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## Optical discrimination of a phytoplankton species in natural mixed populations

### Abstract

Developing optical detection techniques for discriminating particular phytoplankton species in mixed assemblages has long been a goal of aquatic scientists. Previously, a processing algorithm for phytoplankton absorption spectra was reported that suggested detection of the red tide dinoflagellate *Gymnodinium breve* was possible. The algorithm evaluated the fourth derivative of the particulate absorption spectrum of an unknown sample and compared it to a standard fourth derivative spectrum for *G. breve* using a similarity index. We report here the first-time application of this technique to the detection of *G. breve* in natural, mixed phytoplankton communities. Pigment and spectral absorption data were collected from natural blooms of *G. breve* in the eastern Gulf of Mexico. This dinoflagellate is the only species of phytoplankton in the Eastern Gulf of Mexico observed to contain the pigment gyroxanthin-diester, and it appears in constant proportion to cellular chlorophyll *a* (Chl *a*) in *G. breve*. The in vitro absorption spectrum of gyroxanthin-diester is nearly identical to other xanthophylls (including diadinoxanthin, lutein, and 19'-hexanoyloxyfucoxanthin) and is not singularly responsible for imparting a unique absorption signature. Quantifying gyroxanthin-diester and Chl *a* allowed us to estimate the fraction of the biomass in mixed populations associated with *G. breve*. Subsequent regression of the *G. breve* similarity indexes to the *G. breve* biomass fractions yielded a significant linear correlation. Finally, the liquid waveguide capillary cell appears to be a promising technology for automating this technique.

Harmful algal blooms pose a threat that requires efforts to reduce or eliminate their negative impacts and consequences (Boesch et al. 1996). In the Gulf of Mexico, toxic blooms of the dinoflagellate *Gymnodinium breve* Davis regularly lead to untimely restrictions on commercial and recreational shellfish harvesting and deleterious effects on tourism and public health (Steidinger et al. 1973). Mitigation of

some harmful effects from these toxic blooms of *G. breve* can be achieved by early detection of this species in mixed phytoplankton communities. Currently, microscope examination of discrete water samples is the principal detection method for *G. breve*. Unfortunately, this method is slow, labor intensive, and intermittent. Significant benefits can be gained from an automated detection method, and optical methods hold promise for such applications (Cullen et al. 1997).

Over the past two decades, oceanographers have developed optical instrumentation that can collect data in a non-intrusive manner. Optical techniques are amenable to a variety of platforms (satellites, aircrafts, mooring, and profiling instrumentation), allowing researchers to design multiplatform sampling networks capable of collecting data over ecologically relevant scales (Smith et al. 1987; Dickey 1993). Many integrated observing systems currently are under development by the oceanographic community (Glenn et al. 1998). Although promising, optical approaches have been criticized because they provide only bulk composite signals for a given water mass, and the signatures for distinct phytoplankton species are difficult to discriminate (Garver et al. 1994).

Laboratory work suggests that partial discrimination of algal species from cellular absorption is possible. For example, Johnsen et al. (1994), using stepwise discriminant analyses to classify absorption spectra among 31 bloom-forming phytoplankton (representing the four main groups of phytoplankton with respect to accessory chlorophylls; i.e., chlorophyll *b*, chlorophyll *c*<sub>1</sub> and/or *c*<sub>2</sub>, chlorophyll *c*<sub>3</sub>, and no accessory chlorophyll), differentiated toxic chlorophyll *c*<sub>3</sub>-containing dinoflagellates and prymnesiophytes from taxa not having this pigment. However, problematic and toxic taxa could not be further separated from other chlorophyll

