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Relating photosynthetic pigments and *in vivo* optical density spectra to irradiance for the Florida red-tide dinoflagellate *Gymnodinium breve*

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ABSTRACT: The lipophilic pigment content and composition and *in vivo* optical density (OD) spectra for batch cultures of the Florida (USA) red-tide dinoflagellate *Gymnodinium breve* Davis were analyzed after exposure to irradiance treatments representative of an irradiance gradient that cells might experience in a coastal water column. Decreases in total chlorophyll (chl) content (attributable to decreases in chls *a*, c_1/c_2 and c_3) and total carotenoid content (attributable to decreases in total fucoxanthin and 19'-acylofucoxanthins, and gyroxanthin-diester) coincided with exposure to increased irradiance. The relative abundances of chl pigments remained constant whereas decreases in the relative abundances of total fucoxanthin and 19'-acylofucoxanthins, and β-ψ-carotene and an increase in the relative abundance of diadinoxanthin coincided with exposure to increased irradiance. Because gyroxanthin-diester has been observed only in a limited number of toxic dinoflagellates (of which *G. breve* is the only warm-water taxon), was consistently quantifiable throughout various irradiance-induced physiological states, and had a distinctive elution position and absorption maxima in the chromatographic eluent, this carotenoid may serve as a 'biomarker' for *G. breve* within Florida coastal waters. An analysis of variance, performed on fourth-derivative plots derived from normalized OD spectra, identified portions of the OD spectra which differed among irradiance treatments. Designated wavelengths coincided with wave crests in the fourth-derivative plots representative of absorption maxima for total chl *c* (460 to 468, 585 to 590 nm) and total fucoxanthin and 19'-acylofucoxanthins, and diadinoxanthin (490 to 496 nm). Stepwise discriminant analysis identified a set of 6 wavelengths (403, 541, 546, 509, 673 and 663 nm) which optimally classified OD spectra for populations among irradiance treatments. This lack of correspondence between wavelengths identified by the 2 statistical techniques was not unexpected; fourth-derivative analysis identified portions of the OD spectra where differences in the sharpness of curvature (as produced by absorption maxima of component pigments) occurred among treatments whereas discriminant analysis identified portions of the spectra which could be used to classify populations among treatments and did not necessarily have to correspond to absorption maxima. Such detectable differences in the bio-optical 'signatures' of *G. breve* indicate that previous light history, as manifested in the pigment component, could be incorporated into future pigment-based monitoring applications to allow for the detection and/or physiological characterization of problematic taxa prior to bloom status.

KEY WORDS: Chlorophyll · Carotenoid · Stepwise discriminant analysis · Fourth derivative · High-performance liquid chromatography · *In vivo* absorption · Red tide Phytoplankton

INTRODUCTION

The recent increased incidence of harmful algal blooms has been attributed to 'hidden' taxa (Anderson et al. 1993, Hallegraeff 1993) whose growth and toxicity are triggered by a consortium of physical, chemical, and biological factors. Once established, harmful algal blooms typically are mono-specific or near mono-specific, reaching extremely high biomass over small spatial scales. Consequently, the bio-optical 'signatures' of

the blooms are distinct and usually constant for an extended time period, thereby lending themselves to photosynthetic pigment-based applications, such as high-performance liquid chromatograph (HPLC)-derived pigment analyses, *in vivo* absorption assays, and multi-spectral remote sensing.

Pigment-based applications provide for characterization of bloom dynamics in relevant temporal and spatial scales and in response to event-scale processes and/or perturbation affecting such dynamics (e.g. Campbell et al. 1986, Tyler & Stumpf 1989, Millie et al. 1993, Carrick & Worth 1994) and may allow for identification of problematic flora prior to bloom status. However, pigment analyses, *in vivo* absorption assays, and remote sensing collect mutually exclusive, yet complementary, data sets. For example, the future deployment of multi-spectral satellite/airborne sensors and *in situ* transmissometers will evoke improved pigment scanning capabilities, thereby facilitating acquisition of remotely sensed imagery based on pigments other than chlorophyll (chl) *a* (see Millie et al. 1992, 1995, Richardson et al. 1994). The coupling of this information with accurate taxon-specific spectral absorption/reflectance and HPLC-confirmed pigment 'signatures' will be required to provide a previously unrecognized calibration of spectral reflectance data (Millie et al. 1993).

Blooms of the toxic dinoflagellate *Gymnodinium breve* Davis usually develop offshore of western Florida (USA) in the Gulf of Mexico (Steidinger 1975) and may be transported along the western coast of Florida, through the Florida Straits and along the eastern seaboard of the United States via the Loop Current, the Florida Current, and the Gulf Stream, respectively (Murphy et al. 1975, Tester et al. 1989, 1991). Physical forcing events (e.g. water circulation, tides, winds) can transport offshore assemblages to coastal waters (Steidinger & Ingle 1972) where the release of brevetoxins from cells may cause deleterious effects on public health and finfish/shellfish and aquacultural fisheries (Steidinger et al. 1973, Steidinger & Baden 1984, Riley et al. 1989).

Phototactic-induced vertical migration of *Gymnodinium breve* provides for maximum concentrations of cells (as great as 1×10^8 cells l^{-1}; G. J. Kirkpatrick unpubl. data) within surface waters, often discoloring the water in red, brown and/or yellow hues (i.e. 'red tides'). Presumably, differences in bloom color are due to alterations in cell pigment content/composition in response to changing irradiance and/or cell senescence, which affect cell-specific absorption and spectral reflectance. Because knowledge of the alteration in the bio-optical signature of *G. breve* is necessary for accurate pigment-based monitoring applications, the intent of this study was to characterize the lipophilic pigment complex and the *in vivo* optical density (OD) spectra of *G. breve* in response to varying irradiance.

