J. Phycol. **34,** 945–951 (1998)

THE PHYCOBILIN SIGNATURES OF CHLOROPLASTS FROM THREE DINOFLAGELLATE SPECIES: A MICROANALYTICAL STUDY OF *DINOPHYSIS CAUDATA, D. FORTII,* AND *D. ACUMINATA* (DINOPHYSIALES, DINOPHYCEAE)[1]

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ABSTRACT

The absorbance and fluorescence emission spectra for three species of Dinophysis, D. caudata *Saville-Kent,* D. fortii *Pavillard, and* D. acuminata *Claparède et Lachmann, were obtained through an* in vivo *microanalytical technique using a new type of transparent filter. The pigment signatures of these* Dinophysis *species were compared to those of* Synechococcus *Nägeli, a cryptophyte, and two wild rhodophytes, as well as those of another dinoflagellate, a diatom, and a chlorophyte. Phycobilins are not considered a native protein group for dinoflagellates, yet the absorption and fluorescence properties of the three* Dinophysis *species were demonstrated to closely resemble phycobilins and chlorophylls of* Rhodomonas *Karsten (Cryptophyceae). Analyses of* Dinophysis *species using epifluorescence microscopy found no additional nucleus or nuclear remnant as would be contributed by an endosymbiont.*

Key index words: absorbance spectra; Cyclopore filter; Dinophysis; *endosymbiosis; fluorescence spectra; nucleus; phycobilin; toxic algae*

Abbreviations: chl, chlorophyll, DAPI, diamidino-2-phenylindole-2-HCL; FTF, filter–transfer–freeze technique; MeOH, methanol; PEB, phycoerythrobilin; PUB, phycourobilin

Traditionally, diatoms (containing fucoxanthin) can be distinguished from dinoflagellates (containing peridinin), cryptophytes (containing chlorophyll [chl] *c* and C-phycoerythrobilin [PEB]), and red algae (lacking chl *c* but having R-PEB) based on pigment analysis. Spectral data of bulk water samples can be interpreted in terms of the types of phytoplankton that contribute based on these characteristics (Yentsch and Phinney 1982). The types of pigments contained in archetypical algal cells also provide a convenient paradigm to explain evolutionary development involving endosymbiotic acquisition of photosynthetic cellular organelles (Margulis 1970) that resulted in ancestral trees having common and divergent links (Gibbs 1981, McFadden and Gilson 1995, Liaud et al. 1997).

However, relatively recent insights into the dynamics and function of unicellular marine plankton, assisted by tools developed and now used to observe individuals, may require a new evaluation of these classical paradigms. The use of epifluorescence microscopy expanded the perceptions of taxonomists, who began to identify organisms in relation to natural pigmentation and histochemical properties to supplement classifications based on morphology. Today, we now know that what previously had been considered phytoplankton includes both phototrophs and heterotrophs, and a significant number of taxa compose a wide spectrum having mixotrophic or symbiotic lifestyles. Dinoflagellates (considered dinokaryotic) are thought to represent an ancient branching from the evolutionary development toward the eukaryotes (Taylor 1980, Rizzo 1987). Some investigators consider dinoflagellates to be heterotrophic protists that derived their chloroplasts from multiple endosymbiotic events and did not evolve parallel with the evolution of their morphology and general structure of their order (see Dodge 1987).

One of the primitive dinoflagellate genera, *Dinophysis,* has gained recent recognition because it includes toxic species that produce dinophysotoxin responsible for diarrhetic shellfish poisoning, which has closed fisheries in Europe and Asia (Hallegraeff 1995). *Dinophysis* is one of several dinoflagellate genera that contain both photosynthetic and heterotrophic species. Although not all dinophysoid species have been examined with epifluorescence microscopy, approximately half those investigated have been found to be heterotrophic, and many of the remaining, under blue-light excitation, fluoresce yellow to orange, (see cover of *J. Phycol.* 34(6); Lessard and Swift 1986, Geider and Gunter 1988, Hallegraeff and Lucas 1988, Schnepf and Elbrächter 1988, Giacobbe 1995). This is in contrast to the red

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fluorescence that represents chlorophyll–peridinin-containing chloroplasts of ''traditional'' dinoflagellates. Such species were suggested to have phycobilins, and ultrastructural studies indicate the thylakoid structure of their chloroplasts is similar to that found in cryptophytes (Hallegraeff and Lucas 1988, Schnepf and Elbrächter 1988, Lucas and Vesk 1990). Further physiological study on these species has been hampered by the lack of success in obtaining them in culture. Therefore, study of dinophysoid species has been limited to wild forms and restricted by methods where concentration or isolation of individuals from natural populations permitted their investigation.