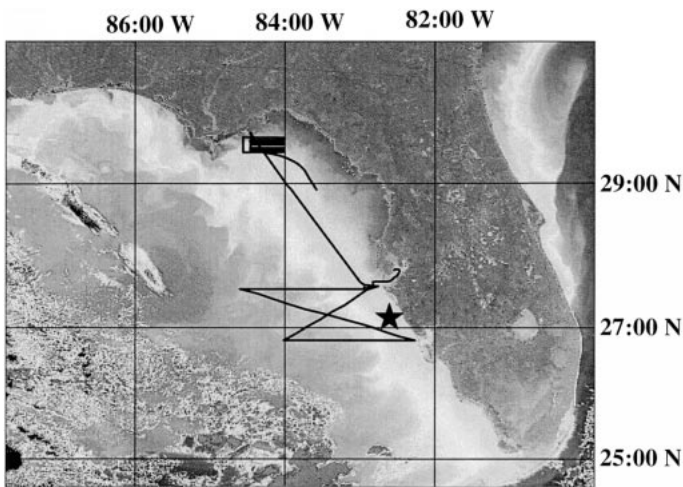


Fig. 1. Field data were collected aboard the OSV *Anderson* during a cruise conducted from 25 to 31 August 1997 in the Gulf of Mexico (bold line). The cruise consisted of a spatially extensive survey leg until a bloom of *G. breve* located off Apalachicola Florida, where a time series study was conducted. A second data set was collected off Sarasota Florida (denoted by a star).

$c_3$ -containing taxa because of the similarities among absorption spectra. Millie et al. (1997) also used stepwise discriminant analyses to differentiate mean-normalized absorption spectra for laboratory cultures of *G. breve* from absorption spectra of a diatom, a prasinophyte, and peridinin-containing dinoflagellates. Therefore, absorption sometimes may provide enough information to distinguish among absorption spectra between phylogenetic groups, and potentially taxa. However, wavelengths delineated by the stepwise techniques were wavelengths associated with the accessory carotenoids. This is problematic as the relative absorption in green, yellow, and orange wavelengths where the carotenoids absorb light is much less than the absorption by chlorophyll in the blue and red wavelengths of light. Furthermore the absorption attributable to unique accessory pigments is difficult to discern due to the dampening of the shoulders on absorption spectra from pigment packaging effects (cf. Morel and Bricaud 1986).

In order to maximize the minor inflections in spectral absorption, fourth derivative analysis (Butler and Hopkins 1970) has been used to resolve the positions of absorption maxima attributable to specific photosynthetic pigments (e.g. Bidigare et al. 1989; Smith and Alberte 1994; Millie et al. 1995). The application of derivative analysis and comparison to a standard derivative spectrum for *G. breve* proved successful at not only discriminating the presence of *G. breve*, but also at providing an estimate of the fraction of the chlorophyll biomass attributable to *G. breve* in laboratory cultures (Millie et al. 1997). This paper describes the application and utility of derivative analysis of absorption spectra in conjunction with spectral similarity analyses to discriminate the presence and dominance of *G. breve* in natural phytoplankton assemblages.

Whole water samples were collected from a variety of sampling stations along the west Florida coast and continental shelf between August 1995 and August 1997 (Fig. 1).

