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Chlorophyll fluorescence from single cells: Interpretation of flow cytometric signals

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Abstract

The relationship between flow cytometric fluorescence signals and photosynthetic pigments was investigated in three species of marine eucaryotic phytoplankton, *Thalassiosira weissflogii, Hymenomonas carterae,* and *Amphidinium carteri.* The species were grown over a range of light intensities to provide a spectrum of pigment compositions for the study. Both interspecific and intraspecific differences were observed. Variations among the species in fluorescence per unit of chlorophyll *a* (Chl *a*) could be explained by differences in the relative abundance of Chl *a* and accessory pigments. *Amphidinium carteri* had much greater fluorescence per unit of Chl *a* than the other two species and it had the highest Chl *c* : Chl *a* ratio. Fluorescence per unit of Chl *a* decreased as Chl *a* per cell increased in *H. carterae* and *A. carteri,* whereas it remained the same in *T. weissflogii.* We interpret these differences to have their origins in the "package" effect on light absorption. This effect seems to be least evident in the diatom because cell size increased as Chl *a* per cell increased in low-light-grown cells. In *H. carterae* and *A. carteri* the opposite was true, such that the intracellular concentration of Chl *a* increased substantially in the low-light cells. Based on these data, we find that to interpret fluorescence signals among species, differences in the relative abundances of Chl *a* and accessory pigments must be considered, while for intraspecific fluorescence differences, the package effect may be more important.

Measurements of phytoplankton pigments via fluorescence signals have contributed greatly to our understanding of the distribution and abundance of primary producers in the sea. Until recently however, these measurements reflected the average properties of the phytoplankton community in a water sample and were thus limited in resolution. With the introduction of flow cytometry to oceanography (Yentsch et al. 1983; Olson et al. 1985) came the ability to measure fluorescence and other optical properties of individual cells. This advent allowed us to recognize populations by their characteristic flow cytometric "signatures," i.e. groups of correlated parameters such as light scatter and fluorescence at specified wavelengths.

With this type of analysis major groups of phytoplankton, such as phycoerythrin-containing *Synechococcus,* and all chlorophyll *a* (Chl *a*)-containing cells can be distinguished according to their characteristic orange and red fluorescence when excited by blue light (Yentsch et al. 1983; Olson et al. 1985; Li and Wood 1988; Chisholm et al. 1988*a*). Recently, resolution has been expanded by the introduction of dual beam analysis, which allows two-point excitation-emission spectra to be measured on individual cells at sea (Olson et al. 1988). This technique allows one to distinguish pigment types of *Synechococcus* and has revealed that their distribution and abundance in the oceans is not at all reflected in their frequency of occurrence in culture collections. It has also played an important role in the recent discovery, identification, and enumeration of prochlorophytes in the deep euphotic zone (Chisholm et al. 1988*b*).

Although progress has been made in identifying different groups by their character-

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istic fluorescence, a theoretical framework for interpreting environmentally induced changes in fluorescence properties *within* a given species is lacking. This framework is essential if we want to translate the fluorescent signals into information about cellular pigment content and to develop methods for using phytoplankton as tracers to detect water-column mixing. It is well known that the pigment content and composition of cells changes with light and nutrient conditions (Prézelin and Sweeney 1978; Falkowski and Owens 1980; Dubinsky et al. 1986) and that these changes are reflected in fluorescence intensity (Olson et al. 1983; Yentsch et al. 1985; Glibert et al. 1986). None of these studies, however, examines fluorescence changes in sufficient detail to support a mechanistic framework for their interpretation.

The goal of this paper is to systematically explore the relationship between flow cytometry-induced fluorescence and Chl *a* per cell in three taxonomically distant species of marine phytoplankton grown over a range of irradiances. We see this effort as a first step toward codifying the ground rules for interpreting and calibrating fluorescence signals measured in sea-going flow cytometry.

Methods

Culture conditions—Cultures of *Thalassiosira weissflogii* (Actin), *Hymenomonas carterae* (Cocco II), and *Amphidinium carteri* (Amphi) were maintained in exponential growth in nutrient-replete f/2 media (Guillard 1975) at 20°C. Constant illumination was supplied by Sylvania "cool-white" fluorescent lamps at intensities ranging from 10 to 1,000 μmol quanta m^{-2} s^{-1}. Three cultures were maintained at each of 7–9 intensities for each species. The various light levels were created with neutral density, plastic window screen, and intensities were measured with a Biospherical Instruments, Inc., QSL-100 quantum scalar irradiance meter. Growth rate was calculated via natural logarithmic transformation of in vivo fluorescence data (*see below*), and adaptation to the various light intensities was considered complete when Chl *a* per cell and fluorescence per cell remained constant. Analysis was not conducted until at least 10 generations had passed in constant conditions.

Pigment extraction and analyses—For analysis of Chl *a*, samples were filtered onto Schleicher and Schuell No. 34 glass-fiber filters and extracted for 48 h in 90% acetone under refrigeration. After homogenization with a Teflon tissue grinder, Chl *a* concentration was determined fluorometrically (Parsons et al. 1984). The method was calibrated spectrophotometrically with the equations of Jeffrey and Humphrey (1975).

Additional aliquots were collected on glass-fiber filters and frozen rapidly in liquid nitrogen for subsequent analysis with high pressure liquid chromatography (HPLC). Samples were extracted and ground in 100% acetone and analyzed for Chl and carotenoids by reverse-phase HPLC (Mantoura and Llewellyn 1983). Data for Chl *a*, Chl *c*, fucoxanthin, and peridinin are presented here; content of other carotenoid accessory pigments quantified by HPLC have been reported for each species by Sosik (1988).

Fluorescence measurements—The in vivo fluorescence of cell suspensions was determined with a Turner Designs fluorometer model 10. Excitation was between 400 and 460 nm and red emission was measured at >640 nm. All fluorescence measurements was made after 15 min of dark exposure, and fluorescence values were normalized by cell concentration for each culture. Cell number and average cell size were measured on a Coulter Electronics model Z_m/C256 electronic particle counter.

Quantum-corrected fluorescence excitation spectra were measured on suspensions of cells with an SLM Aminco SPF-500C spectrofluorometer. Within a species, cell concentration was adjusted to give the same optical density at 440 nm for each culture. The excitation source was a xenon arc-lamp and excitation wavelength was varied in 1-nm increments from 360 to 600 nm with a 2-nm band width. Emission was monitored at 680 nm with a 20-nm width.