Here, we report *in vivo* absorbance and fluorescence emission spectra for three species of yellow-fluorescing *Dinophysis*: *D. fortii, D. acuminata,* and *D. caudata.* They represented a minor component of the Southern California Bight plankton communities (<1 cell/L) at the time of sampling. Absorbance and fluorescence emission spectra of individual cells were determined *in vivo* by spectral microphotometry to examine and compare pigment compositions. Epifluorescence microscopical technique was employed to further determine whether a cryptophycean endosymbiont's nucleus or nuclear remnants could be found. Methodology for the spectral analysis of pigments on very rare individuals within a population is described, presenting a potentially important *in vivo* technique for assessing other species-specific characteristics of algae. The *in vivo* spectral signatures of these *Dinophysis* species are compared to those of other algae of both cultured and wild types.

METHODS

Natural phytoplankton surface samples were collected by dipping a 20 μm mesh plankton net into surface waters off the Scripps Institution of Oceanography pier in La Jolla, California (32°50′ N, 117°10′ W). Samples were filtered through either a 100 μm or 50 μm Nitex mesh to remove larger particulates and organisms. The concentrate was then filtered onto a 3.0 μm Cyclopore™ filter (Whatman International Ltd.) until a slight green color was observed. The filter was laid onto a glass coverslip to sandwich the particles. The coverslip with filter was placed on a glass microscope slide with ~0.2 μL filtered seawater as necessary to prevent air bubbles. Microscopical analysis followed immediately. An Olympus AX 70 microscope, coupled with a Nanometrics Nano-500 spectrophotometer, was used to analyze absorbance and fluorescence emission spectra for individual cells. The absorbance procedure is similar to that reported by Graham and Mitchell (in press). For all absorbance spectra, a minimum of four different individuals of each taxa was obtained. The individual absorbance spectrum was first zeroed at 750 nm then normalized to the absorption at 678 nm; replicates were averaged at each wavelength and finally smoothed by 5 nm running averages to obtain representative spectra.

Epifluorescence microscope examination and spectrofluorometric analyses were made using 420–480 nm bandpass excitation (Olympus U-MSWB) and >550 nm long pass emission (Oriel #51302) filters. This configuration effectively blocked the excitation energy allowing the fluorescence emission spectra to be determined using the Nano-500 spectrophotometer. Emission spectra were obtained from a minimum of three individuals. Relative fluorescence intensities for individuals of each species were normalized at 680 nm then averaged and smoothed, as were absorbance spectra.

Taxa examined were the dinoflagellates *D. fortii, D. acuminata, D. caudata,* and *Prorocentrum micans* (Ehrenberg); a centric diatom *Eucampia sp.*; and both the individual cells composing thalli (Red Alga 1) and an unidentified unicellular type (Red Alga 2; 5–10 μm diameter) from the class Rhodophyceae. All natural samples were collected mid-September through mid-October 1997. Numerous attempts to quantify *Dinophysis* species abundance using filtration (up to 1 L seawater) and settling (150 mL) techniques (Reid 1983) were made without success.

Synechococcus cultures of WH-8103, WH-7803, and WH-7805, grown using standard culture plating techniques (Toledo and Palenik 1997), were also examined for pigmentation. Colonies were scooped from agar and squeezed between a coverslip and microscope slide. Our microanalytical system cannot resolve spectra of individual particles <3 μm in diameter accurately; therefore, absorbance and fluorescence emission spectra were determined for aggregates of *Synechococcus* cells. Native wild *Synechococcus* sp. present in our net samples were sometimes found in small aggregates devoid of nonphycoerythrin photosynthetic cells. These aggregates did not provide enough material to obtain good absorbance spectra, but fluorescence emission spectra were obtained.

The nuclear contents of dinoflagellates were examined by epifluorescence microscopy using 4′,6′-diamidino-2-phenylindole-2-HCL (DAPI)-stained samples prepared by a modified filter–transfer–freeze (FTF) technique (Hewes and Holm-Hansen 1983). Net phytoplankton samples were filtered onto polycarbonate filters until a thin film of seawater remained. Methanol (MeOH) was added to extract chlorophyll in a gradient of up to 100% by continuously filtering and slowly adding increased concentrations (starting at 25%) of 4° C MeOH. Cells were left in contact with 100% MeOH for at least 10 min. Rehydration of the cells was made by continuously filtering and slowly adding chilled deionized water. Cells were stained with DAPI, then washed again with distilled water and prepared for combined epifluorescence and transmitted light microscopy using 10% glycerine for the mounting medium.

