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# Photophysiological responses of the toxic red-tide dinoflagellate *Gymnodinium breve* (Dinophyceae) under natural sunlight

**TERENCE J. EVENS[1], GARY J. KIRKPATRICK[2], DAVID F. MILLIE[2,3], DAVID J. CHAPMAN[4] AND OSCAR M. E. SCHOFIELD[5]**

[1]US DEPARTMENT OF AGRICULTURE–AGRICULTURAL RESEARCH SERVICE, SOUTHERN REGIONAL RESEARCH CENTER, 1100 ROBERT E. LEE BLVD, NEW ORLEANS, LA 70124, [2]MOTE MARINE LABORATORY, 1600 KEN THOMPSON PARKWAY, SARASOTA, FL 34236, [3]SOUTHERN REGIONAL RESEARCH CENTER, AGRICULTURAL RESEARCH SERVICE, US DEPARTMENT OF AGRICULTURE, NEW ORLEANS, LA 70179, [4]DEPARTMENT OF ECOLOGY, EVOLUTION AND MARINE BIOLOGY, AND THE MARINE SCIENCE INSTITUTE, UNIVERSITY OF CALIFORNIA AT SANTA BARBARA, SANTA BARBARA, CA 93106 AND [5]INSTITUTE OF MARINE AND COASTAL SCIENCES, RUTGERS UNIVERSITY, NEW BRUNSWICK, NJ 08901-8521, USA

*Because Gymnodinium breve Davis exhibits positive phototaxis and often accumulates at or near the air–water interface, the diurnal, in situ physiological responses of this red-tide toxic dinoflagellate were investigated in both cultured and field populations. Cultures were incubated outdoors in temperature-controlled waterbaths, and allowed to acclimate to attenuated (i.e. without UV) natural irradiance. Five-liter aliquots of these photoacclimated cultures were placed in cubitainers fitted with either a quartz window or a quartz window covered with acetate [providing exposure to photosynthetically available radiation (PAR) + UV or PAR-only, respectively], incubated in Sarasota Bay, Florida (USA), and then assessed for diurnal variations in in vivo fluorescence and in vitro pigmentation, lipid, carbohydrate and protein contents over three sinusoidal photoperiods in weather varying from overcast to partly cloudy to extremely sunny. In addition, surface, field populations were assessed for diurnal variations in in vivo fluorescence and in vitro pigmentation over two sinusoidal photoperiods in extremely sunny weather. The maximum quantum yield for stable charge separation at photosystem II (measured by variable fluorescence/maximum fluorescence) exhibited depressions that were roughly symmetrical about solar noon on the overcast and partly cloudy days, but exhibited a pronounced hysteresis on the sunny days for both the cultured and field populations. Induction and relaxation of the xanthophyll cycle over the course of the photoperiod during the partly cloudy and sunny days resulted in stoichiometrically inverse cellular accumulation of the xanthophyll cycle pigments diadinoxanthin and diatoxanthin. Generally, only minor adjustments occurred in the cellular chlorophyll a and fucoxanthin contents. No differences occurred between cultures exposed to PAR-only or PAR + UV treatments in the epoxidation state of the xanthophyll cycle pigments or in the maximum quantum yield for stable charge separation at photosystem II. Differences in oxygen production rates and other biochemical parameters between cultures exposed to PAR-only or PAR + UV treatments were not directly attributable to UV, indicating that G. breve possesses an inherent UV resistance and a robust photosynthetic capability.*

## INTRODUCTION

The Florida red-tide toxic dinoflagellate *Gymnodinium breve* Davis [recently renamed *Karenia brevis* Davis, but referred to here as *G. breve* for clarity; (Daugbjerg *et al.*, 2000)] forms large blooms in the coastal waters of the Southeastern USA and throughout the Gulf of Mexico (Holligan, 1985; Vargo *et al.*, 1987). The economic and public health impacts due to the release of brevetoxins during blooms that may persist for weeks to months can be quite significant (Steidinger *et al.*, 1973; Riley *et al.*, 1989; Shumway *et al.*, 1990; Hallegraff, 1993; Tester and Fowler, 1993; Anderson *et al.*, 2000). Consequently, the biogeography of *G. breve* has been the focus of numerous studies (Marshall, 1982; Fraga and Sanchez, 1985; Churchill and Cornillon, 1991; Tester and Steidinger, 1997). However, it is only recently that the ecology and physiology of these dinoflagellates have begun to be examined in any detail [cf. (Paerl, 1988; Smayda, 1997)].