From each culture, measurements of single cell fluorescence were collected with each of two flow cytometers (*see below*). As discussed by Olson et al. (1988), fluorescent beads were used as a standard. The beads

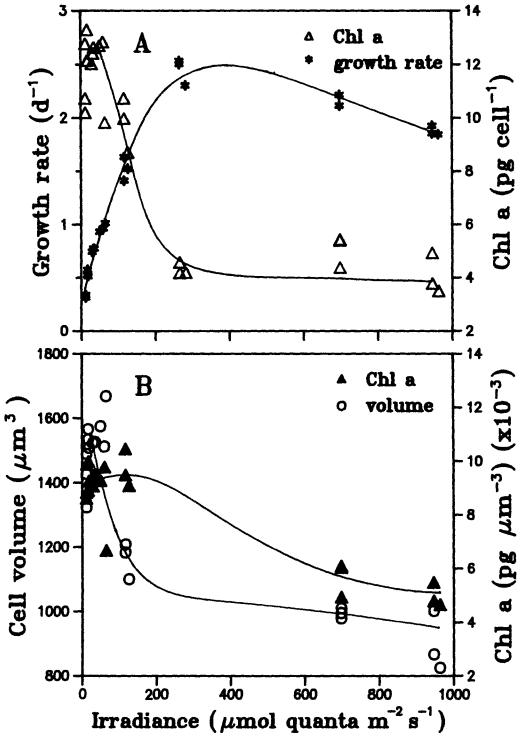

Fig. 1. Growth rate, cell volume, and Chl *a* as a function of growth light intensity in the diatom *Thalassiosira weissflogii*. A. Growth rate and Chl *a* per cell. B. Cell volume and Chl *a* per unit of cellular volume.

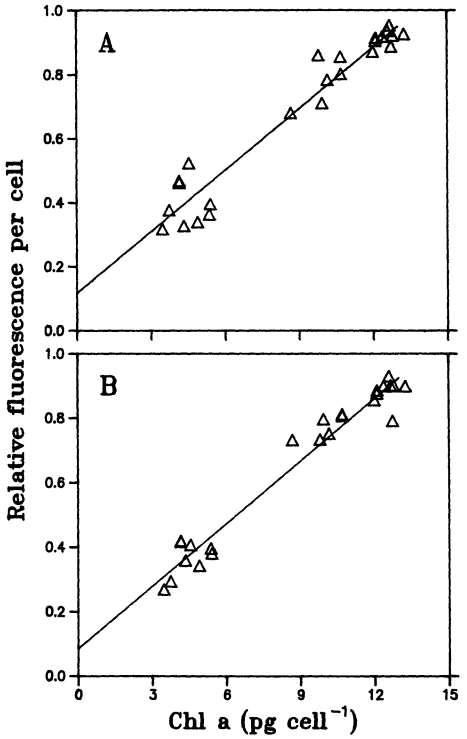

Fig. 2. Fluorescence per cell as a function of Chl *a* per cell in *Thalassiosira weissflogii*. A. Fluorescence measured on individual cells with the Cytomutt flow cytometer. B. Bulk in vivo fluorescence measured in a fluorometer and normalized by cell concentration.

were added to each cell suspension before analysis, and the mean fluorescence value for the cell distribution was divided by the mean value for the bead distribution. This normalization allows the accurate comparison of relative fluorescence among different cultures.

All samples were first analyzed with a microscope-based flow cytometer or "Cytomutt" (Shapiro 1985), described by Olson et al. (1983). Excitation illumination consisted of the blue lines of a mercury arc-lamp where the majority of the emission intensity is at 436 nm. Fluorescence was measured as emitted light passing a 600-nm, long-pass filter. The standards used were 2.1-μm-diameter, Nile Red beads (Pandex) for *T. weissflogii* and 2.24-μm-diameter Fluoresbrite beads (Polysciences, Inc.) for *H. carterae* and *A. carteri*.

All samples were also analyzed on a Coulter EPICS V flow cytometer (Coulter Electronics) with the 488-nm laser line as the excitation source. Fluorescence was measured as emitted light passing a 680-nm bandpass filter (±20-nm bandwidth). For these data, which will be used to compare fluorescence values among the three species, the standard was Nile Red beads for all samples.

Results

Thalassiosira weissflogii—As previously reported for this species (Dubinsky et al. 1986; Falkowski et al. 1985; Post et al. 1984), Chl *a* per cell increased threefold as growth rate decreased due to light limitation (Fig. 1A). Unlike the other two species (*see below*), cell volume also increased at low light intensities, resulting in rather small changes in Chl *a* per unit of cellular volume over the range of light intensities examined (Fig. 1B). The pigment increase at low irradiance resulted in a more than twofold change in the fluorescence per cell measured by flow