METHODS

Culture conditions and experimental design. Batch cultures of *Gymnodinium breve* (clone W53DB, Mote Marine Laboratory) were maintained in f/20 growth medium (Guillard & Ryther 1962) within culture flasks at 25 ± 1°C. A mixture of cool-white, and Vita-Lite fluorescent lamps provided the culture flasks with 60 µE m^{-2} s^{-1} of photosynthetic active radiation (PAR) in a 12 h light : 12 h dark cycle (lights on at 06:00 h). All PAR measurements were made with a Biospherical Instruments, Inc., model QSL-100 quantum scalar irradiance sensor placed in culture flasks filled with growth medium. A sheet of 0.13 mm clear acetate was placed between the lamps and the flasks to minimize culture exposure to UV-B radiation produced by the lamps. To reduce changes in light transmission characteristics of the plastic during experimental treatment periods (see below), the acetate sheet was placed in position 100 h prior to the beginning of the irradiance treatment.

Nine 2.8 l Pyrex Fernbach flasks, each containing 1 l of growth medium, were inoculated with an equal volume of an exponentially growing maintenance culture. The cultures were exposed to maintenance light conditions and incubated for 9 to 14 d until cell densities reached approximately 2.17 to 2.39×10^7 cells l^{-1} (see Table 1). The length of this incubation was chosen to ensure adequate biomass for bio-optical analyses and to ensure relatively equivalent physiological states among the cultures.

The 9 flasks were randomly divided into 3 groups; each group then was randomly assigned to 1 of 3 irradiance treatments (60 and 165 µE m^{-2} s^{-1} PAR and 220 µE m^{-2} s^{-1} PAR + UV irradiance). These treatments were chosen to represent an irradiance gradient that cells might experience at distinct depths within a coastal water column (previous culture experience indicated that laboratory cultures of *Gymnodinium breve* would not sustain growth at irradiances greater than the greatest irradiance tested). A Phillips Corp. model F40UVB sun lamp, which had been preconditioned with a 72 h burn-in period, provided the UV irradiance. The UV lamp was controlled by a separate timer from that controlling the fluorescent lamps and was on from 10:00 to 14:00 h daily. The relative UV-exposure level was determined using a Hamamatsu Corp. model G3614 UV-sensitive photodiode detector placed within an empty culture flask. Using this configuration, the experimental UV-exposure level was approximately 5% of the solar, sea-surface UV irradi-

ance measured at noon in late spring. This method of comparison, using a broad-band UV detector (>10% peak response between 260 to 400 nm) and a lamp with peak emission at approximately 310 nm, overemphasizes the UV-B component relative to the natural spectrum. UV-B radiation from the fluorescent lamps was attenuated by an acetate sheet for all flasks except those assigned to the 220 µE m^{-2} s^{-1} PAR + UV irradiance treatment.

On the initial day of the irradiance treatments (Day 0), aliquots from the 3 flasks for each treatment were pooled. This pooled volume was subsampled for culture cell density and bio-optical characterization (see below). The cultures then were exposed to their respective irradiance treatment. On Days 4 and 8 after exposure to irradiance treatments, cultures were analyzed for biomass and bio-optical characteristics (see below). Five replicate experiments were performed sequentially.

Biomass and bio-optical analyses. All sampling was performed between 13:00 and 14:00 h local time. For determination of culture biomass, cells of culture aliquots were preserved immediately with Utermöhl's solution and later enumerated using a hemacytometer (Guillard 1973). For analyses of lipophilic pigments and *in vivo* OD spectra, culture aliquots were filtered under low vacuum (<75 mm Hg) onto GF/F glass-fiber filters. Filters for lipophilic pigments were immediately frozen in liquid nitrogen and stored at −20°C until analysis. Filters for *in vivo* absorption were immediately analyzed after filtration.

For pigment analyses, frozen filters were placed in 3 ml of 100% acetone, sonicated and extracted in darkness at −20°C for at least 1 h. Filtered extracts (75 to 125 µl) were injected directly into a Hewlett Packard model 1090 HPLC equipped with a ODS-Hypersil C_{18} column (200 × 4.6 mm, 4 µm) and a diode array detector set at 440 nm. The mobile phase followed the tertiary gradient system described by Wright et al. (1991). Solvent flow rate was 1 ml min^{-1}. The column temperature was 40°C. Pigment peaks were identified and quantified by comparison of retention times and absorption spectra to those of authentic crystalline standards, including chl *a* (Sigma Chemical Company), fucoxanthin (Hoffman-LaRoche & Company), and β-carotene (Sigma Chemical Company). All other pigments were identified by comparison to pigments extracted from phytoplankton cultures (see Wright et al. 1991) and quantified using the appropriate extinction coefficients (from Mantoura & Llewellyn 1983).

In vivo OD spectra of cells collected on glass-fiber filters were recorded using a Varian model DMS-80 UV-VIS spectrophotometer (after Yentsch 1962; for a review see Cleveland & Weidemann 1993). To minimize the loss of scattered light, filter holders were fabricated to position the filters as close as possible to the detector windows. The OD was zeroed at 750 nm for each sample. Prior to each sample scan, a glass-fiber filter, wetted with filtered media, was scanned to establish a 'blank' baseline. This baseline was subsequently subtracted from all sample scans. Because an objective of the study was to compare the OD spectra among treatment cultures of *Gymnodinium breve* only, OD data were not corrected for pathlength amplification (β).