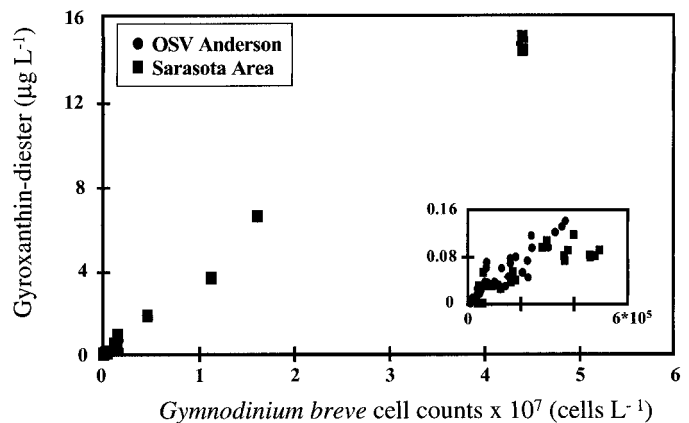


Fig. 2. Linear relationship between the concentration of gyroxanthin-diester and the number of *G. breve* cells in natural water samples. Inset shows a magnified scale where OSV *Anderson* cruise data falls and regression lines for both data sets. High linear correlation is evident for Sarasota coastal area ( $r^2 = 0.98$ ) and OSV *Anderson* cruise ( $r^2 = 0.94$ ). Slopes of regression lines differ by less than 1%. The equation of the best-fit line for the OSV *Anderson* data is Gyroxanthin-diester =  $0.008 + (3.34 \times 10^{-7} \times G. breve \text{ cell count})$  ( $r^2 = 0.87$ ,  $n = 45$ ,  $r$  significant at  $p < 0.01$ ). The equation of the line for the Sarasota data is Gyroxanthin-diester =  $0.003 + (3.37 \times 10^{-7} \times G. breve \text{ cell count})$  ( $r^2 = 0.99$ ,  $n = 36$ ,  $r$  significant at  $p < 0.01$ ).

Environments ranged from estuarine to offshore, oligotrophic waters. The samples were grouped into those collected in the Sarasota area over the entire 2-yr period and those collected during a cruise of the OSV *Anderson* covering shelf waters from Charlotte Harbor to Apalachicola in the last week of August 1997. Aliquots were filtered, under low vacuum ( $< 10 \text{ cm Hg}$ ), through GFF (Whatman) glass-fiber filters to concentrate the particles for pigment and absorption determinations. No special effort was made to acquire a predetermined amount of particulate matter. These filters were processed, as described below, either immediately or after storage in liquid nitrogen. In addition, unfiltered water samples were used for particulate absorption determinations in a liquid waveguide capillary cell (LWCC, World Precision Instruments) during the cruise of the OSV *Anderson*.

Photosynthetic and photoprotective pigment complements were determined using high-performance liquid chromatography (HPLC). Pigment analyses were conducted according to Wright et al. (1991) using a C-18 Hypersil reverse-phase column. Chromatographic peaks were detected by a photodiode array UV-VIS detector (SPD-M6A, Shimadzu) and identified by retention time and comparison of absorbance spectra with spectra of pigments from standard microalgae cultures.

Particulate absorption spectra were measured using the quantitative filter technique (QFT) (Kiefer and Soohoo 1982; Kishino et al. 1986; Mitchell and Kiefer 1988; Bricaud and Stramski 1990). Sample and reference filters were placed directly in front of the detector windows of a scanning dual-beam spectrophotometer (DMS80, Varian) to minimize scattering loss. Optical density spectra were acquired by a desktop computer interfaced to the analog output of the spectrophotometer. These spectra were corrected for path-

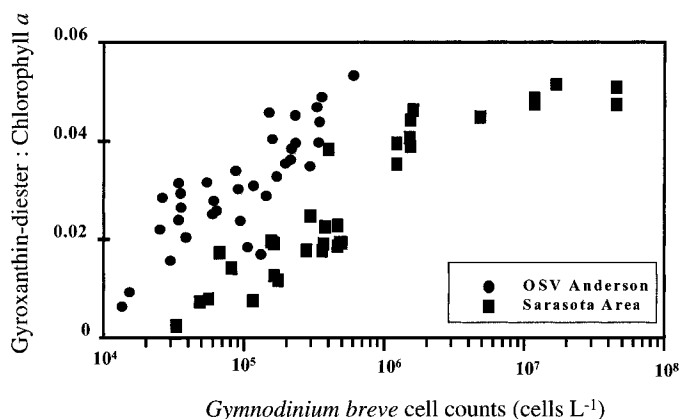


Fig. 3. Ratio of gyroxanthin-diester to chlorophyll *a* in whole water. At low *G. breve* cell counts other (non-gyroxanthin-diester) species contributed chlorophyll *a*, yielding lower ratios. However, at high *G. breve* cell counts the ratio represents the cellular ratio for near monospecific *G. breve* blooms. The asymptote of the fitted exponential curve matches the ratio observed in *G. breve* cultures and previous monospecific blooms.

length amplification (Mitchell 1990) and then normalized to the mean value between 400 and 700 nm (Roesler et al. 1989).