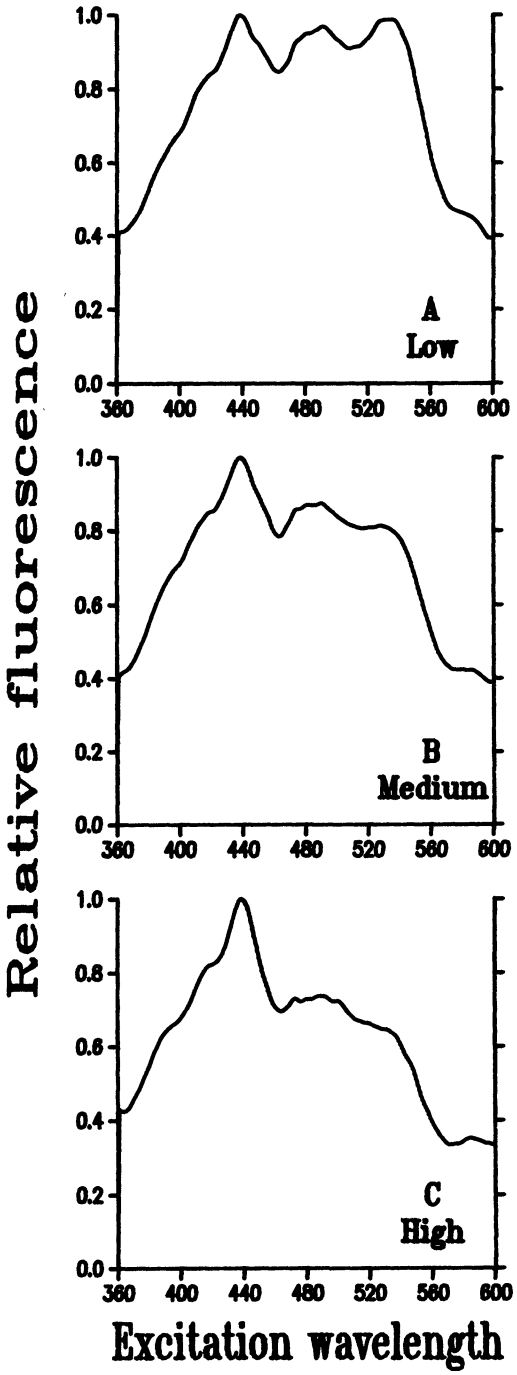

Fig. 3. Representative in vivo fluorescence excitation spectra for *Thalassiosira weissflogii* grown over a range of irradiances. Fluorescence emission was measured at 680 nm. A. Low light: 30 μmol quanta m^{-2} s^{-1}. B. Medium light: 120 μmol quanta m^{-2} s^{-1}. C. High light: 950 μmol quanta m^{-2} s^{-1}.

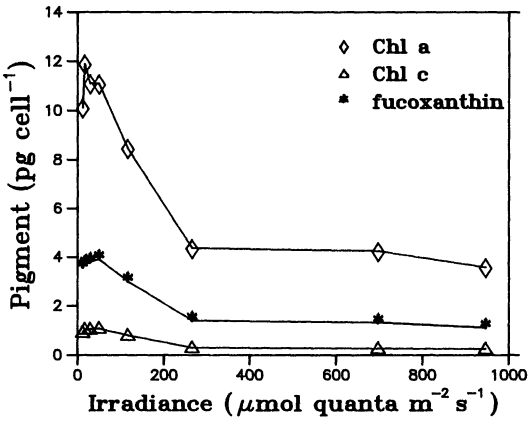

Fig. 4. Cellular Chl *a*, Chl *c*, and fucoxanthin quantified by HPLC in *Thalassiosira weissflogii* grown at different light intensities.

cytometry (Fig. 2A). Fluorescence was related linearly to Chl *a* per cell, and a similar trend was observed for bulk in vivo fluorescence normalized by cell concentration (Fig. 2B).

Fluorescence excitation spectra on whole cells consistently showed three main peaks in fluorescence emission measured at 680 nm (Fig. 3). They correspond to excitation at about 440, 490, and 535 nm and are indicative of absorption by Chl *a*, Chl *c*, and fucoxanthin, respectively (Jeffrey 1980; Prézelin and Boczar 1986). The relative magnitude of these peaks changed markedly as a function of growth light intensity (Fig. 3). The fluorescence intensity was similar for excitation at the three peak wavelengths when cells were grown at low light. As the light intensity for growth increased, however, the peaks in emission due to excitation at 490 and 535 nm decreased relative to excitation at 440 nm.

HPLC analysis of cell extracts showed that Chl *a*, Chl *c*, and the carotenoid fucoxanthin decreased on a cellular basis as growth irradiance increased over subsaturating light intensities (Fig. 4). As noted in work with other species (Chan 1978; Falkowski 1980; Prézelin and Sweeney 1978), these pigments did not continue to increase in cells grown at the lowest photon flux densities, nor did they change over a range of saturating light intensities. The molar ratio of Chl *a* to Chl *c* was near 7 for cells under growth-limiting

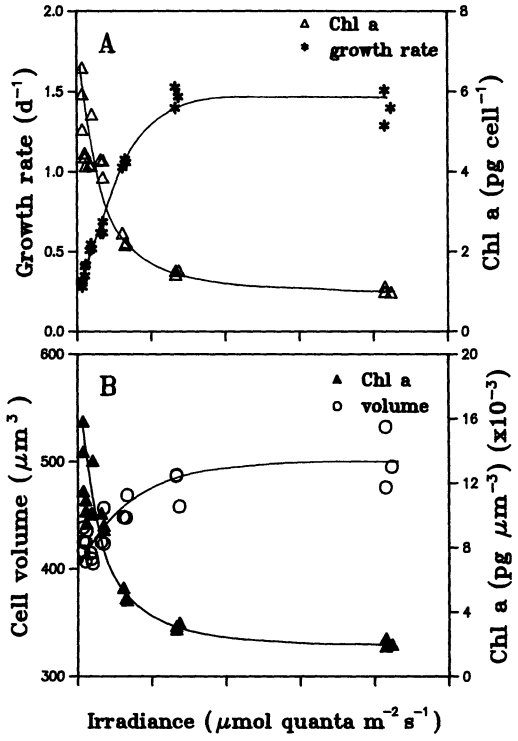

Fig. 5. As Fig. 1, but for the coccolithophorid *Hymenomonas carterae.*

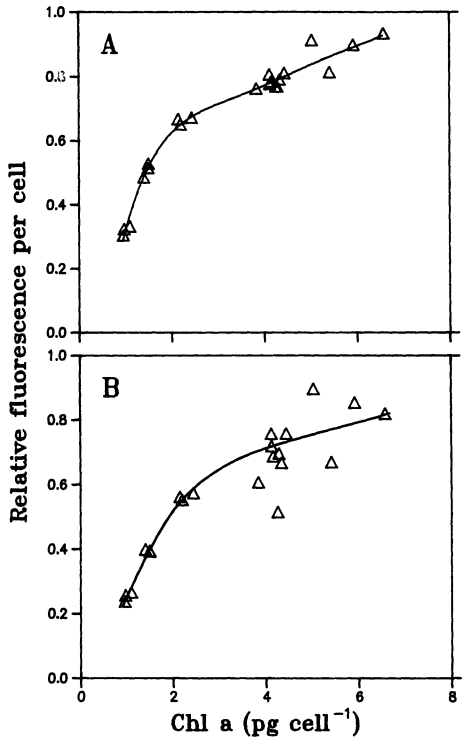

Fig. 6. As Fig. 2, but in *Hymenomonas carterae.*

light conditions and rose to 10 after light became saturating; Chl *a* to fucoxanthin was nearly constant at a molar ratio just over 2 regardless of growth light intensity.