Cultures of the green alga *Dunaliella* sp. and a cryptophyte, *Rhodomonas* sp., were processed for microanalysis using three methods. Cells were filtered onto 3.0-μm-pore Cyclopore filters, frozen to about −60° C with aerosol freezing spray, allowed to thaw, and subsequently examined for absorbance spectra. These cells also were prepared using FTF with and without 0.5% glutaraldehyde fixation (30 s fixation, followed by FSW wash for 3 min) and were mounted in 10% glycerine. Absorbance spectra of five individual cells for each species and preparation were zeroed at 750 nm then averaged to provide representative spectra. Additionally, both absorbance and fluorescence emission spectra were made of living *Rhodomonas* sp. filtered onto 3-μm-pore Cyclopore filters and processed as described for natural samples.

RESULTS

Glutaraldehyde fixation lowered chl *a* absorption (440 nm and 678 nm) for both *Dunaliella* sp. (not shown) and *Rhodomonas* sp. but most significantly reduced the PEB absorption band between 540 and 580 nm (Fig. 1). Although the baseline-corrected spectra for both FTF- and Cyclopore-filtered material (frozen then thawed since this was a necessary step for FTF) demonstrated the same spectral structure, there were differences in the relative magnitude for peaks and valleys (note in Fig. 1 that the 440-nm chl *a* peaks are about the same, but the 678-nm peaks are different). If one assumes that the FTF preparation mounted in water provided a ''true'' absorbance spectrum of marine particles (Allali et al. 1995), the Cyclopore filter changed the magnitude for absorption but did not add or delete absorption

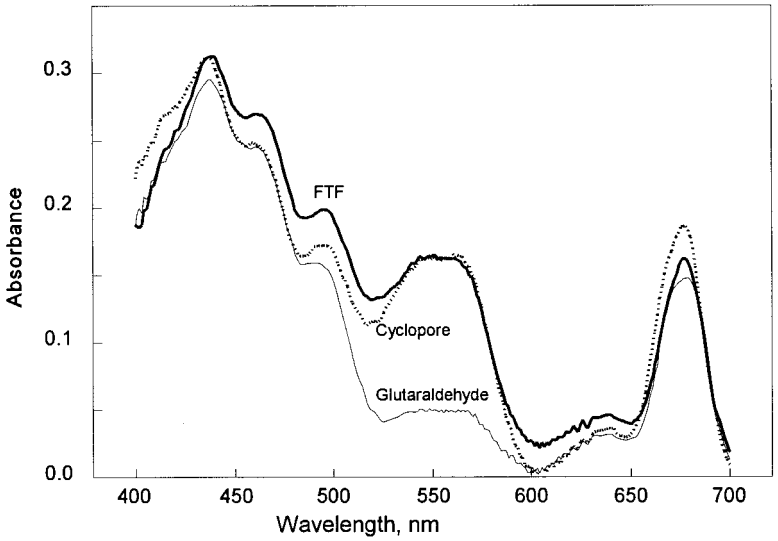

FIG. 1. Absorbance spectra for *Rhodomonas* sp. using FTF and Cyclopore filters without fixation and FTF with ~30-s fixation with glutaraldehyde (identified as glutaraldehyde). All samples were frozen prior to analysis. Glutaraldehyde fixation reduced the phycoerythrin absorption band (centered at ~550 nm). Cyclopore filters cause some spectral distortion relative to FTF; note that the 440 nm chl *a* peaks are about the same for all methods, but the 678 nm chl *a* peak for the Cyclopore filter is higher than that for FTF.