*Gymnodinium breve* is flagellated, negatively geotactic and positively phototactic (Kamykowski *et al.*, 1998). As such, blooms of this organism tend to concentrate at or

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near the air–water interface during prolonged periods of weak mixing, and are exposed to strong and highly variable amounts of photosynthetically available radiation (PAR; 400–700 nm) and ultraviolet radiation (UVR; 295–400 nm). Seemingly, this taxon has evolved a vigorous photoprotective response to PAR and UVR, and/or a robust repair capability. However, very little is known concerning the autecology of *G. breve* [cf. (Paerl, 1988; Smayda, 1997)].

Generally, chronic photoinhibition [*sensu* (Osmond, 1994)] of photosynthesis results from excessive excitation of the photosynthetic apparatus by PAR and/or through direct damage by UVR (Lorenzen, 1979; Smith *et al.*, 1980; Osmond, 1981). The primary manifestation of chronic photoinhibition involves photo-induced damage to some part of photosystem I (PSI) and/or PSII; it often results in a decrease in the efficiency of both oxygen evolution and carbon fixation, and requires *de novo* protein/pigment/enzyme synthesis to reverse the effects (Prasil *et al.*, 1992).

Dynamic photoinhibition, referred to hereafter as 'photoprotection', may be the result of rapidly reversible defenses (xanthophyll cycling, state transitions, etc.), which serve to 'down-regulate' the photosynthetic process to avoid the build-up of potentially deleterious, oxidative radicals resulting from excessive photon fluxes. The xanthophyll cycle, which is essential to the photoprotective capacity of *G. breve* and many other algae, is induced under saturating irradiance when the pH of the thylakoid membrane lumen decreases. In diatoms and dinoflagellates, it involves the reversible de-epoxidation of the monoepoxide diadinoxanthin (DD) to diatoxanthin (DT). When irradiance becomes subsaturating, an epoxidase enzyme converts DT back to DD, thereby completing the cycle. The exact mechanism(s) by which the xanthophyll cycle mediates energy dissipation has been the subject of some debate. Some researchers have suggested that the de-epoxidized pigments (DT in the case of *G. breve*, antheraxanthin and zeaxanthin in the case of terrestrial plants and chlorophytes) have extended $\pi$ conjugations that lower the energy of the lowest excited singlet state, which directly quenches chlorophyll singlet and/or triplet states [cf. (Demmig-Adams, 1990; Owens *et al.*, 1993; Foyer and Mullineaux, 1994; Frank *et al.*, 1994; Gilmore *et al.*, 1996a,b)]. However, in the absence of zeaxanthin, quenching may also be activated by lutein (in those phototrophs that possess it), albeit more weakly (Gilmore and Yamamoto, 1991). Thus, others [cf. (Horton *et al.*, 1991; Horton and Ruban, 1994; Ruban *et al.*, 1997; Ruban and Horton, 1999)] have postulated that the xanthophyll cycle pigments are responsible for allosteric regulation of the aggregation states of the major light-harvesting complexes in PSII, rather than acting as direct quenchers.

Whatever the actual mechanism of protection offered by the xanthophyll cycle, it is extremely important for regulation of the photoprotective capabilities of those algae that possess it. Given that *G. breve* often blooms at the water's surface, accurate characterization of this alga's photoprotective mechanisms is central to our understanding of the physiological/biochemical strategies that underlie the growth and bloom dynamics of this organism. It is equally important to examine the inherent photoinhibitory responses that may have synergistic effects with the photoprotective responses upon the photophysiology of *G. breve*. Our study characterizes some of the physiological responses of both cultured and natural *G. breve* populations to variable, 'natural' surface irradiance.