Hymenomonas carterae—Trends of growth rate and Chl *a* per cell as a function of light intensity in the coccolithophorid *H. carterae* were similar to those of the diatom (Fig. 5A). Growth saturated at about the same light level as in *T. weissflogii,* although at a slower rate. Unlike *T. weissflogii,* growth of the coccolithophorid was not inhibited at high irradiances, and cell volume increased with light intensity, contributing to a wider range of Chl *a* per unit of cellular volume (Fig. 5B). Despite similar values of Chl *a* per unit of cellular volume, under all conditions the Chl *a* per cell was lower for *H. carterae* than observed for *T. weissflogii.* Correspondingly, bulk in vivo fluorescence per cell and average fluorescence per cell measured by flow cytometry were consistently lower for the coccolithophorid. Although each of these fluorescence measurements was linear with Chl *a* per cell for *T. weissflogii,* the relationship is less simple for *H. carterae* (Fig. 6). For Chl *a* values $\gtrsim 3$ pg cell^{-1}, relative fluorescence per cell increased less steeply than at lower Chl *a* values. This result was consistent for both bulk in vivo fluorescence and flow cytometry (Fig. 6).

In vivo fluorescence excitation spectra with emission monitored at 680 nm show three broad peaks at about 440, 470, and 530 nm (Fig. 7). As for the diatom, these peaks are indicative of absorption by Chl *a,* Chl *c,* and fucoxanthin. Also consistent with the results for *T. weissflogii,* the relative peak heights shift dramatically for cultures at different light intensities (Fig. 7). High-light cells showed relatively less fluorescence due to excitation at the longer wavelengths than low-light cells.

As expected, Chl *a,* Chl *c,* and fucoxanthin per cell decreased in cells grown in high light (Fig. 8). The molar ratio of Chl *a* to Chl *c* was 7–8 for all light intensities except the highest, where it increased to nearly 12,

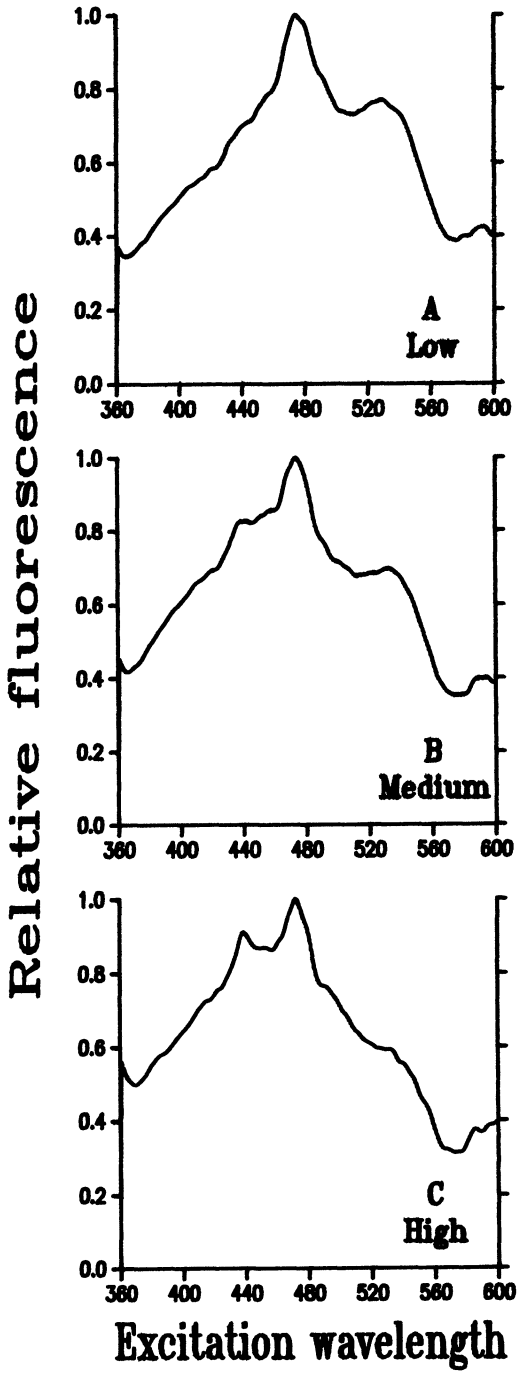

Fig. 7. As Fig. 3, but for whole cells of *Hymenomonas carterae*. A. Low light: 20 μmol quanta m^{-2} s^{-1}. B. Medium light: 120 μmol quanta m^{-2} s^{-1}. C. High light: 850 μmol quanta m^{-2} s^{-1}.

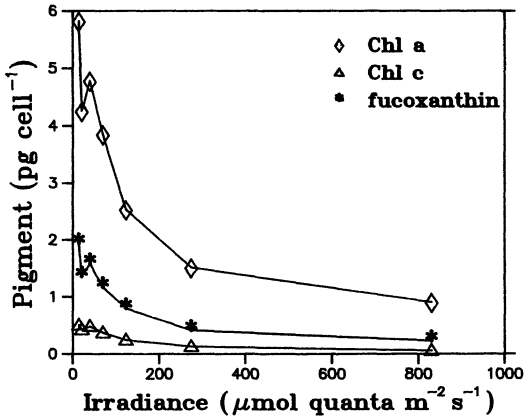

Fig. 8. As Fig. 4, but in *Hymenomonas carterae*.

while Chl *a* to fucoxanthin varied from 2.1 at the lowest irradiance to 2.9 at the highest.

Amphidinium carteri—Growth rate of the dinoflagellate *A. carteri* saturated at a characteristically low (Chan 1978) light intensity (<100 μmol quanta m^{-2} s^{-1}), and the major increases in Chl *a* per cell occurred at <200 μmol quanta m^{-2} s^{-1} (Fig. 9A). As in *H. carterae*, cell volume was generally lower in low-light cells, and the range of Chl *a* per unit of cellular volume was broad relative to *T. weissflogii* (Fig. 9B). Also consistent with results in *H. carterae*, the relationship between fluorescence and Chl *a* was nonlinear over the full range of cellular pigment concentrations (Fig. 10). At Chl *a* concentrations of >2 pg cell^{-1}, fluorescence per cell changed only slightly. As in both of the other species, flow cytometry and bulk fluorescence measurements yielded the same trends (Fig. 10).