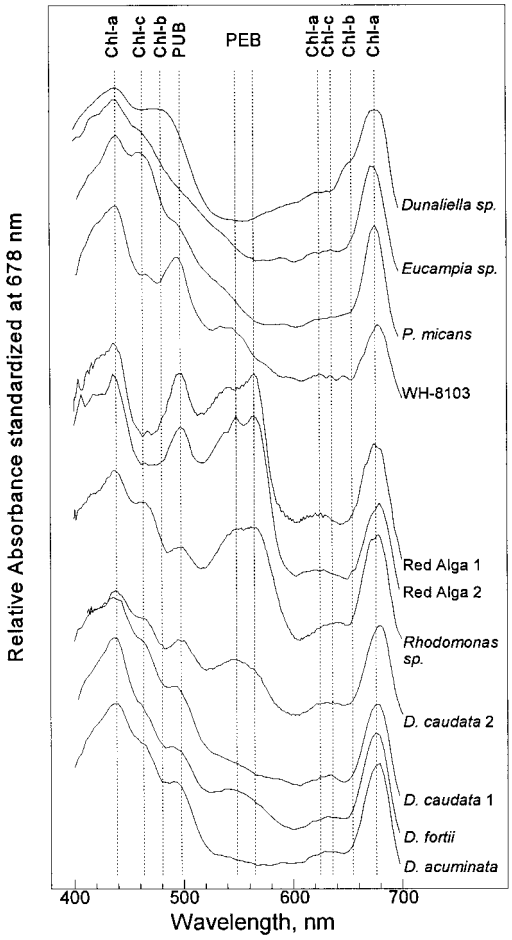

FIG. 2. Relative absorbance spectra for various taxa of chlorophyll-containing unicellular plankton with absorption peaks for chls *a, b, c,* PUB (for *Synechococcus* but alloxanthin in *Rhodomonas*), and PEB (vertical stippled lines). *Dinophysis caudata* was analyzed on two separate occasions and variability in the phycoerythrin (550 nm) absorption is apparent.

peaks or shoulders. The advantage of Cyclopore filtering over FTF methods was that live organisms could be examined, opening up the possibility for physiological studies on individual cells obtained from natural habitats. The disadvantage was that the pores of Cyclopore filters were clearly resolved with phase and differential interference contrast microscopy, thus introducing a background noise that was distracting when examining nano- and picoplankton and possibly causing spectral aberration due to scattering. The optical quality of these filters is excellent, and permits different types of light microscopy to be used (see cover of *J. Phycol.* 34(6)).

The three *Dinophysis* species had absorption peaks at 440, 460, 495, 545, 620, and 678 nm (Fig. 2). The peaks at 440 and 678 nm were attributed to chl *a* and were present in all the other algal specimens as well. The peaks at 460 and 620 nm were attributed mainly to chl *c*. These peaks could also be observed for *P. micans, Eucampia* sp., and *Rhodomonas* sp. but not for the red algae or *Synechococcus* sp. The peak at 495 nm was related both to carotenoids, such as lutein in *Dunaliella* sp.; diadinoxanthin and diatoxanthin in *Eucampia* sp. and *P. micans*; and alloxanthin in *Rhodomonas* sp., whereas in the red alga and *Synechococcus* sp., the peak at 495–498 nm is attributed to phycourobilin (PUB; Glazer et al. 1982, Rowan 1989), as well as the carotenoid zeaxanthin (Grysmski et al. 1997).

High absorption was observed in the yellow region of the spectrum 510–590 nm in *Synechococcus* sp., in the red algae, and also in *D. fortii* and *D. caudata* 1 but was less remarkable for *D. acuminata* and *D. caudata* 2 (Fig. 2). The presence of PEB, in particular, was responsible for absorption in this region for the red algae (Smith and Alberte 1994) and *Rhodomonas sp.* (MacColl and Guard-Friar 1987). The *in vivo* phycobilin absorption peaks in Red Alga 1 were at 498, 534, and 565 nm, whereas in Red Alga