Statistical analyses. The effects of irradiance and exposure time on pigment content and ratio and the relative abundances of chl and carotenoid fractions within the total chl and carotenoid pigments were compared by an analysis of variance (ANOVA). The significance between pairs of variable means was analyzed by a least significant difference (LSD) analysis. LSD analyses were considered applicable only if the ANOVA indicated significant differences between variable means among irradiance and days (Snedecor & Cochran 1980). Because only cells that had been exposed to 60 µE m^{-2} s^{-1} PAR were used as inoculum for the experimental flasks, no comparison among irradiance treatments could be made for Day 0. Therefore, the ANOVA only compared values for Days 4 and 8. To increase the variance associated with binomial proportion (percentage) data, relative abundances of chls and carotenoids were square-root- then arcsin-transformed prior to statistical analysis (see Snedecor & Cochran 1980).

In vivo OD spectra first were normalized to each respective spectral mean OD (after Roesler et al. 1989). The spectral fourth derivative (Butler & Hopkins 1970) then was computed for each normalized spectrum to resolve the position of the absorption peaks (see Owens et al. 1987, Bidigare et al. 1989, Smith & Alberte 1994). An ANOVA was performed for each wavelength (391 to 725 nm) in the fourth-derivative plots for cultures 8 d after exposure to irradiance treatments in order to detect wavelengths where the different irradiance treatments produced detectable differences in the shape of the OD spectra.

Stepwise discriminant analysis (Tabachnick & Fidell 1983) was used to identify wavelengths allowing optimal classification of OD spectra for cultures exposed to distinct irradiance treatments (cf. Johnsen et al. 1994b). Normalized OD spectra of cultures 8 d after exposure to irradiance treatments were discriminated for every spectral wavelength between 400 and 700 nm. Classification error rates, calculated by cross-validation and re-substitution techniques, were used to determine which set of wavelengths sufficiently differentiated culture populations among irradiance treatments. These classification rates were obtained from a separate discriminant analysis using the wavelengths selected by the stepwise analysis.

RESULTS

Daily cell counts of cultures in 2 of the experimental replicates indicated that the cultures were in logarithmic growth at the beginning of the treatment period and had transitioned to early stationary phase by the end of the experiment. On Day 4, biomass was relatively similar among populations exposed to irradiance treatments (Table 1). However, on Day 8, the biomass of populations exposed to 60 µE m^{-2} s^{-1} PAR were ca 1.4-fold greater than those of populations exposed to 220 µE m^{-2} s^{-1} PAR + UV irradiance (due to termination of growth after Day 4 in populations exposed to 220 µE m^{-2} s^{-1} PAR + UV irradiance). Additionally, the dramatic decreases in the amounts of chls and carotenoids per unit cell on Days 4 and 8 from those on Day 0 (see Tables 1 & 2) were most likely attributable to the rapid cell division occurring in the culture populations.

Quantifiable lipophilic pigments within *Gymnodinium breve* determined by HPLC included chls *a*, c_1/c_2, and c_3 and the carotenoids 19'-butanoyloxyfucoxanthin, fucoxanthin, 19'-hexanoyloxyfucoxanthin, diadinoxanthin, gyroxanthin-diester and β-ψ-carotene (Fig. 1). Because chls c_1 and c_2 co-elute when using the HPLC solvent system employed here (see Wright et al. 1991), the presence of either (or both) pigment(s) was reported as chl c_1/c_2. Additionally, because baseline separations of 19'-butanoyloxyfucoxanthin, fucoxanthin and 19'-hexanoyloxyfucoxanthin were not consistently achieved, these pigments are (hereafter) reported collectively as total fucoxanthin and 19'-acylofucoxanthins.

Decreases in total chl content ($p \leq 0.0309$), attributable to decreases in chl *a* ($p \leq 0.0358$), chls c_1/c_2 ($p \leq 0.0069$) and chl c_3 ($p \leq 0.0097$) contents, coincided with increasing irradiance (Table 1). On both Days 4 and 8, the chls *a*, c_1/c_2 and c_3 contents of populations exposed to 60 µE m^{-2} s^{-1} PAR were ca 1.7-, 1.9- and 1.7-fold greater, respectively, than those in populations exposed to 165 µE m^{-2} s^{-1} PAR and 220 µE m^{-2} s^{-1} PAR + UV irradiance.

Decreases in total carotenoid content ($p \leq 0.0187$), attributable to decreases in total fucoxanthin and 19'-acylofucoxanthin ($p \leq 0.0129$) and gyroxanthin-diester ($p \leq 0.0180$) contents, coincided with increasing irradiance (Table 2). On Day 4, the total fucoxanthin and 19'-acylofucoxanthins and gyroxanthin-diester contents of populations exposed to 60 µE m^{-2} s^{-1} PAR were ca 1.4- and 1.7-fold greater than those of populations exposed to 165 µE m^{-2} s^{-1} PAR and/or 220 µE m^{-2} s^{-1} PAR + UV irradiance. By Day 8, the gyroxanthin-diester contents of populations exposed to 60 µE m^{-2} s^{-1} PAR were ca 1.7-fold greater than populations exposed to 165 µE m^{-2} s^{-1} PAR and 220 µE m^{-2} s^{-1} PAR + UV irradiance. Diadinoxanthin and β-ψ-carotene contents were not (statistically) altered with increasing irradiance.