During August 1997, on the OSV *Anderson* cruise, absorption spectra also were measured using a 0.5-m path-length LWCC coupled to a fiberoptic spectrometer (SD2000, Ocean Optics) and a fiberoptic incandescent light source (F-O-Lite, World Precision Instruments). The spectrometer was interfaced to a notebook computer through a PCMCIA A/D converter (DAQCard-700, National Instruments) and controlled through software provided by the spectrometer manufacturer (OOIBase, Ocean Optics). The resulting absorption spectra were also normalized to the mean absorption between 400 and 700 nm.

Absorption spectra were compared using a similarity index (from Shimadzu Scientific Instruments) described previously by Millie et al. (1997). Briefly, fourth derivative spectra (Butler and Hopkins 1970) initially were computed for the normalized absorption spectra described above. This yielded spectra detailing the wavelength position and magnitude of curvature in the parent absorption spectra (Millie et al. 1995, 1997). The spectral similarity index was determined by computing the angle between the vectors comprising the fourth derivative spectra of a standard *G. breve* sample (culture or monospecific bloom sample) and the unknown natural, mixed-population sample as

$$SI = 1 - \left( \frac{2 \times \arccos \left[ \frac{A_{std} \cdot A_{unk}}{|A_{std}| \times |A_{unk}|} \right]}{\pi} \right) \quad (1)$$

where  $A_{std}$  and  $A_{unk}$  are the normalized particulate absorption spectra of the standard and unknown samples respectively. The arc-cosine transformation and division by  $\pi/2$  converts from a nonlinear result (cosine of the angle) to a linear result between zero and one. All empirical relationships presented are model II regressions (cf. Laws 1997).

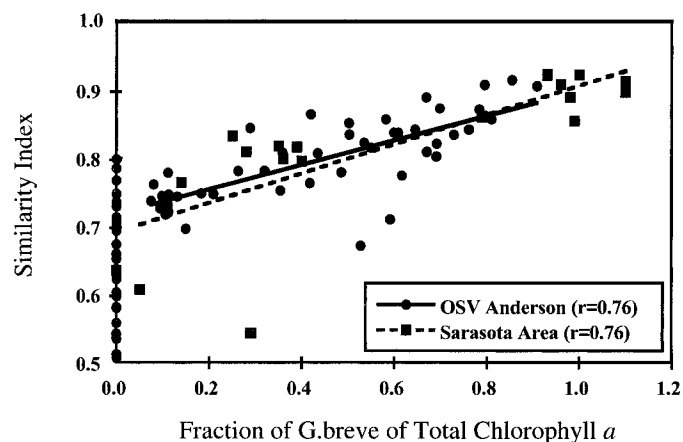


Fig. 4. Linear relationship between *G. breve* fraction of chlorophyll *a* biomass and the similarity index derived from particulate absorption spectra measured using the quantitative filter technique (QFT). Data were grouped and analyzed as offshore (OSV *Anderson*) and nearshore (Sarasota Area). The *G. breve* fraction of biomass exceeds 1.0 for several samples due to the error associated with HPLC quantification of pigments and the variability in the ratio of gyroxanthin-diester to chlorophyll *a*. Linear regressions include only samples with nonzero *G. breve* biomass fractions. The equation of the best-fit line is similarity index =  $0.72 + (0.18 \times \text{fraction of } G. \text{ breve})$  ( $r^2 = 0.57$ ,  $n = 46$ ,  $r$  significant at  $p < 0.01$ ). The equation of the line for the Sarasota data set is similarity index =  $0.69 + (0.21 \times \text{fraction of } G. \text{ breve})$  ( $r^2 = 0.58$ ,  $n = 18$ ,  $r$  significant at  $p < 0.01$ ).