## METHOD

### Culture conditions (Mote Marine Laboratory)

Semi-continuous batch cultures of *G. breve* were grown in 20 l polycarbonate carboys in enriched seawater growth medium with 4 mM Tris buffer [f/2+t; (McLachlan, 1973)]. Fluorescent lamps provided the original growth irradiance at ~50 μmol quanta $m^{-2}$ $s^{-1}$. After the cultures had attained sufficient biomass (~$1 \times 10^7$ cells $l^{-1}$), the carboys were relocated outdoors in a temperature-controlled water bath and allowed to acclimate to visible sunlight (without UV) for 15 days. Initially, four layers of neutral density screening were placed over the carboys, which reduced the PAR irradiance by ~90%. One layer of screening was removed every 5 days until only two layers remained. The cultures were then relocated to the study site in Sarasota Bay, Florida, and allowed to further acclimate for another 5 days. Every fifth day of the acclimation period, ~3 l of each culture were removed and replaced with an equal volume of f/2+t medium.

UVR has been shown to have significant photoinhibitory effects on photosynthesis [e.g. (Cullen *et al.*, 1992; Smith *et al.*, 1992; Cullen and Neale, 1993; Schofield *et al.*, 1995)], and is a major concern for surface-blooming algae such as *G. breve*. However, it can be difficult to distinguish photoinhibitory responses due to UV-induced damage from photoinhibitory/photoprotective responses due to (super)-saturating irradiance. Therefore, in order to distinguish between these two conflating conditions, we designed the culture-based experiments to expose aliquots of the same cultures to natural sunlight with and without UVR. Thus, for each of the three experiments conducted from 3 to 6 November 1998, 10 l of culture were removed from one of the carboys and split between two 5-l cubitainers fitted with either a quartz window (50% transmittance cut-off at 220 nm; PAR + UV) or a quartz

window covered with a layer of acetate plastic (50% transmittance cut-off at 411 nm; PAR-only; Figure 1). The cubitainers were suspended just below the water's surface in Sarasota Bay 8–9 h before dawn on each day of sampling. Every 3 h from just before sunrise to well after sunset, aliquots were removed from the cubitainers and analyzed for maximum quantum yield of fluorescence ($\phi_{IIe}°$), photopigments, lipids, carbohydrates, proteins, pH, dissolved $O_2$ (DO), cell counts and supplemental dithiothreitol (DTT) incubations (see below).

Cell enumeration was performed in pseudo-triplicate with a Coulter Multisizer IIe utilizing AccuComp software (Version 1.19). DO and hydrogen ion concentrations were determined on an Orion 525A pH meter fitted with either an Orion 81-72 ROSS pH probe or an Orion Research Model 97-08 $O_2$ electrode. For each of the fluorescence, pigment, lipid, carbohydrate and protein analyses, two 15-ml volumes from each treatment were filtered under low vacuum (<75 mm Hg) onto GF/C glass fiber filters. All but the fluorescence samples were frozen in liquid $N_2$, and stored in darkness at –70°C until analysis.

PAR data were obtained with an LI190A 2π, cosine-corrected sensor and an LI-1000 recording light meter (LiCor, Inc., Lincoln, NE) mounted 2 m above the surface near the cubitainers. Pyranometer data were obtained from the Mote Marine Laboratory weather station, which incorporates an LI192 sensor. UVR values were obtained from the USDA UV-B Monitoring Program's Everglades National Park site (~110 miles southeast of Sarasota).