Fluorescence excitation spectra for the dinoflagellate as a function of growth irradiance showed trends similar to those observed for the other two species. As growth light intensity decreased, the contribution to fluorescence from absorption by the accessory pigments increased relative to Chl *a* (Fig. 11). Peaks in fluorescence emission occur at excitation wavelengths of about 440, 475, and 530 nm. In contrast to results for *T. weissflogii* and *H. carterae*, the 440-nm Chl *a* peak appears generally as a shoulder and the Chl *c* peak dominates the spectrum. This difference is due to the relatively higher Chl *c* to Chl *a* content of *A. carteri*. The

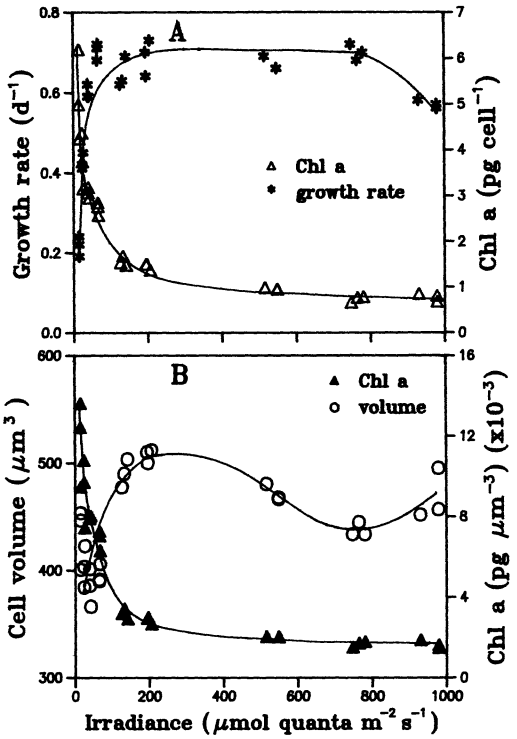

Fig. 9. As Fig. 1, but in the dinoflagellate *Amphidinium carteri.*

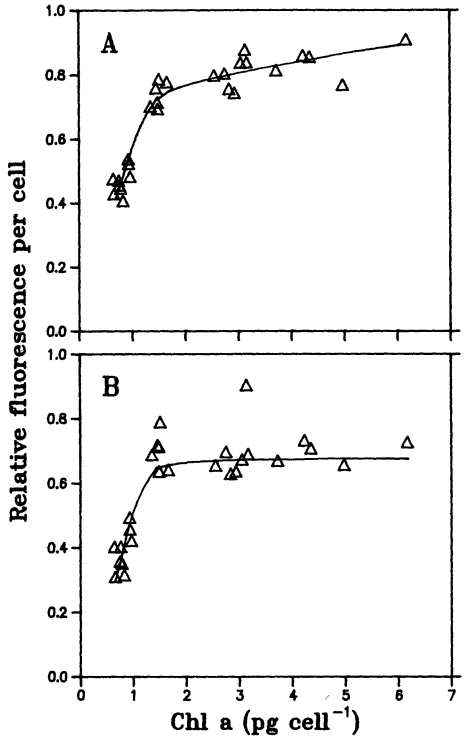

Fig. 10. As Fig. 2, but in *Amphidinium carteri.*

530-nm peak in the excitation spectrum corresponds to absorption by the carotenoid peridinin.

HPLC pigment analysis revealed increases in the chlorophylls and peridinin at low growth irradiances (Fig. 12). Molar ratios of Chl *a* to Chl *c* were 2.1–2.3 for irradiances of 200 μmol quanta m^{-2} s^{-1} or less and increased to just over 3 at the highest light level; the Chl *a* to peridinin ratio was consistently about 1.3 with low-light values as high as 1.5.

Discussion

The three species examined have different relationships between Chl *a* content and fluorescence intensity per cell (Fig. 13). For the same amount of Chl *a* per cell, interspecific differences in the amount of cellular fluorescence were observed. In addition, intraspecific variability between Chl *a* content and fluorescence was also evident. In *T. weissflogii,* the relationship between fluorescence and Chl *a* was roughly linear; i.e. the fluorescence intensity per cell, normalized to Chl *a,* was constant regardless of the light intensity at which the cells were grown. In *H. carterae* and *A. carteri,* however, the relationship was more complex. In these species, fluorescence per unit of Chl *a* decreased markedly as Chl *a* per cell increased due to photoadaptation.

The observed changes in fluorescence per unit of Chl *a* could be due to changes in the efficiency of photon absorption or to changes in the quantum yield of fluorescence. It is not possible to infer from our data whether changes in the quantum yield of fluorescence may be important. We can consider, however, the effects of two factors that change the absorptive properties of the cells: differences in the relative abundances of Chl *a* and accessory pigments and changes in absolute abundances of absorbing molecules and cell size as they contribute to the effects of pigment packaging in cells.

The variability in fluorescence per unit of Chl *a* among species can be explained largely by examination of the accessory pigment