The relative abundances of chls and carotenoids exhibited non-uniform responses to irradiance treatments. Chls *a*, c_1/c_2 and c_3 accounted for ca 90, 6, and 4%, respectively, of the total chl within *Gymnodinium breve* (Table 3). The relative abundances of chls

Table 1. Biomass and chlorophyll (chl) content of *Gymnodinium breve* populations exposed to irradiances of 60 and 165 µE m^{-2} s^{-1} photosynthetic active radiation (PAR) and 220 µE m^{-2} s^{-1} PAR + 5% of surface ultraviolet (UV) irradiance. Data are means ± standard errors (n = 5). Day (D) and irradiance (I) effects (as determined by analysis of variance) are significant ($p \leq 0.05$) if designated. Variable means within and between Days 4 and 8 with different superscripted letters are significantly different ($p \leq 0.05$). Least significant difference analyses based on 17 degrees of freedom

Variable	Effects	Irradiance	Day 0	Day 4	Day 8
Biomass (cells × 10^7 l^{-1})	D	60	2.17 ± 0.19	2.48 ± 0.04 [a]	3.68 ± 0.53 [b]
		165	2.18 ± 0.10	2.73 ± 0.03 [a]	3.11 ± 0.18 [a,b]
		220 + UV	2.39 ± 0.25	2.80 ± 0.05 [a,b]	2.57 ± 0.33 [a]
Chl *a* (pg $cell^{-1}$)	I	60	8.68 ± 0.60	5.00 ± 0.89 [a]	3.66 ± 1.28 [a,b]
		165	7.02 ± 1.01	3.31 ± 0.09 [a,b]	2.08 ± 0.31 [b]
		220 + UV	7 58 ± 0.71	2.75 ± 1.69 [a,b]	1.99 ± 0.52 [b]
Chl c_1 and c_2 (pg $cell^{-1}$)	D, I	60	0.59 ± 0.03	0.35 ± 0.04 [a]	0.23 ± 0.03 [b]
		165	0.50 ± 0.08	0.21 ± 0.00 [b]	0.13 ± 0.08 [b]
		220 + UV	0.52 ± 0.06	0.16 ± 0.01 [b]	0.11 ± 0.06 [b]
Chl c_3 (pg $cell^{-1}$)	D, I	60	0.34 ± 0.18	0.23 ± 0.01 [a]	0.13 ± 0.03 [b,c]
		165	0.25 ± 0.01	0.15 ± 0.02 [b]	0.08 ± 0.01 [c]
		220 + UV	0.52 ± 0.08	0.12 ± 0.01 [b,c]	0.08 ± 0.02 [c]
Total chl (pg $cell^{-1}$)	I	60	9.61 ± 0.63	5.58 ± 0.93 [a]	4.02 ± 1.38 [a,b]
		165	7.77 ± 1.10	3.68 ± 0.10 [a,b]	2.29 ± 0.33 [b]
		220 + UV	8.41 ± 0.85	3.02 ± 0.19 [b]	2.17 ± 0.57 [b]

Table 2. Carotenoid content and chlorophyll/carotenoid ratio of *Gymnodinium breve* populations exposed to irradiances of 60 and 165 μE m^{-2} s^{-1} PAR and 220 μE m^{-2} s^{-1} PAR + 5% of surface UV irradiance. Data are means ± standard errors (n = 5). Day (D) and irradiance (I) effects (as determined by analysis of variance) are significant ($p \leq 0.05$) if designated. Variable means within and between Days 4 and 8 with different superscripted letters are significantly different ($p \leq 0.05$). Least significant difference analyses based on 17 degrees of freedom

Pigment	Effect	Irradiance	Day 0	Day 4	Day 8
Fucoxanthin + 19'-acylofucoxanthins (pg $cell^{-1}$)	D, I	60	6.34 ± 0.13	3.78 ± 0.36 ^{a}	2.37 ± 0.63 ^{b}
		165	5.25 ± 0.62	2.45 ± 0.15 ^{b}	1.50 ± 0.24 ^{b}
		220 + UV	5.53 ± 0.13	2.02 ± 0.13 ^{b}	1.40 ± 0.30 ^{b}
Diadinoxanthin (pg $cell^{-1}$)	D	60	0.99 ± 0.02	0.64 ± 0.07 ^{a}	0.39 ± 0.08 ^{b,c}
		165	0.92 ± 0.10	0.62 ± 0.00 ^{a}	0.37 ± 0.04 ^{b,c}
		220 + UV	0.97 ± 0.12	0.56 ± 0.04 ^{a,b}	0.33 ± 0.07 ^{c}
Gyroxanthin-diester (pg $cell^{-1}$)	D, I	60	0.61 ± 0.01	0.32 ± 0.01 ^{a}	0.26 ± 0.043 ^{a,b}
		165	0.51 ± 0.06	0.26 ± 0.02 ^{a,b}	0.16 ± 0.10 ^{c}
		220 + UV	0.57 ± 0.08	0.22 ± 0.01 ^{b,c}	0.15 ± 0.04 ^{c}
β-ψ-carotene (pg $cell^{-1}$)		60	0.12 ± 0.02	0.06 ± 0.04 ^{a}	0.04 ± 0.03 ^{a}
		165	0.11 ± 0.01	0.04 ± 0.01 ^{a}	0.01 ± 0.01 ^{a}
		220 + UV	0.09 ± 0.01	0.01 ± 0.00 ^{a}	0.01 ± 0.00 ^{a}
Total carotenoids (pg $cell^{-1}$)	D, I	60	6.79 ± 0.18	4.79 ± 0.46 ^{a}	3.07 ± 0.67 ^{b,c}
		165	8.06 ± 0.80	3.37 ± 0.16 ^{b}	2.04 ± 0.18 ^{b,c}
		220 + UV	7.17 ± 0.83	2.81 ± 0.29 ^{b,c}	1.90 ± 0.41 ^{c}
Total chlorophyll/total carotenoid		60	1.19 ± 0.05	1.16 ± 0.08 ^{a}	1.27 ± 0.17 ^{a}
		165	1.14 ± 0.03	1.09 ± 0.02 ^{a}	1.12 ± 0.06 ^{a}
		220 + UV	1.17 ± 0.02	1.08 ± 0.04 ^{a}	1.13 ± 0.05 ^{a}