## Fluorescence measurements

Fluorescence measurements were performed with a Pulse-Amplitude-Modulated (PAM) fluorometer (Heinz-Walz, Effeltrich, Germany). The maximum quantum yield for stable charge separation at PSII was measured via the saturation pulse method (Schreiber *et al.*, 1986; Schreiber and Bilger, 1993). Maximum quantum yields were calculated as variable fluorescence over maximum fluorescence [$F_V/F_M$; (Genty *et al.*, 1989; Haveaux *et al.*, 1991)], and were achieved using an 800 ms flash from a Schott lamp (Schott Inc., Germany). Values were calculated according to the following equation:

$$F_V/F_M = (F_M - F_0)/F_M \quad (1)$$

where $F_M$ and $F_0$ are the maximum and minimum fluorescence, respectively (Butler, 1978). All samples were dark adapted for 20 min prior to analysis, and then filtered onto GF/C filters. Adjusting the flash intensity and/or flash duration did not increase the maximum fluorescence level.

## Dithiothreitol experiments

A series of supplemental incubations with and without DTT was conducted to examine the interplay between xanthophyll cycle-mediated photoprotection and chronic photoinhibition due to UVR and/or excessive PAR. DTT is an inhibitor of the de-epoxidation reaction of the xanthophyll cycle, but does not interfere with either the epoxidation reaction or the formation/maintenance of the photosynthesis-dependent pH gradient across the thylakoid membrane (Yamamoto and Kamite, 1972; Sokolave and Marscho, 1976; Olaizola *et al.*, 1994).

A single aliquot from each treatment (PAR + UV and PAR-only) was split into two equal volumes and placed into four 75-ml plastic culture vessels (97% maximum transmittance, 50% transmittance cut-off at 304 nm; Figure 1). Two culture vessels (i.e. PAR-only treatments) were wrapped with the same acetate plastic used for the PAR-only cubitainer and two vessels (i.e. PAR + UV) were left unwrapped. DTT (100 μM final concentration) was added to one of each of the two vessels in each group to provide four irradiance–DTT combinations: (1) PAR + UV + DTT; (2) PAR + UV – DTT; (3) PAR-only + DTT; and (4) PAR-only – DTT. After the addition of DTT, these four culture vessels were immediately placed in the dark for ~30 min to allow the xanthophyll cycle to relax. At the end of the 30 min period, the vessels were removed from the dark, and aliquots for pigment analysis (see below) were removed; immediately after removing the aliquots, all vessels were placed into the water next to the cubitainers. At the end of a further 30 min irradiance exposure,

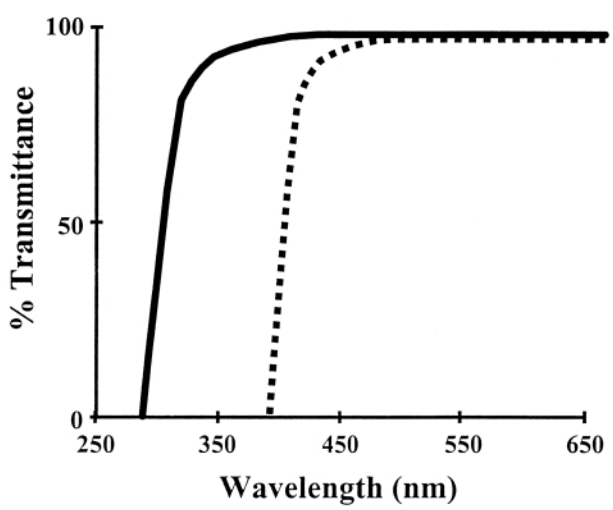


**Fig. 1.** Transmittance of the plastics used in this study. The acetate (dotted line) had a 50% transmittance cut-off at 411 nm and the culture flask used in the supplemental DTT incubations (solid line) had a 50% transmittance cut-off at 304 nm. The maximum transmittance values were 95 and 97%, respectively.

aliquots were obtained for photopigment analysis and quantum yields (designated $F_V/F_{M\text{-}D}$ in order to distinguish measurements performed on the supplemental DTT incubations from those performed on samples removed directly from the cubitainers).