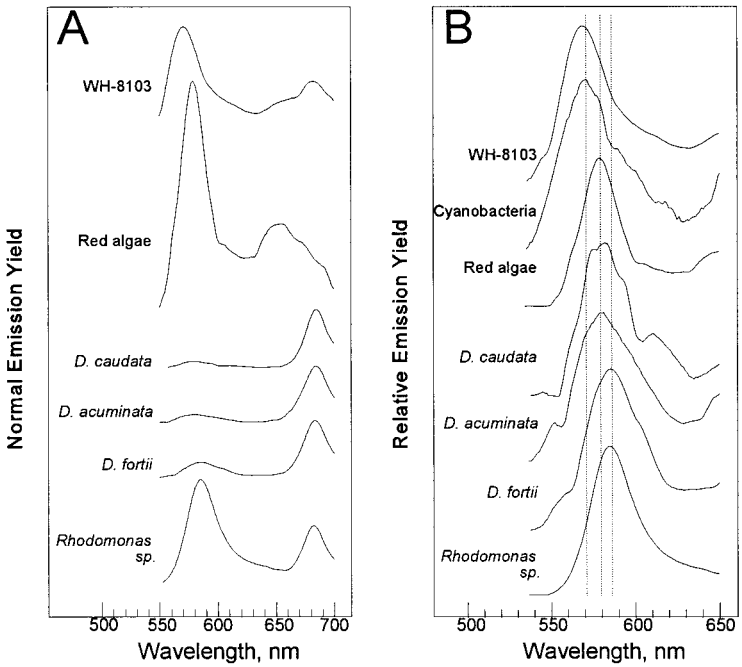

FIG. 3. Fluorescence emission spectra of different taxa normalized to (A) chl *a* emission peak and (B) maximum PEB emission peak. In (B), vertical stippled lines indicate primary emission peaks of cyanobacteria, red algae, and *Rhodomonas* sp., and small secondary shoulders and peaks (*Dinophysis* and cyanobacteria) are considered artifactual due to low signal:noise ratios. Note that cyanobacteria and eukaryotic cells have different wavelengths for their phycoerythrin fluorescence peak.

2 they were at 498, 538 (shoulder), 547, and 565 nm. The absorption in *Rhodomonas* sp. was observed at 545 nm. In *Synechococcus* WH-8103, the peaks for absorption were 493 ± 1 and 544 ± 2 nm. Other *Synechococcus* sp. (data not included) had absorption maxima in this region (WH-7803 at 498 and 546 nm, WH-7805 at 498 and 569 nm; Toledo and Palenik 1997). Finally, the absorption peak in *Dinophysis* sp. was at ~545 nm. However, the cellular concentrations of phycobilin in *D. caudata,* as indicated by the magnitude of 545 nm absorbance, varied on different sampling dates (Fig. 2).

The peaks of the phycoerythrin fluorescence emission spectra for Red Alga 1 (578 ± 1 nm), *Rhodomonas* sp. (585 ± 9 nm), *D. acuminata* (580 ± 0 nm), *D. caudata* (581 ± 1 nm), and D. *fortii* (584 ± 2 nm) were similar (Fig. 3). The fluorescence emission peak for *Synechococcus* WH-8103 was 569 ± 1 nm (WH-7803 was 575 ± 4 nm; WH-7805 was 578 ± 1 nm, not shown in Fig. 3; Toledo and Palenik 1997). A similar difference between peak fluorescence emission of a *Dinophysis* and a cyanobacterium has been reported by Geider and Gunter (1988). Fluorescence emission was also observed at 685 nm, corresponding to chl *a.* In addition, the red algae had fluorescence emissions centered at 655 nm (Fig. 3A), presumably from phycocyanin (Gantt 1981).

The relative *in vivo* fluorescence emission spectra varied considerably in magnitude between species, between individuals of the same species, and over time, necessitating the integration of signals for up to 2 s and averaging 10 scans per individual. For weak signals, such as that obtained from the wild *Synechococcus* sp. and *D. caudata* (Fig. 3B), this resulted in secondary spectral emission structure, which we considered artifact. Due to these limitations, we restricted our discussion of differences in PEB to the spectral position of the primary emission peaks. Epifluorescent microscopical examination of individual cells for each *Dinophysis* species revealed a highly variable color of fluorescence, ranging from bright yellow to orange to red—apparently the result of variations between the relative concentration of PEB to red fluorescing chlorophyll.

Dinokaryotic nuclei were clearly resolved with DAPI staining and combined phase contrast and epifluorescence microscopy. Most of the chlorophyll had been extracted from chloroplast-containing cells (glutaraldehyde or formalin fixation prior to MeOH extraction retained a greater amount of red fluorescence). MeOH was not a particularly good fixative for microscopical purposes, but nuclei for most organisms with a rigid cell casing (diatoms, dinoflagellates, ciliates, etc.) were intact (see Dass and Alfert 1977). Removal of chlorophyll from the samples permitted epifluorescence examination of DAPI-stained nuclei without obstruction from chlorophyll fluorescence. Numerous (10+) observations on individuals for each of the three *Dinophysis* species revealed that they contained a single dinokaryotic nucleus. Chromosomes were condensed, although not as much as found for *P. micans* or *Ceratium* species. In comparison, *D. rotundata* (Claparède et Lachmann), a predatory species not containing chloroplasts, was also present in these samples and contained multiple eukaryotic nuclei, which we attributed to engulfed prey items.

