, \( \beta \)-carotene and zeaxanthin, behaved differently (Table II). The intracellular concentration of \( \beta \)-carotene decreased with increasing irradiance (\( \sim 3 \) fold higher at LL than at HL), the opposite pattern to that seen by Kana et al. (Kana et al., 1989). Thus our results support the idea that \( \beta \)-carotene serves a mainly light-harvesting function, as proposed by...
Goedheer (Goedheer, 1969). The intracellular concentration of zeaxanthin, which is the most abundant PP, remained almost constant (~1% difference between LL and HL), consistent with the observation of a constant concentration of zeaxanthin per cell found for varying light intensities by Kana et al. (Kana et al., 1988). It has been shown that in the cyanophyte *Anacystis nidulans* most of the zeaxanthin is located in the cytoplasmic membrane and the cell envelope (Murata et al., 1981; Omata and Murata, 1983), which certainly is a strategic location for a photoprotective pigment.

**Variations in the absorption and fluorescence spectra in relation to the organization of the PSU**

It should be noted that when we examined differences in the spectra at LL and HL, we used normalized spectra. Hence, changes in absorption or fluorescence spectra are all relative to a fixed value at 545 nm, which is close to the maximum of absorption of phycocyanin and is on the tail end of absorption spectra of photosynthetic carotenoids. In contrast, for all species, the increase in absorption at HL at the red peak (~676 nm) may be interpreted as a change in the packaging effect at different irradiances, as shown by changes in the specific absorption coefficients in diatoms (Figure 2).

To explain variations in the absorption and fluorescence spectra for the different species, under different light conditions, we examined the actual difference spectrum absorption – fluorescence, $\alpha_a - \phi_f$ (Figure 4). In *T. weissflogii*, the difference between absorption and fluorescence excitation spectra ($\alpha_a - \phi_f$) was similar to the shape of absorption by photoprotective pigments at HL, and hence there was no significant difference between absorption and fluorescence at LL when the concentration of PP was low. In *Chlorella* sp. at HL, $\alpha_a - \phi_f$ was much larger than the absorption spectrum of PP. This might indicate that there was some difference in the Chl a distribution between the photosystems or that the packaging effect was influencing absorption and fluorescence to different extents. In *Synechococcus* sp., it was evident that the fluorescence excitation spectrum, both at LL and HL, showed an extremely low contribution of Chl a (Figure 4), so that $\alpha_a - \phi_f$ cannot be entirely accounted for by PP absorption, even though the proportion of PP to Chl a increased with irradiance (see the pigments section). In cyanophytes, as mentioned above, the main LHCs serving PSII are phycobilins, while most of the Chl a-protein complexes are associated with PSI, which contributes <5% of total fluorescence at ambient temperature. Thus, significant differences between absorption and fluorescence spectra are expected. One interesting feature seen in the difference spectra ($\alpha_a - \phi_f$) of the diatoms was a conspicuous peak at the red end of the spectrum.

This shift of the red peak between absorption and fluorescence might be an indication of absorption by subantenna Chl a from PSI, which hardly fluoresces, and which is known to absorb in the far red [at ~700 nm (Prézelin, 1981; Johnsen et al., 1997)].

In order to distinguish the effect of PP from that of the differential distribution of Chl a between photosystems, on the ($\alpha_a - \phi_f$) difference, we studied at the ratios of absorption to fluorescence at the red [8676] and the blue [8459] peaks. The ratio PP/ca increased with growth irradiance in the three species (Table III). Because PP/ca was much higher in *Synechococcus* sp. than in the two diatoms, we expected $S(439)$ to be greater for the former than for the latter, as is seen in Figure 5a. The magnitude of increase in $S(439)$ for *Synechococcus* sp., however, was far higher than one would have expected from extrapolating from the data for *T. weissflogii*. If we had pooled the data on variations in $S(439)$ with changes in PP/ca for *Synechococcus* sp. with those for the diatoms (shown in Figure 5a), we would have found a high coefficient of determination ($r^2 = 0.96$). This does not mean, however, that all the changes in $S(439)$ were due to changes in PP. We found that a better way to distinguish the effect of PP on the difference ($\alpha_a - \phi_f$) was to examine the relationship between $[S(439)/S(676)]$ and PP/ca (Figure 5b). Indeed, providing there is no bias due to the normalization at 545 nm, the ratio S(676), should represent a measure of the effect of unequal distribution of Chl a between PSII and PSI.