For each light treatment, the $F_V/F_{M\text{-}D}$ value in the PAR-only – DTT sample (i.e. no UV present and the xanthophyll cycle active) was used as a reference to calculate the fractional inhibition ($FI_D$) in the other treatments as:

$$FI_D = 1 - (F_V/F_{M\text{-}DX})/(F_V/F_{M\text{-}DPAR})$$

where $F_V/F_{M\text{-}DX}$ is the $F_V/F_{M\text{-}D}$ measured in one of the other three treatments. Thus, we were able to estimate $FI_D$ circumvented by the xanthophyll cycle, the $FI_D$ due to UV, and the combined effects of both of these at each time point.

## Photopigment analysis and identification

Photopigment analysis and identification were performed following procedures outlined in Wright *et al.* (Wright *et al.*, 1991). At the time of analysis, filters were macerated in cold (4°C) 100% HPLC-grade MeOH and allowed to extract in the dark at 2°C for 2 h. After this initial extraction, enough cold 0.25 M ammonium acetate solution was added to achieve a MeOH:ammonium acetate ratio of 90:10; the samples were then allowed to extract in the dark at 2°C for a further 22 h. After extraction, the samples were centrifuged at ~2000 *g* for 5 min in a benchtop centrifuge, and enough 0.25 M ammonium acetate solution was again added to achieve a final MeOH:ammonium acetate ratio of 70:30. Pigment extracts were injected directly into a Waters HPLC (996 photodiode array; two 510 pumps; one 515 pump; 712 WISP auto-injector) equipped with a Waters Spherisorb™ ODS2 (4.6 × 250 mm, 5 μm sphere size), which was preceded in-line by a 25 mm guard column of the same specifications.

## Total lipid analysis

Total lipid analysis was performed according to the procedure outlined in Kochert (Kochert, 1978). At the time of analysis, filters were washed twice with cold, dilute perchloric acid. This was followed by duplicate chloroform/methanol extractions, which were combined after centrifugation at ~2000 *g*. After a cold water wash of the combined organic solvent extracts, the aqueous layer was removed, and the organic layer was reduced to dryness by vacuum evaporation. After exposure to dichromate at 100°C for 45 min, the sample was cooled, and the absorbance at 350 nm was read against a palmitic acid standard, using 1 cm semi-microcuvettes on a Beckman DU650 spectrophotometer.

## Carbohydrate analysis

Carbohydrate analysis was adapted from Dubois *et al.* (Dubois *et al.*, 1956). At the time of analysis, the filters were lyophilized at –40°C for ~24 h, after which the dried filters were then resuspended in 2 ml of distilled water. One milliliter of 5% phenol solution was added, followed by the slow addition of 5 ml of concentrated sulfuric acid inside a fume hood. The samples were vortexed and allowed to remain at room temperature for 45 min. After centrifugation at 2000 *g* for 10 min, the supernatant was removed to a semi-microcuvette, and the absorbance was read at 485 nm on a Beckman DU650 spectrophotometer against a distilled water blank. Standards were prepared from a dilution series of dextrose solution (starting point at 200 mg dextrose $ml^{-1}$ distilled $H_2O$).

## Protein analysis

Protein analysis utilized the Sigma Diagnostics protein assay kit (Procedure No. P-5656) based on Lowry *et al.* (Lowry *et al.*, 1951) and modified by Peterson (Peterson, 1977). At the time of analysis, the filters were lyophilized at –40°C for ~24 h, after which the dried filters were resuspended in 1 ml of distilled water. One-tenth of a milliliter of 1 M sodium deoxycholate solution and 0.1 ml of 1 M trichloroacetic acid were added to induce precipitation and isolate the proteins from any contaminants. After centrifugation at 2000 *g* for 10 min, the supernatant was removed, the pellet was dissolved in 1 ml of modified Lowry reagent (alkaline cupric tartrate:distilled $H_2O$) and resuspended in 1 ml of distilled $H_2O$. After a 20 min incubation, 1 ml of 5% phenol was added, and the samples were placed in darkness for 30 min at room temperature. The sample absorbance was read at 750 nm on a Beckman DU650 spectrophotometer against a distilled $H_2O$ blank. Standards were prepared from a dilution series of bovine serum albumin.