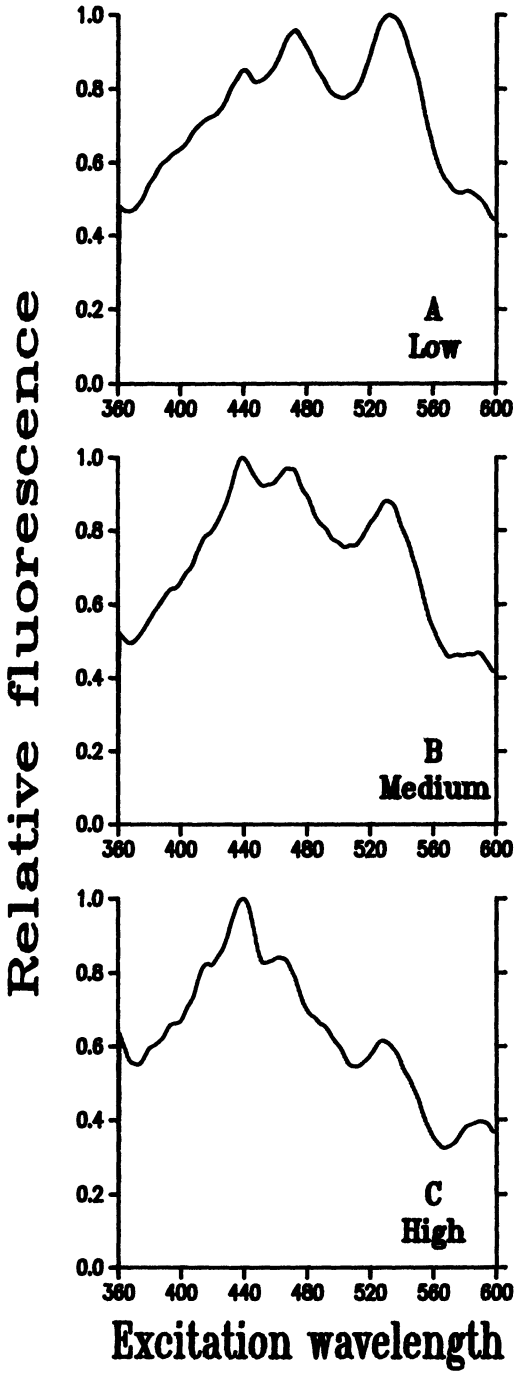

Fig. 11. As Fig. 3, but for *Amphidinium carteri*. A. Low light: 20 μmol quanta m^{-2} s^{-1}. B. Medium light: 200 μmol quanta m^{-2} s^{-1}. C. High light: 930 μmol quanta m^{-2} s^{-1}.

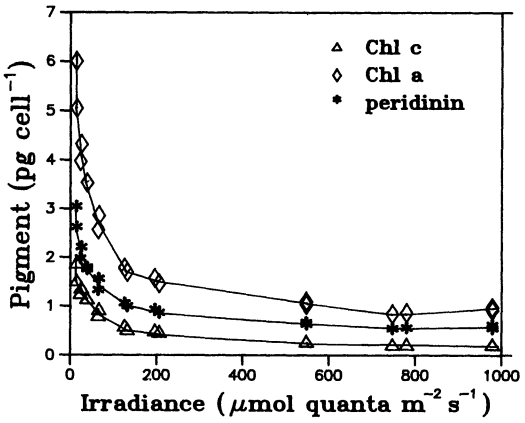

Fig. 12. As Fig. 4, but of cellular Chl *c*, and peridinin in *Amphidinium carteri*.

content of the different species (Fig. 14). For the same amount of Chl *a*, *A. carteri* showed higher flow cytometric fluorescence than the other two species (Fig. 13). This result was also found when fluorescence was measured on the Turner fluorometer. Under all growth conditions, *A. carteri* had more Chl *c* and peridinin relative to Chl *a* than either of the other two species, which had similar Chl *a*: Chl *c* and Chl *a*: fucoxanthin ratios. These pigments absorb blue light and pass energy to Chl *a*, likely explaining the enhanced fluorescence per unit of Chl *a* in *A. carteri*. Changes in relative amounts of accessory pigments cannot explain, however, the changes in fluorescence per unit of Chl *a* as a function of growth light intensity within each individual species.

Accessory pigments may also be the sources of differences in the fluorescence trends between the two flow cytometers. The most pronounced difference is evident for *A. carteri* in which fluorescence per unit of Chl *a* decreases more steeply at high Chl *a* per cell on the Cytomutt than on the EPICS (Figs. 10A, 13). Excitation on the Cytomutt is primarily at 436 nm while the EPICS laser line is at 488 nm. Comparison of these two wavelengths on the fluorescence excitation spectra for *A. carteri* (Fig. 11) shows a dramatic increase in the accessory pigment peaks relative to the Chl *a* peak at low light intensities. This relative increase in fluorescence due to light absorption by accessory pigments is expected to have a larger

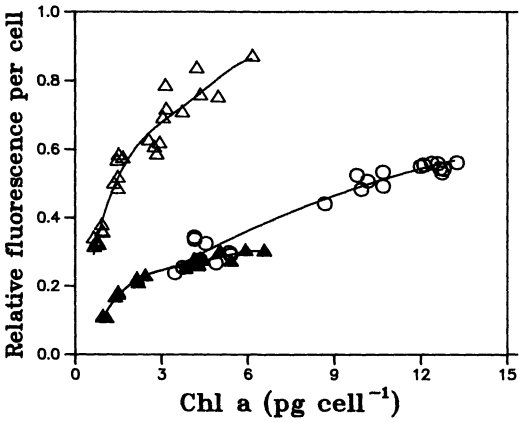

Fig. 13. Fluorescence per cell measured on the EPICS flow cytometer in *Thalassiosira weissflogii* (○), *Hymenomonas carterae* (▲), and *Amphidinium carteri* (△) as a function of Chl *a* per cell. Chl *a* variation was achieved by growing cultures of each species over similar ranges of light intensity.

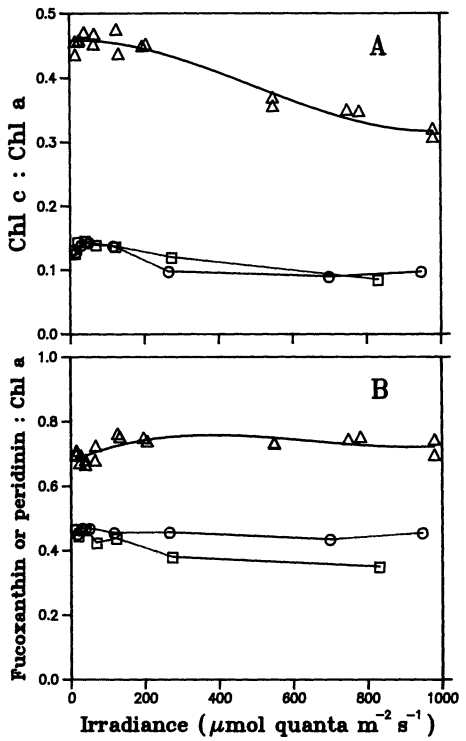

Fig. 14. Molar pigment ratios in *Thalassiosira weissflogii* (○), *Hymenomonas carterae* (□), and *Amphidinium carteri* (△) grown over a range of irradiances. A. Chl *a* : Chl *c*. B. Chl *a* : fucoxanthin in *T. weissflogii* and *H. carterae*; Chl *a* : peridinin in *A. carteri*.

effect on EPICS signals since these pigments are more efficiently excited at 488 than 436 nm.