remained constant among irradiance treatments within a particular sampling day. Total fucoxanthin and 19'-acylofucoxanthins, diadinoxanthin, gyroxanthin-diester and β-ψ-carotene accounted for ca 76, 15, 8, and 1%, respectively, of the total carotenoids (Table 3). Decreases in the relative abundances of total fucoxanthin and 19'-acylofucoxanthins ($p \leq 0.0001$) and β-ψ-carotene ($p \leq 0.0016$) and an increase in the relative abundance of diadinoxanthin ($p \leq 0.0001$) coincided with increasing irradiance. On Days 4 and 8 in populations exposed to 165 μE m^{-2} s^{-1} PAR and 220 μE m^{-2} s^{-1} PAR + UV irradiance, relative abundances of total fucoxanthin and 19'-acylofucoxanthins, and diadinoxanthin decreased and increased, respectively, ca 7% from that of populations exposed to 60 μE m^{-2} s^{-1} PAR. On Day 8, the relative abundance of β-ψ-carotene was ca 3-fold greater in populations exposed to 60 μE m^{-2} s^{-1} PAR than that of populations exposed to 220 μE m^{-2} s^{-1} PAR + UV irradiance.

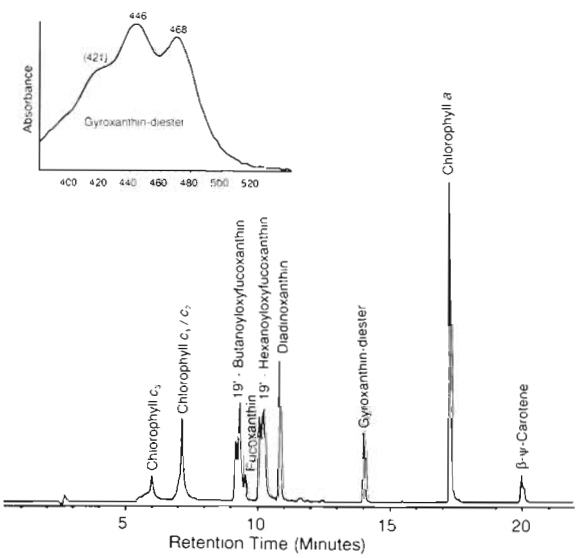

Fig. 1. Representative, reverse-phase HPLC chromatogram of lipophilic pigments extracted from *Gymnodinium breve*. The spectral plot for gyroxanthin-diester (insert) identifies the distinct absorption maxima for this carotenoid

Fourth-derivative plots of the OD spectra for *Gymnodinium breve* (Fig. 2) produced wave crests indicating points within OD spectra where shoulders or peaks occurred. Conversely, wave troughs indicated points within the spectra where valleys (between shoulders and peaks) occurred. The occurrence of wave crests was representative of absorption maxima of the component pigments whereas wave troughs delineated regions of weaker absorption between the peak maxima. Wave crests in the fourth-derivative plots (and the pigments to which they corresponded; after Bidigare et al. 1990, Johnsen et al. 1994a, b, Smith & Alberte 1994) occurred at 412 nm (chl *a*), 438 nm (chl *a*, diadinoxanthin), 466 nm (total chl *c*, diadinoxanthin), 495 nm (total fucoxanthin and 19'-acylofucoxanthin, diadinoxanthin), 589 nm (total

Table 3. Percent relative abundance of chlorophyll and carotenoid fractions in total chlorophyll and carotenoid pigments, respectively, of *Gymnodinium breve* populations exposed to irradiances of 60 and 165 μE m^{-2} s^{-1} PAR and 220 μE m^{-2} s^{-1} PAR + 5% of surface UV irradiance. Data are means ± standard errors (n = 5). Day (D) and irradiance (I) effects (as determined by analysis of variance) are significant ($p \leq 0.05$). Variable means within and between Days 4 and 8 with different superscripted letters are significantly different ($p \leq 0.05$) if designated. Least significant difference analyses based on 17 degrees of freedom

Pigment	Effect	Irradiance	Day 0	Day 4	Day 8
Chlorophylls					
a	D	60	89.89 ± 0.40	89.55 ± 0.65 [a]	90.31 ± 0.66 [a]
		165	89.96 ± 0.34	89.63 ± 0.51 [a]	90.61 ± 0.34 [a]
		220 + UV	89.97 ± 0.51	89.96 ± 0.96 [a]	90.93 ± 0.52 [a]
c_1 and c_2	D,I	60	6.52 ± 0.27	6.42 ± 0.41 [a]	5.98 ± 0.17 [a,b]
		165	6.71 ± 0.34	6.11 ± 0.19 [a]	5.68 ± 0.32 [a,b]
		220 + UV	6.44 ± 0.31	5.64 ± 0.43 [a,b]	5.11 ± 0.16 [b]
c_3		60	3.58 ± 0.09	4.03 ± 0.48 [a]	3.71 ± 0.34 [a]
		165	3.33 ± 0.15	4.27 ± 0.19 [a]	3.71 ± 0.18 [a]
		220 + UV	3.59 ± 0.34	4.40 ± 0.54 [a]	3.95 ± 0.37 [a]
Carotenoids					
Fucoxanthin and 19'-acylofucoxanthins	I	60	79.64 ± 0.96	78.67 ± 0.15 [a]	77.89 ± 0.67 [a]
		165	78.54 ± 1.21	73.59 ± 1.16 [b]	74.64 ± 1.18 [b]
		220 + UV	78.62 ± 1.41	72.17 ± 0.81 [b]	72.92 ± 0.95 [b]
Diadinoxanthin	I	60	11.76 ± 0.52	13.25 ± 0.15 [a]	12.67 ± 0.17 [a]
		165	12.81 ± 0.81	18.13 ± 0.65 [b,c]	17.00 ± 0.96 [b]
		220 + UV	12.68 ± 0.86	19.78 ± 0.39 [b,c]	18.69 ± 1.09 [b,c]
Gyroxanthin-diester	D	60	7.32 ± 0.29	6.79 ± 0.47 [a]	8.11 ± 0.65 [a]
		165	7.22 ± 0.30	7.16 ± 0.45 [a]	7.81 ± 0.23 [a]
		220 + UV	7.51 ± 0.43	7.48 ± 0.53 [a]	7.92 ± 0.20 [a]
β-ψ-carotene	I	60	1.27 ± 0.18	1.27 ± 0.45 [a,b]	1.32 ± 0.39 [a]
		165	1.43 ± 0.11	1.12 ± 0.19 [a,b]	0.55 ± 0.13 [a,b]
		220 + UV	1.19 ± 0.19	0.56 ± 0.16 [a,b]	0.46 ± 0.02 [b]