## Experimental conditions for field-based experiments (aboard R/V 'Pelican')

In addition to the culture-based experiments described above, two consecutive diel studies were performed in the Gulf of Mexico aboard the R/V 'Pelican' during 2 and 3 October 2000. These experiments were carried out on natural, surface populations that were heavily dominated by a *G. breve* bloom. Near-surface, Davis/CODE-type drifters (Brightwaters Instrument Corp., Model V104) were used to track the movements of the bloom (Davis, 1985). These Lagrangian drifters were drogued to track the bloom population entrained in the upper meter of the water column. They broadcast their position (GPS derived) by VHF radio every 15 min. This made it

possible to locate them and the bloom patch when it was time to sample the water column without having to remain in continuous visual contact. The position broadcasts were logged internally and captured by computer on the ship. With this approach, it was possible to track and sample the bloom patch for 89 h as it was advected by surface currents.

Over the course of 2 days, at 1.5 h intervals during the daylight hours (3 h intervals after sunset), water samples were taken within 0.5–1.5 m of the surface in 5 l Go-Flo™ bottles that were attached to a Sea-Bird Electronics, Inc. Model SBE 911 Plus CTD, and analyzed for pigmentation, cell counts and $F_V/F_M$. Total cell enumeration of preserved (Utermöhl's), whole-water samples was performed in pseudo-triplicate with a Coulter Multisizer IIe utilizing AccuComp software (Version 1.19). Additional cell counts for establishing *G. breve* numbers were performed immediately after water collection. One milliliter of water was dispensed into a counting well that contained 1 drop of Utermöhl's solution in a 24 well plate. Cells were allowed to settle for at least 10 min and then counted using an Olympus CK-40 inverted-stage compound microscope. The sample was diluted by a factor of 10 using filtered sample water when concentrations of cells were too high to count directly (>1000 cells $ml^{-1}$). PAR data were obtained as described above for the culture-based experiments.

## RESULTS

### Irradiance during culture-based experiments

Pyranometer data indicate that the weather on the days immediately preceding and during the three culture-based experiments varied from extremely cloudy to very sunny (Figure 2A). The impact of Hurricane Mitch on the Sarasota area on 3 and 4 November created two consecutive days of lower than normal irradiance levels. Although the UVR levels were obtained from the USDA UV-B Monitoring Program's Everglades National Park site (~120 miles from Sarasota Bay), the pyranometer data between the two sites are directly comparable, and give an estimate of the comparative magnitudes of UVR that the *G. breve* cultures were most likely experiencing during the experiments (Figure 2B and C).

On 3 and 6 November, the PAR + UV treatment lipid concentrations (Figure 3) were significantly lower than the PAR-only values (*t*-test, $p < 0.005$) from the midday samples to the end of the photoperiod. Also, during the relatively high irradiance of 6 November, the PAR + UV carbohydrate and protein values were significantly lower than the PAR-only concentrations at midday (*t*-test, $p < 0.001$). At all other times, there was no discernible difference between the PAR-only and PAR + UV treatments.