**Comparison between field and laboratory results**

These experimental results are consistent with previous field results from a study in the North Atlantic (Lutz et al., 1998). For example, it is obvious that for the pooled data for the three individual species, the variation in the shape of the fluorescence excitation spectra should be higher than the variation in the shape of the absorption spectra (Figure 3), as was the case in the field (Lutz et al., 1998). In the laboratory, *Synechococcus* sp. showed the largest difference between absorption and fluorescence spectra, which is due to the particular distribution of Chl a inside the PSU. Thus, in cyanophytes, energy distribution within the PSU appears to correspond to an extreme case of the ‘puddle model’. These laboratory results are consistent with the highest values of $S(439)$ being found in nature in areas dominated by cyanophytes (Lutz et al., 1998). The two species of diatoms studied showed some variations in the difference between absorption and fluorescence spectra. In *T. weissflogii*, this difference ($\alpha_a - \phi_f$) could be attributed mainly to absorption by PP, implying that in
this diatom the energy is distributed according to the ‘lake model’, where both photosystems receive energy from all the LHCs in the PSU. In the case of Chaetoceros sp., however, part of the difference ($\Delta$ at $f$) was not due to absorption by PP. This unexplained portion of $\Delta$ might have been caused in part by errors in the methods, but it also seems reasonable to suggest that in this species perhaps some of the LHCs were associated exclusively with PSI (paddle model), so that they did not contribute to the fluorescence spectrum. This suggestion is also consistent with field results from areas dominated by diatoms or prymnesiophytes where only some of the differences in the ratio S(439)/S(685) could be explained by the presence of PP (Lutz et al., 1998). Recently, Culver et al. have also suggested that differences between absorption and fluorescence spectra (normalized at the red peak) of diatom-dominated field samples from Puget Sound (Washington, USA) might have been due to differential distribution of Chl $a$ between PSI and PSII (Culver et al., 1999). As mentioned above, we still lack information on how all the different species of phytoplankton manage the energy flow inside the PSU, but it is possible that, besides the extremes of the lake model (all the energy shared by PSI and PSII, e.g. T. weissflogii), and the paddle model (each photosystem has its own LHC, e.g. cyanophytes), there could be intermediate cases where only some of the LHCs are unique to PSI or PSII.

More detailed studies of the type performed by Johnson et al. (Johnson et al., 1994, 1997) separating different pigment–protein complexes, or those carried out by Goedheer (Goedheer, 1969) and Subramaniam et al. (Subramaniam et al., 1999), investigating changes in the fluorescence spectra at ambient temperature (caused mainly by PSI), and low temperature (~196°C; showing also the contribution of PSII), should help us to understand how different LHCs distribute energy to PSI and PSII in different species of phytoplankton and under different physiological conditions.

CONCLUDING REMARKS

It has been shown in laboratory experiments with different species of phytoplankton that differences between absorption and fluorescence excitation spectra are not exclusively due to the presence of photoprotective pigments, as was also suggested by earlier field results (Lutz et al., 1998). Differences in the arrangement of the photosynthetic apparatus may contribute to differences between absorption and fluorescence spectra. Cyanophytes, which are extensively distributed and abundant in the open ocean (Johnson and Sieburth, 1979; Li et al., 1983; Lewis et al., 1988; Capone et al., 1997, 1998), represent an extreme case where the fluorescence spectrum differs significantly from the absorption spectrum. Other species, including diatoms (this study) and dinoflagellates (Johnson et al., 1994, 1997), also show some degree of difference between absorption and fluorescence due to unequal distribution of Chl $a$ between photosystems. This influence of the arrangement of the photosynthetic apparatus on the fluorescence spectrum should be considered by those who wish to use the fluorescence spectrum as a proxy of the action spectrum of photosynthesis by phytoplankton.

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