chl *c*), 639 nm (total chl *c*, total fucoxanthin and 19'-acylofucoxanthin), and 677 nm (chl *a*).

The ANOVA identified wavelengths of the fourth-derivative plots derived from OD spectra which differed among the irradiance treatments ($p \leq 0.05$). Because it was our intent only to identify portions of the OD spectra which differed, the significance between pairs of wavelength means was not determined. Wavelengths having a significant *F*-value (Fig. 3) directly coincided with the apex of wave crests in the fourth-derivative plots representative of absorption maxima for total chl *c* (460 to 468, 585 to 590 nm) and total fucoxanthin and 19'-acylofucoxanthins and diadinoxanthin (490 to 496 nm) (see Fig. 2). The ANOVA also identified significant *F*-values for 409 and 682 nm, wavelengths which coincided with the shoulders of wave crests in the fourth-derivative plots representative of absorption maxima for chl *a*.

The stepwise discriminant analysis identified 13 wavelengths as optimal classifiers of OD spectra for populations exposed to distinct irradiance treatments. The set of 6 wavelengths (403, 541, 546, 509, 673 and 663 nm; in the cumulative order of wavelength selection) fully optimized (100%) the classification of cultures (Table 4, Fig. 4). Obviously, the remaining wavelengths chosen by the analysis (400, 483, 407, 405, 589, 507 and 586 nm; in the cumulative order of wavelength selection of 7 through 13) could not improve on this optimization and, therefore, were not considered.

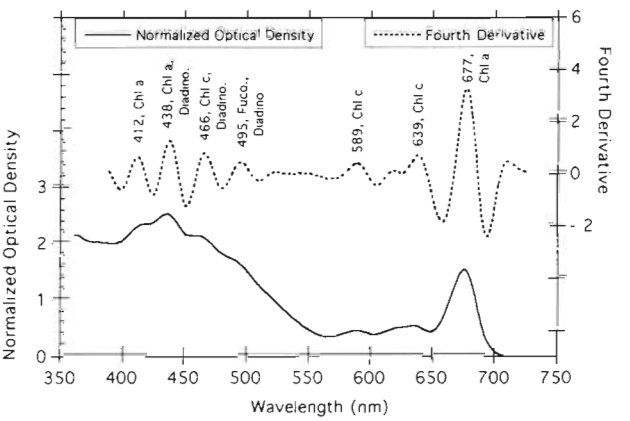

Fig. 2. A representative, normalized optical density (OD) spectrum and associated fourth-derivative plot for *Gymnodinium breve*. The wave crests of the fourth-derivative plot correspond to peaks and shoulders of the OD spectrum representative of absorption maxima for component pigments. The approximate wavelength of and the primary chlorophyll (Chl) and carotenoid (Fuco: total fucoxanthin and 19'-acylofucoxanthins; Diadino: diadinoxanthin) pigment(s) responsible for the absorption maxima are indicated

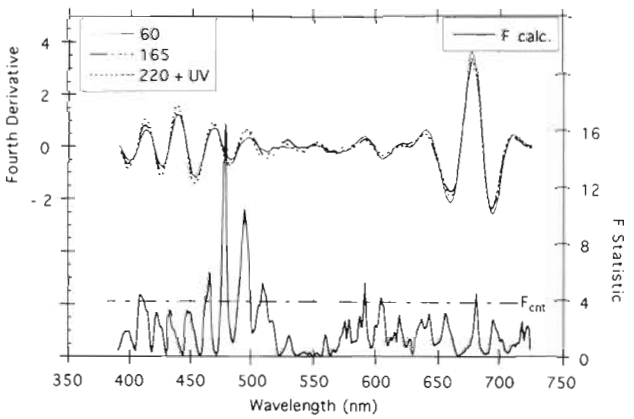

Fig. 3. Mean fourth-derivative plots of OD spectra (n = 5) for *Gymnodinium breve* populations 8 d after exposure to irradiances of 60 and 165 µE m^{-2} s^{-1} PAR and 220 µE m^{-2} s^{-1} PAR + 5% of surface UV irradiance. The wavelength-dependent *F*-statistic (F_{calc}) and the significant *F*-value (F_{crit}) from the analysis of variance of the fourth derivatives are overlain to depict the regions of the OD spectra to be distinct among irradiance treatments