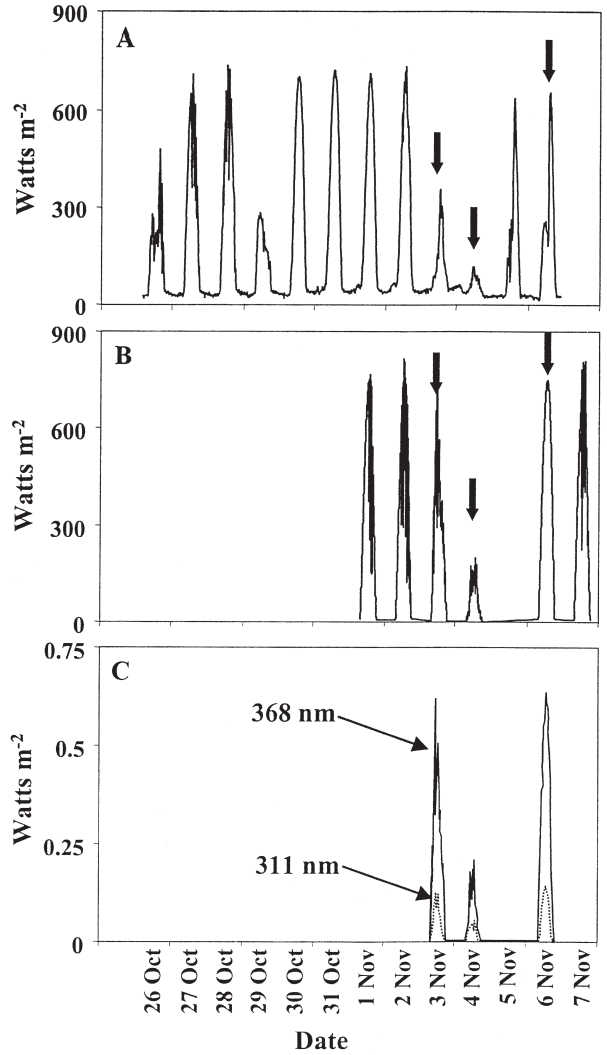


**Fig. 2.** Pyranometer data (380–1100 nm) from Mote Marine Laboratory (**A**) and pyranometer data (380–1100 nm) and UVR (311 and 368 nm) from the USDA UV-B monitoring site in Everglades National Park (**B** and **C**, respectively) for the days immediately preceding and during the culture-based experiments. The experimental photoperiods are indicated by arrows. Note that there are no data for 5 November from the USDA site.

DO levels were lower in the PAR + UV treatments over the course of the photoperiod for all three culture-based experiments. Data normalized to the pre-dawn $O_2$ concentrations (Figure 4A) indicate rapid increases in DO from 09:00 to 12:00 h, with moderate increases afterwards. The smallest increases in DO occurred in the 6 November samples. The addition of Tris to the media helped stabilize the pH, provide a more constant concentration of $CO_2$ and mitigate carbon limitation effects.