DISCUSSION

Both absorbance and fluorescence emission spectra coincide with those measured in bulk with tra-

ditional spectrophotometers and/or spectrofluorometers (Jeffrey 1980, Rowan 1989, Jeffrey et al. 1997). Glutaraldehyde fixation modified the spectral composition of algal cells, most dramatically those with phycobilin absorption (Fig. 1). Freezing unpreserved cells disrupts their cellular structure (pers. observ.); therefore, the spectral analysis of PEB-containing organisms is best done *in vivo*. The absorption peaks of native rhodophytes suggest R-PEB Type III for Red Alga 1 and R-PEB Type I for Red Alga 2. This identification is tentative due to the change in peak height and thus a different relationship between PUB and PEB peaks when measured *in vivo,* compared to extracted phycobilins. Spectral signatures of both unicellular and multicellular red algae were similar and easily distinguished from those of cryptomonads. Cryptophytes can contain any one of three types of cryptophycean-specific phycoerythrins (absorption maxima at 545, 555, or 568 nm). In this study, *Rhodomonas* sp. had an absorption peak at 545 nm (Fig. 2), which seems to correspond to PEB-545 (MacColl and Guard-Friar 1987).

Dinophysis sp. absorption maxima correspond to those of chl *a,* chl *c,* alloxanthin, and PEB-545 present in *Rhodomonas* sp. (Fig. 2). In addition, the fluorescence emission peak at 580–584 nm corresponds to the 585-nm autofluorescence peak due to PEB-545 in *Rhodomonas* sp. (Fig. 3B). The fluorescence emission normalized to the chl *a* peak (Fig. 3A) shows low autofluorescence of PEB-545 compared to *Rhodomonas* sp. The low autofluorescence could be due to high energy transfer between PEB and chl *a* or low PEB concentration in the cell. Irrespective of the cause, the presence of PEB in *Dinophysis* is confirmed by the fluorescence emission normalized to the PEB peak (Fig. 3B). This emission peak was similar to the one in red algae but differs from those of native wild cyanobacteria present in our samples and WH-8103 (Fig. 3B). Thus, the overall characteristics for absorption of chlorophylls, carotenoids, and phycoerythrins indicates that the pigments found in *Dinophysis* sp. are similar to those of *Rhodomonas* sp. The presence of cryptophycean-like pigmentation in *Dinophysis* chloroplasts has been suspected for some time based on qualitative epifluorescence microscopy (Lessard and Swift 1986, Schnepf and Elbrächter 1988) or the fluorescence emission spectra (Geider and Gunter,1988), although spectral analysis of pigment absorption bands in single cells was not previously reported. Because *D. caudata, D. fortii,* and *D. acuminata* pigments include phycobilins and parallel those of PEB-545-containing cryptophytes, questions arise concerning the origin of these chloroplasts in a dinoflagellate genus.

The only evidence of symbiosis we found were yellow-fluorescing enucleated chloroplasts. Our repeated efforts to find multiple nuclei in photosynthetic *Dinophysis* species using MeOH/DAPI staining methods were unsuccessful. Because the nonphotosynthetic cells of *D. rotundata* were found with numerous nuclear inclusions (derived from eukaryotic prey) and the photosynthetic *Dinophysis* species we observed had only a single nucleus per cell, we conclude the latter were neither mixotrophic nor had an intact symbiont. Only a single nucleus having dinokaryotic morphology was present in each cell, confirming other reports that were based on fewer observations of individuals. Previous studies (Hallegraeff and Lucas 1988, Schnepf and Elbrächter 1988, Lucas and Vesk 1990) provided electron microscope evidence that the chloroplasts of *Dinophysis* species were similar to those found in cryptophytes (e.g. the thylakoids are composed of paired stacks). These studies found that other features of cryptophycean chloroplast ultrastructure were absent, namely the second set of paired membranes enclosing the nucleomorph, the nucleomorph itself, and chloroplast endoplasmic reticulum.