DISCUSSION

The carotenoid peridinin is the primary light-harvesting pigment and also is considered the diagnostic pigment for most autotrophic dinoflagellates (Jeffrey et al. 1975, Millie et al. 1993) *Gymnodinium breve* does not have peridinin, but rather has fucoxanthin and 19'-acylofucoxanthins as the primary light-harvesting pigments, a condition observed in several prymnesiophytes and chrysophytes and a few dinoflagellates (e.g. Jeffrey et al. 1975, Tangen & Bjørnland 1981, Haxo 1985, Bjørnland et al. 1988, Hooks et al. 1988, Johnsen et al. 1992, Johnsen & Sakshaug 1993). The accessory chls observed in *G. breve* included chls c_1/c_2 and c_3. However, because the HPLC method used here cannot differentiate between chls c_1 and c_2, we were unable to definitively indicate whether one or both pigments occur. Jeffrey (1976) reported that chl c_1 is present in almost all chromophytes (except the peridinin-based dinoflagellates) and that chl c_2 is present in several fucoxanthin-based dinoflagellates. Bidigare et al. (1990) noted the presence of chl c_3 in common bloom-forming chromophytes, including *G. breve*. These lines of evidence support the hypothesis that the fucoxanthin-based dinoflagellates arose from endosymbiosis of colorless flagellates with chromophytes, such as chrysophytes and/or prymnesiophytes (Jeffrey 1976, Bjørnland et al. 1988).

Vertical migration of phototactic cells within the water column provides for variable exposure to photon flux density and spectral quality. Presumably, differences in the color of 'red tide' assemblages are due to distinct pigment alterations in response to photoacclimation and/or pigment degradation due to cell senescence. Although culture populations of *Gymnodinium breve* had transitioned to early stationary phase by the end of the experiment, cell senescence was not observed as (1) cells continued diurnal vertical migrations throughout the entire experiment under all treatment irradiances, (2) cell sediment deposits (such as those observed in maintenance culture carboys when the populations 'crash') were not observed in any experimental flasks, and (3) chlorophyll-degradation products (such as pheophytin) commonly associated with cell senescence were not observed in any pigment chromatogram. Consequently, differences in photosynthetic pigments and/or *in vivo* OD spectra among treatment cultures were attributed to differences in irradiance.

Table 4. Wavelengths derived from stepwise discriminant analysis allowing optimal classification of optical density spectra for *Gymnodinium breve* populations among irradiance treatments

Cumulative no. of wavelengths	Wavelength (nm)	Percent successful classifications (%)
1	403	40
2	541	60
3	546	73
4	509	93
5	673	93
6	663	100

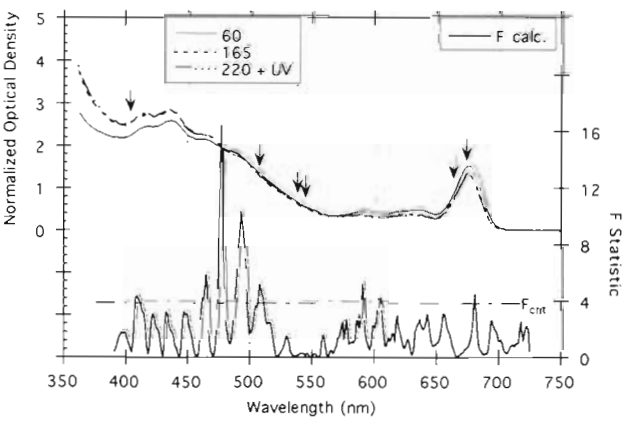

Fig. 4. Mean, normalized OD spectra (n = 5) for *Gymnodinium breve* cultures 8 d after exposure to irradiances of 60 and 165 µE m^{-2} s^{-1} PAR and 220 µE m^{-2} s^{-1} PAR + 5% of surface UV irradiance. The wavelength-dependent *F*-statistic (F_{calc}) and the significant *F*-value (F_{crit}) from the analysis of variance of the fourth derivatives are overlain to depict the regions of the OD spectra to be distinct among irradiance treatments. The wavelengths of OD spectra identified by the stepwise discriminant analysis to be distinct among irradiance treatments are indicated by arrows

Lipophilic pigments within *Gymnodinium breve* exhibited non-uniform responses to irradiance treatments, most likely resulting from alterations in the size of the light-harvesting components associated within each photosynthetic unit rather than alterations in the cellular concentrations of the photosynthetic unit (after Prézelin 1976, Schofield et al. 1990). Culture populations exposed to 60 $\mu E\ m^{-2}\ s^{-1}$ PAR had greater chls *a* and *c*, total fucoxanthin and 19'-acylofucoxanthins, and gyroxanthin-diester contents than populations exposed to greater irradiance. Because chls *a*, c_2 and c_3, fucoxanthin, and the 19'-acylofucoxanthins are photosynthetically active (Haxo 1985, Schofield et al. 1990, Johnsen et al. 1994a), alterations in these pigments would be expected. Diadinoxanthin, the second major carotenoid component in *G. breve*, is considered a photo-protectant pigment (Demers et al. 1991, Brunet et al. 1993) and the content of this pigment would be expected to increase with increasing irradiance. Although the cell contents of diadinoxanthin and β-ψ-carotene were not (statistically) altered, the relative abundance of these pigments to the total carotenoid pool increased and decreased, respectively, upon exposure to increased irradiance.

The exact environmental conditions leading to harmful algal blooms are poorly understood (see Paerl 1988). Consequently, it is extremely difficult to effectively predict the occurrence and magnitude of a bloom, thereby ensuring an 'after-the-fact' management strategy dependent upon accurate water-quality evaluation (H. Paerl, Institute of Marine Sciences, University of North Carolina, Morehead City, NC 28557, USA, pers. comm.). Current monitoring efforts rely on microscopic identification and enumeration of harmful taxa in water samples. However, such a microscopic-based monitoring system requires a high level of taxonomic skill, usually takes considerable time, and can be highly variable among personnel (Millie et al. 1993). As such, the implementation of an alternative and/or complimentary evaluation system for predicting bloom occurrence and dynamics is highly desirable. Diagnostic pigment signatures and *in vivo* OD spectra can effectively differentiate among most phylogenetic groups of micro- and macroalgae and, sometimes, taxa within a variety of habitats (Millie et al. 1993, Johnsen et al. 1994b, Smith & Alberte 1994). If such diagnostic pigments and/or spectra would allow for detecting the presence of harmful taxa prior to bloom status, a rapid, objective, and economical 'biomarker' protocol easily could be deployed for monitoring applications over temporal and spatial scales relevant to fisheries and water-quality management.