The relatively similar fluorescence-Chl *a* trends measured on the two flow cytometers and the Turner fluorometer are interesting in light of recent work by Neale et al. (1989). They suggest that although bulk fluorescence measured in a Turner fluorometer corresponds to a low-level fluorescence yield (F_0), excitation with the EPICS laser system produces a yield between F_0 and the maximum yield (F_{max}). In addition, Cytomutt excitation conditions (similar to a FACS analyzer) should produce a yield closer to F_0 than observed with the EPICS. It is difficult to assess how these different fluorescence yields may change relative to one another as cells are acclimated to various growth irradiances. In interpreting our data, however, we rely only on the assumption that the fluorescence yield does not vary greatly for a given species under one set of excitation conditions.

We now turn attention to intraspecific differences in the relationship between pigments and fluorescence. For each species the general trends were the same: as pigment concentration increased under low light, the amount of accessory pigments increased or remained relatively constant with respect to Chl *a* (Fig. 14) and the height of the fluorescence excitation peaks corresponding to absorption by the accessory pigments increased relative to the Chl *a* peak (*see Figs. 3, 7, and 11*). These results suggest that an increase in fluorescence per Chl *a* might be expected in low-light cells, since under these conditions there should be relatively more light absorbed by accessory pigments. We have observed the opposite trend, however: fluorescence per unit of Chl *a* actually decreased in cells grown in low light. This pattern suggests that the changes reflected in the pigment ratios are not of primary importance in determining the relationship between fluorescence per cell and cellular pigment content within a given species.

We hypothesize that the decreased fluorescence per unit of Chl *a* in low-light-grown cells is a result of decreased light absorption per unit of Chl *a* in these highly pigmented cells. The effects of discrete packaging of pigment molecules on light absorption were

Significant symbols

Q_a	Absorption efficiency for a single cell, dimensionless
ρ	Optical thickness along the cell diameter, dimensionless
a_{cm}*	Specific absorption coefficient for cell matter, $m^2\ mg^{-1}$
c_i	Intracellular concentration of absorbing cell matter, $mg\ m^{-3}$
d	Cell diameter, m
A_a	Absorption cross-section of a cell, m^2
s	Geometric cross-section of a cell, m^2

predicted some time ago (Duysens 1956; Morel and Bricaud 1981; Kirk 1983). More recently, spectral absorption changes and changes in Chl *a* specific absorption cross-sections have been measured in cells grown at different light intensities (Dubinsky et al. 1986; Geider and Osborne 1987; Iturriaga et al. 1988; Mitchell and Kiefer 1988; Berner et al. 1989), and the results of these studies have been consistent with predictions of the package effect.

Since we did not measure absorption in this study, we have drawn on the model of Morel and Bricaud (1981, 1986) to help us interpret the patterns we see in our fluorescence data. Collins et al. (1985) and Mitchell and Kiefer (1988) have proposed that this model is appropriate for interpreting cellular fluorescence when factors reflecting the quantum yield for fluorescence and intracellular reabsorption of fluorescence are incorporated. Our goal is simply to calculate the absorption cross-section of our cells at the different growth irradiances and examine the qualitative relationship between changes in this parameter and changes in fluorescence per cell as a function of Chl *a* per cell.

Morel and Bricaud (1986) have shown that the red absorption cross-section can be predicted from the Chl *a* concentration in individual cells. Although our work was done entirely with blue excitation, we have chosen to calculate red absorption cross-sections—recognizing that the comparison should be conservative. Any pigment packaging effects at red wavelengths would be greater in the blue because of the more strongly absorbing blue Soret bands. In addition, since only Chl *a* absorbs at red wavelengths, the relative abundances of the various absorbing accessory pigments are not reflected in these calculations of absorption cross-section. As explained above, however, we have reason to believe that these accessory pigment differences are not of primary importance in interpreting intraspecific fluorescence changes.

From the work of Morel and Bricaud, absorption efficiency at a single wavelength (Q_a), defined as the ratio of the number of photons absorbed by a cell to the number of photons incident on the geometrical cross-section of the cell, can be expressed as a function of the dimensionless parameter ρ:

$$Q_a = 1 + \frac{2[\exp(-\rho)]}{\rho} + \frac{2[\exp(-\rho) - 1]}{\rho^2} \quad (1)$$

where ρ is the product of the specific absorption coefficient (a_{cm}*) of a solution of cell matter, the intracellular concentration of absorbing matter (c_i) and the cellular diameter (d):

$$\rho = a_{cm}^* \times c_i \times d. \quad (2)$$

(Units given in list of symbols.) For cells with relatively low values of the product $d \times c_i$, Q_a varies linearly with ρ. At higher $d \times c_i$, however, the absorption efficiency approaches saturation at $Q_a = 1$, which represents complete absorption (a black body).

Values of ρ and thus Q_a at the red absorption peak (~675 nm) were calculated for each species and light intensity. We used the equivalent spherical diameter measured with the Coulter counter for d, and Chl *a* normalized by cell volume for c_i. The Chl *a* specific absorption coefficient in acetone was used as the value for a_{cm}* (Morel and Bricaud 1986). Since fluorescence intensity depends only indirectly on absorption efficiency (Q_a), being proportional to the number of photons absorbed, we then calculated the absorption cross-section (A_a) of the cell, which is the product of Q_a and the geometrical cross-section of the cell (s):

$$A_a = s \times Q_a. \quad (3)$$

If we plot this calculated absorption cross-section as a function of Chl *a* per cell (Fig.

15), we find that *T. weissflogii* shows a different trend than do *H. carterae* and *A. carteri*. For *T. weissflogii*, A_a (and thus photons absorbed) varies linearly with Chl *a* per cell throughout the measured range, while for the other two species A_a increases more steeply over low values of Chl *a*. These patterns are qualitatively similar to the relationship between fluorescence intensity per cell and Chl *a* per cell we measured in these species (Figs. 2, 6, 10). If we assume that for a given species and excitation method the quantum yield of fluorescence does not change as a function of growth light intensity, the similarities between these two relationships are consistent with the hypothesis that changes in absorption cross-section due to changes in pigment content and cell size are important in predicting flow cytometrically induced fluorescence intensity.