Classical evolutionary concepts incorporate endosymbiosis as the mechanism by which eukaryotes evolved organelles (Margulis 1970), although this hypothesis has been considered a rather conservative process that occurred rarely in the distant past. Cryptophytes represent the result of an endosymbiosis with a red alga ancestor (containing the remnant of that ancestor's nucleus), and red algae are considered to be the more ancient result of endosymbiosis with a blue-green alga (Liaud et al. 1997). Membrane systems are thought to be retained during this type of evolutionary development and provide a record of the ancestral symbiosis (see Gibbs 1981, McFadden and Gilson 1995). Cryptophycean (and other algal taxa) endosymbionts are not uncommon for dinoflagellate species (Dodge 1987). However, a few species lack the membranes and organelles normally associated with cryptophyte endosymbiosis, and several ideas have been presented to account for this (Wilcox and Wedemayer 1984, Schnepf and Elbrächter 1988, Lucas and Vesk 1990). For the several species of *Dinophysis* that have now been examined, the reduced membrane and enucleated chloroplasts incorporating PEB-545-like photosystems seem to be common among them. This genus is considered primitive for dinoflagellates (Taylor 1980), but these mutually atypical chloroplasts provide evidence to suggest that these *Dinophysis* species stem from a common ancestor that evolved after PEB-545 types of cryptophytes. Evidently, the acquisition of cellular organelles may be more common and may have occurred more recently, as well as being of more varied origins than generally understood. Furthermore, because only the dinokaryotic nucleus is present in *Dinophysis* species, we question if this should be considered symbiosis at all.

Our qualitative observations from epifluorescence microscopy suggested that PEB:chl *a* concentrations were highly variable for each of the three *Dinophysis*

species, since the range of emission colors for individuals varied between yellow and red. Such variability in fluorescence color has been noted elsewhere (Lessard and Swift 1986, Giacobbe 1995). The concentration of phycobilins relative to chlorophylls and associated pigments for other taxa has been found quite variable as related to light intensity (Kana and Glibert 1987, Grysmski et al. 1997) and nutrient concentration (Collier et al. 1994) and is dependent on the physiological, ecological, and genetic backgrounds of those organisms that contain them (Grossman et al. 1993a, b). Therefore, the variability in pigmentation for *Dinophysis* that we found is not remarkable, although the differences found in pigment content for individuals of a species in the same sample would suggest that large intraspecies diversity or environmental acclimation response may occur within a population. Quantification of the range of PEB:chl *a* values for a species in natural samples is only possible, however, by use of microspectrophotometry (Fig. 2).

The distinct spectral differences in the 500–600-nm region evident in Figure 2 between PEB-containing species and those lacking this pigment would suggest that such features should be easily discriminated in the ocean. There are examples of phycobilins making large contributions to total bulk particle absorption, as seen in blooms of cryptophytes in the Antarctic (Vernet 1992) and coccoid cyanobacteria in the equatorial and northern Atlantic (Morel 1997). Despite the ubiquitous presence of coccoid cyanobacteria (Li et al. 1983), phycoerythrins are not a prominent feature of bulk pigment absorbance spectra in much of the ocean; dominant spectral types are more similar to traditional chl *a*/chl *c* or chl *a*/chl *b Prochlorococcus*-type taxa, especially in deep offshore waters (Mitchell and Kiefer 1988, Garver et al. 1994, Sosik and Mitchell 1995). In the open ocean, *Synechococcus* types tend to be PUB-dominant rather than PEB-dominant (Campbell and Iturriaga 1988). PEB-545 has a unique spectral signature that might be identified from bulk water samples as an indicator of the presence of photosynthetic *Dinophysis* species. Several of the photosynthetic *Dinophysis* species are toxic (Hallegraeff 1995), and because these are retained by ~20-μm screens that would otherwise pass most cryptophytes, water samples could be easily processed and analyzed for their presence. Based on our study, some programs for coastal monitoring of toxic algae could benefit by incorporating epifluorescence microscopy and phycobilin analysis. Microspectrophotometry, as demonstrated by our analysis of *Dinophysis,* can provide the description of optical and pigment variability for a species within a diverse community that is not feasible with other methods.

Support for C.D.H. and B.G.M. was from ONR grant N00014-91-J-1186, support for T.A.M. was from NASA Graduate Fellowship NGT 5-30036, and support for M.V. was from NASA NAGW-3795. The microspectrophotometric system was obtained through funding from ONR grant N00014-94-1-0951. O. Holm-Hansen donated office equipment and supplies to C.D.H. The authors thank M. Graham for introducing us to Cyclopore filters and G. Toledo for providing us with cultures. We also thank two reviewers for their constructive comments.

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