To be adequate biomarkers, pigment signatures and OD spectra must be unique to an organism or small group of closely related organisms, be relatively stable throughout various physiological states of the organism, and have distinct chromatographic and/or absorption characteristics to allow for definitive recognition. The major carotenoids and accessory chl pigments within *Gymnodinium breve* also are found in other chromophytes (see above) and have absorption maxima within HPLC eluent in a 4 to 10 nm range, characteristics which eliminate these pigments as possible biomarkers. However, gyroxanthin-diester may be a diagnostic pigment for *G. breve* within Florida coastal waters. This pigment only has been reported from the toxic dinoflagellates *Gyrodinium aureolum* Hulbert, *Gymnodinium galatheanum* Braarud and *G. breve*, all of which have fucoxanthin and/or 19'-acylofucoxanthins as the major light-harvesting carotenoid(s) (Johnsen & Sakshaug 1993). Of these taxa, only *G. breve* can be considered a warm-water taxon (see Andersen et al. 1991) and would be expected to occur in Florida coastal waters. Additionally, gyroxanthin-diester was a minor, yet stable, component of the total carotenoids in *G. breve*, being consistently detectable and quantifiable in populations exposed to all irradiance treatments. Finally, the distinct elution position and absorption maxima in the HPLC eluent (see Fig. 1) for gyroxanthin-diester allow for it to be distinguished easily from other carotenoids in chromatographic separations.

Fourth-derivative analysis identified portions of the OD spectra which had the greatest curvature as produced by absorption of light-harvesting pigments. A large wave crest in the fourth-derivative plot did not necessarily correspond to a large amount of pigment. Rather, differences in the height of the wave crests indicated differences in the shape (sharpness of curvature) of the OD spectra, thereby indicating qualitative alterations of the light-harvesting pigments. Consequently, this sensitivity to differences in shape makes the fourth-derivative analysis a useful tool for rapid assessment of pigment alterations within blooms. For example, wavelengths having a significant *F*-value directly corresponded with absorption maxima for chl *c* and total fucoxanthin and 19'-acylofucoxanthins, pigments whose cell contents were significantly altered by increasing irradiance. The ANOVA also identified significant *F*-values for 409 and 682 nm, suggesting that alterations in chl *a* may have been responsible for differences among fourth-derivative plots. However, these wavelengths did not directly correspond to the absorption maxima of chl *a* (i.e. apex of wave crests in the fourth-derivative plots) and no differences among fourth-derivative plots at 438 nm, the wavelength representative of the strongest absorption maxima for chl *a*, were observed.

On the other hand, stepwise discriminant analysis selected a set of wavelengths which could be used for

differentiating among spectra. Stepwise discriminant analysis selects wavelengths individually and does not take into account potential relationships among wavelengths (e.g. distinct portions of the OD spectrum having distinct pigment absorption, yet similar light-harvesting functions). Wavelengths identified by stepwise discriminant analysis may not necessarily correspond to absorption peaks of the component light-harvesting pigments, but more likely, should fall between the peaks where differences among OD spectra are maximum. Indeed, in comparisons of the irradiance treatments for *Gymnodinium breve*, wavelengths identified by the ANOVA for the fourth-derivative plots did not correspond to wavelengths identified by stepwise discriminant analysis for the *in vivo* OD spectra (see Fig. 4).

Utilizing stepwise discriminant analysis for differentiating among normalized *in vivo* OD spectra, Johnsen et al. (1994b) successfully grouped phylogenetically related species of phytoplankton. Their ability to differentiate among taxa decreased slightly when spectra from populations of the same taxa acclimated to distinct irradiances were included in the analysis. Obviously, their inclusion of intra-specific variability attributable to physiological state with the variability attributable to inter-specific differences effectively increased the variance of the discriminant. The wavelengths of the OD spectra denoted by Johnsen et al (1994b) to be taxonomically dependent (481, 535, 586, 628 and 649 nm) were different from the wavelengths identified by stepwise discriminant analysis for *Gymnodinium breve* to be irradiance dependent. This difference, most likely, occurred because the former wavelengths were intended for differentiating among 31 taxa belonging to 11 distinct phylogenetic groups whereas the latter wavelengths only were intended to differentiate among irradiance-induced physiological states for a single taxon. Using fourth-derivative analysis on *in vivo* OD spectra, Smith & Alberte (1994) also were able to detect differences among macrophytes acclimated to distinct irradiances. These results indicate that phylogenetically distinct algal taxa can be differentiated based on bio-optical characteristics.

In this study, cultures of *Gymnodinium breve* exposed to distinct irradiances exhibited quantitative differences in lipophilic pigment 'signatures' and in the shape of the *in vivo* OD spectrum. This suggests that for mono-specific blooms, previous light history, as manifested in the pigment component, is detectable through pigment-based applications such as HPLC-based pigment and *in vivo* OD analyses. In the future, the coupling of data acquired through *in situ* multi-wavelength transmissometers and/or hyperspectral satellite/airborne-based sensors with such bio-optical characterizations may permit the determination of early bloom history, thereby leading to a greater understanding of the mechanisms triggering and forcing harmful algal blooms.

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