A linear relationship existed ( $r^2 = 0.99$ ,  $n = 36$ ,  $r$  significant at  $p < 0.01$ ) between whole water concentrations of the carotenoid, gyroxanthin-diester and *G. breve* cell number (Fig. 2). A similar observation was made for cultures and natural assemblages by Millie et al. (1997). A constant cellular ratio has been previously noted between gyroxanthin-diester and chlorophyll *a* (Chl *a*) in both natural blooms (when the assemblage was nearly monospecific) and cultures of *G. breve* (Millie et al. 1997). During this study a positive linear relationship was observed for *G. breve* cell number and low values of the ratio gyroxanthin-diester to Chl *a* (Fig. 3). This largely reflected the variable amount of Chl *a* associated with *G. breve* and other algal species present within these mixed natural assemblages. At high gyroxanthin-diester to Chl *a* ratios, where *G. breve* was the dominant species present, the ratio showed little variability, consistent with laboratory studies. The high ratio values observed in the field populations (0.05) were very similar to laboratory cultures of *G. breve* (0.05–0.06, Millie et al. 1995). Given this the carotenoid pigment, gyroxanthin-diester appears to be a reliable indicator of the presence of the red tide organism *G. breve* in the eastern Gulf of Mexico. The constant ratio of gyroxanthin-diester to Chl *a* in *G. breve* also is a fortuitous feature that allows for the estimation of *G. breve* biomass in mixed populations. Estimates of the fraction of Chl *a* biomass contributed by *G. breve* in this study were based on this constant ratio between gyroxanthin-diester and Chl *a* applied to pigment complements from the natural phytoplankton communities. Although gyroxanthin-diester was a reliable indicator for *G. breve*, its analytical detection by



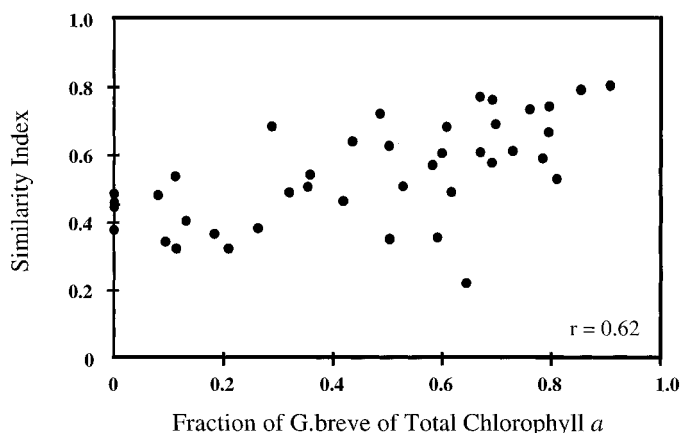


Fig. 5. Linear relationship between *G. breve* fraction of chlorophyll *a* biomass and the similarity index derived from particulate absorption spectra measured using the liquid waveguide capillary cell (LWCC). The best-fit line is similarity index =  $0.39 + (0.33 \times \text{fraction of } G. breve)$  ( $r^2 = 0.38$ ,  $n = 41$ ,  $r$  significant at  $p < 0.01$ ).

HPLC is not a viable approach for real-time, unattended detection of this harmful algae species.