This analysis helps reveal how the influence of changes in cell volume on absorption cross-section may be the key factor which sets *T. weissflogii* apart from the other two species. Although Chl *a* increased in low-light cells in all three species, cell volume decreased with decreasing light intensities in *H. carterae* and *A. carteri* and increased in *T. weissflogii* under the same conditions (*see Figs. 1B , 5B, and 9B*). As a result, intracellular Chl *a* concentration increased twofold in *T. weissflogii* and roughly 10-fold in the other two species as light intensity was decreased over the range of intensities used in this study. This pattern results in a smaller range of values in calculated absorption efficiency (Q_a) for the diatom (Eq. 1, 2), and in a linear relationship between absorption cross-section (A_a) and Chl *a* per cell in this species (Fig. 15). By contrast, the compounding effects of increased Chl *a* per cell and reduced size at low light intensities in the other two species result in curvature of the A_a vs. Chl *a* relationship.

In contrast to our results, Dubinsky et al. (1986) found twofold variation in Chl *a* specific absorption for *T. weissflogii* grown at irradiances within the range presented here. This apparent discrepancy can be explained if cell volume data are considered. Unlike our study, they (Falkowski et al. 1985; Dubinsky et al. 1986) observed that *T. weissflogii* cells grown in low light were smaller than those grown in high light, in addition to having more Chl *a* per cell. Under these conditions, the Chl *a* per unit of cell volume varies widely, and evidence of pigment

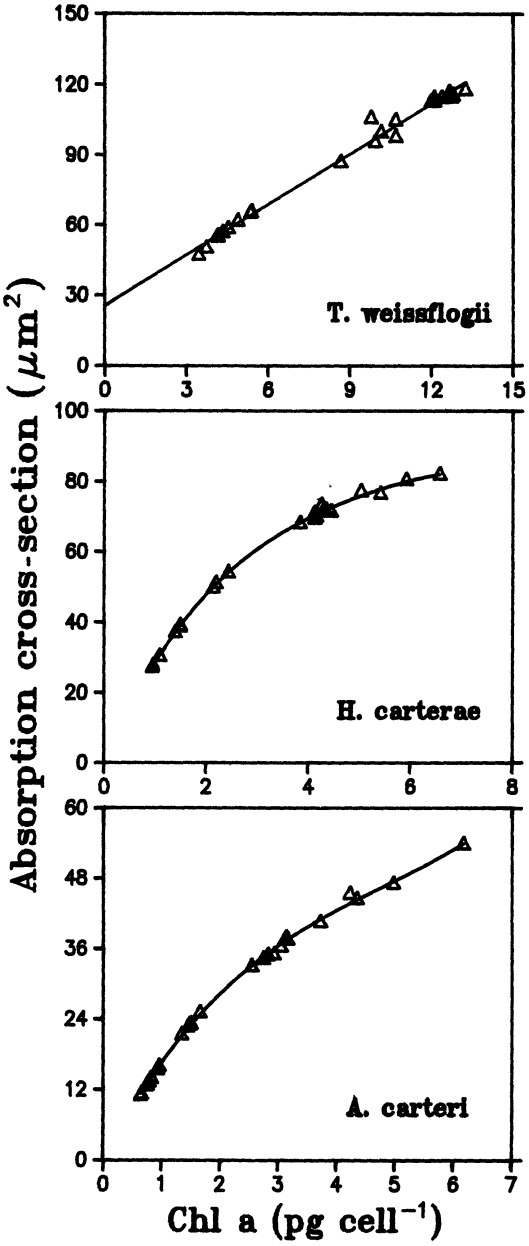

Fig. 15. Absorption cross-section (A_a), the product of absorption efficiency (Q_a) and geometrical cross-section (s), as a function of Chl *a* per cell for each culture.

packaging should be observed. We do not know why the cell volume response in our cells was different from theirs. It is possible that it had to do with how long the various clones had been growing at different light intensities, because average cell volume in diatoms is related to the number of generations that have occurred since auxospore formation.

Although our results strongly support the hypothesis that the package effect plays a role in the fluorescence-pigment relationships in these species, questions remain. To compare rigorously absorption and fluorescence, it is necessary to measure absorption cross-sections at the wavelength of fluorescence excitation. For the case of blue excitation, doing so will include the combined effects of changes in relative pigment abundance and pigment packaging. In addition, it is possible that the transfer efficiency of absorbed photons is generally lower or that the ratio of Chl *a* in photosystem II to Chl *a* in photosystem I is lower in highly pigmented cells. Each of these conditions could contribute to reduced fluorescence per unit of Chl *a* in these cells. The significance of these types of effects on fluorescence yield must be evaluated by comparing the number of photons absorbed and emitted for individual cells. This comparison is also a simplification, however, since enhanced reabsorption of fluorescence is expected in highly pigmented cells (Collins et al. 1985; Mitchell and Kiefer 1988). Finally, changes in chloroplast size and structure, in addition to cell size, should be measured for a rigorous analysis of the package effect (Kiefer 1973; Geider and Osborne 1987; Berner et al. 1989).

From the perspective of interpreting fluorescence signals via sea-going flow cytometry, these results present a set of caveats and opportunities. It is clear that among species, we can make no simple generalizations about the relationship between fluorescence intensity and Chl *a* without considering the role of accessory pigments. Within a given species, however, it appears to be safe to associate higher fluorescence with higher Chl *a* per cell. Although fluorescence intensity need not be directly proportional to pigment content, light-adapted cells can be distinguished from shade-adapted cells. The introduction of dual laser systems, by allowing fluorescence excitation of the same cell at two different wavelengths, could greatly enhance the ability to make this distinction. For instance, a higher ratio of fluorescence excited at the Chl *a* peak to fluorescence excited at the Chl *c* peak would be expected for high-light relative to low-light cells. This type of information could eventually allow us to reconstruct the light history of individual cells and in turn develop models of mixed-layer dynamics.

The key to fully exploiting this type of information lies in our ability to distinguish individual species at sea with flow cytometry. This discrimination has been successful with specific groups of procaryotic picoplankton (Olson et al. 1985, 1988; Chisholm et al 1988*b*; Li and Wood 1988), but we are just beginning to propose schemes for differentiation among the eucaryotes (Yentsch et al. 1983; Olson et al. 1989). Here again, multiple excitation parameters with multiple-beam flow cytometers appear to be the keys to resolution. In the end, the expansion of applications for this technology will depend on the degree to which the optical parameters of individual cells can inform us about properties of the cells and their environment.

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