A total of 99 samples were processed using the QFT over the period of this study. Samples were collected at a variety of locations. *G. breve* cell counts ranged from zero cells  $L^{-1}$  to  $4 \times 10^7$  cells  $L^{-1}$  and water-column Chl *a* concentrations ranged from  $0.08 \mu\text{g } L^{-1}$  to  $324 \mu\text{g } L^{-1}$ . A linear relationship existed ( $r^2 = 0.58$ ,  $n = 64$ ,  $r$  significant at  $p < 0.01$ ) between the similarity index and the fraction of Chl *a* biomass contributed by *G. breve* for the complete data set (Fig. 4). This suggests that the discrimination of *G. breve* in mixed populations of phytoplankton is feasible using the absorption-based analyses. The success of the technique results from the high sensitivity of derivative analysis to uncover subtle characteristics in the curvature of the absorption spectrum. This represents the first-time application of this technique on natural assemblages over a wide range of conditions spanning the majority of the marine environments of West Florida.

The similarity index provides a means to quantify the spectral variability in the absorption spectra and is independent of biomass as it is derived from mean-normalized absorption spectra. As such, the similarity index is related to the overall community structure reflecting the phytoplankton pigments present. This might suggest that similarity index and the fraction of biomass contributed by *G. breve* as derived from gyroxanthin-diester are not independent. However, it is unlikely that gyroxanthin-diester was responsible for producing the distinct absorption signatures for *G. breve* because (1) it has an absorption spectrum that is very similar to other common algal pigments (including diadinoxanthin, lutein, and 19'-hexanoyloxyfucoxanthin), (2) gyroxanthin-diester contributes only 6–8% of the total carotenoid complement in the *G. breve* (Millie et al. 1997), and (3) the similarity index quantifies the spectral variability in the entire absorption spectrum. The distinct absorption properties in *G. breve* therefore more likely reflect its ecological niche. *G. breve* is positively phototactic (Heil 1986; Steidinger

1975), which results in cells concentrating (as great as  $1 \times 10^8$  cells  $L^{-1}$ ) at the air-sea interface. Cells must therefore cope with high irradiances (both ultraviolet and visible) resulting in photoacclimation to minimize potential light-induced damage.

Absorption spectra were also measured using the LWCC at 50 locations during the August 1997 cruise in the Eastern Gulf of Mexico. Those locations had *G. breve* cell counts that ranged from zero cells  $L^{-1}$  to  $5 \times 10^5$  cells  $L^{-1}$  and Chl *a* concentrations that ranged from  $0.4 \mu\text{g } L^{-1}$  to  $4.4 \mu\text{g } L^{-1}$ . The fraction of the Chl *a* biomass that was attributable to *G. breve* ranged from zero to 0.91. The similarity index was linearly related to the fraction of Chl *a* biomass contributed by *G. breve* ( $r^2 = 0.38$ ,  $n = 41$ ,  $r$  significant at  $p < 0.01$ ) (Fig. 5).

The LWCC was successful in discriminating *G. breve* from other phytoplankton in natural assemblages. Discrimination results derived from the LWCC were less robust than those derived from the QFT, which was not unexpected since it was the first test of a prototype configuration. During post-processing it was discovered that the manufacturer's software was set to truncate absorbance values to just three decimal places. This approach substantially degraded the resolution of the absorbance data collected during the cruise, but notably did not nullify their application to discriminating *G. breve*. Additionally, the tungsten-halogen light source was deficient in violet and blue light, which resulted in loss of absorbance signal in a critical portion of the spectrum. Further effort on refining this instrument will yield a very useful field survey system capable of spectral absorption measurements even for oligotrophic waters. The great advantage of the LWCC is that the long pathlength of the system (0.25 m and up) results in sufficient sensitivity to measure absorption spectra even under ambient conditions in the oligotrophic waters of the Gulf of Mexico.

In conclusion, considering the wide range of bloom conditions sampled over 2 yr by a number of personnel using several different techniques, the significant correlation between the fraction of chlorophyll biomass contributed by *G. breve* and the absorption-based similarity index suggests this is a robust approach for discriminating this toxic red tide species. Also, the LWCC is a promising new technology that lends itself to conducting this species-discrimination method in an automated unattended mode that could be used to provide early warning to the presence of *G. breve*.

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