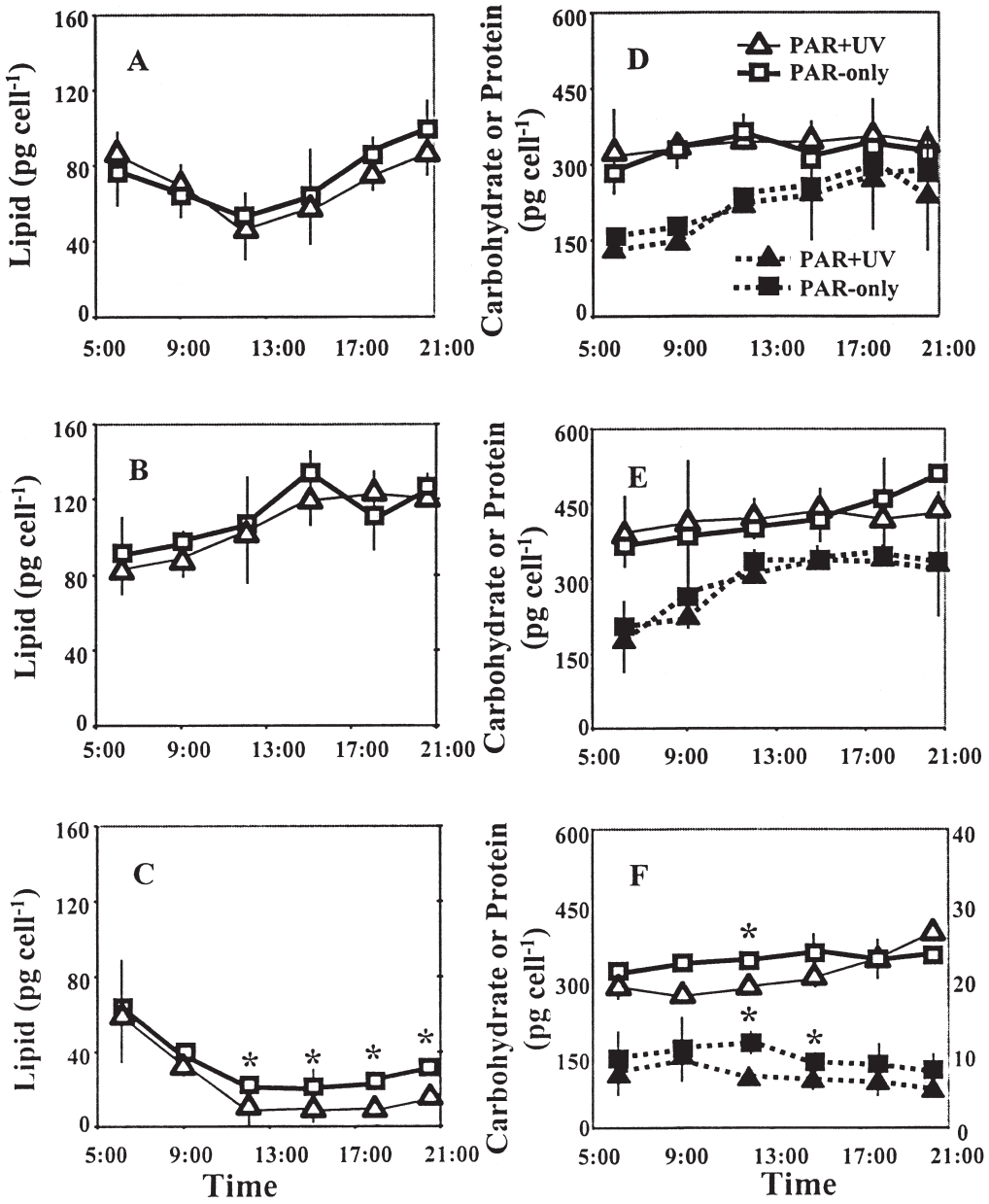


**Fig. 3.** Lipids, carbohydrates and proteins for the PAR-only and PAR + UV treatments. (**A**–**C**) Lipid concentrations for PAR-only (squares) and PAR + UV (triangles) for 3, 4 and 6 November (**A**, **B** and **C**, respectively). (**D**–**F**) Carbohydrate (open symbols) and protein concentrations (filled symbols) for PAR-only (squares) and PAR + UV (triangles) on 3, 4 and 6 November 1998 (**D**, **E** and **F**, respectively).

Thus, the pH stayed constant over the course of the photoperiod (Figure 4B) during all three experiments.

## PSII quantum yields of cultures

Pre-dawn samples yielded $F_V/F_M$ values from 0.5 (3 November) to 0.6 (6 November). At times of maximum irradiance for the day, values decreased to ~75% of the maximum pre-dawn values on 4 November, to ~50% on 3 November, and to ~15% of the maximum values for $F_V/F_M$ on 6 November (Figure 5A–C). By 20:30 h on 3 and 4 November, $F_V/F_M$ had recovered to nearly 90% of the pre-dawn values. However, on 6 November, $F_V/F_M$ at 20:30 h had recovered to only ~44% of the pre-dawn values. $F_V/F_M$ varied inversely with irradiance for both treatments on both 3 and 4 November ($r^2 = 0.86$, $p \leq 0.001$), but did not exhibit the same inverse relationship on 6 November ($r^2 = 0.36$, $p \geq 0.1$), perhaps because $F_V/F_M$ continued to decrease after the 12:00 h samples.

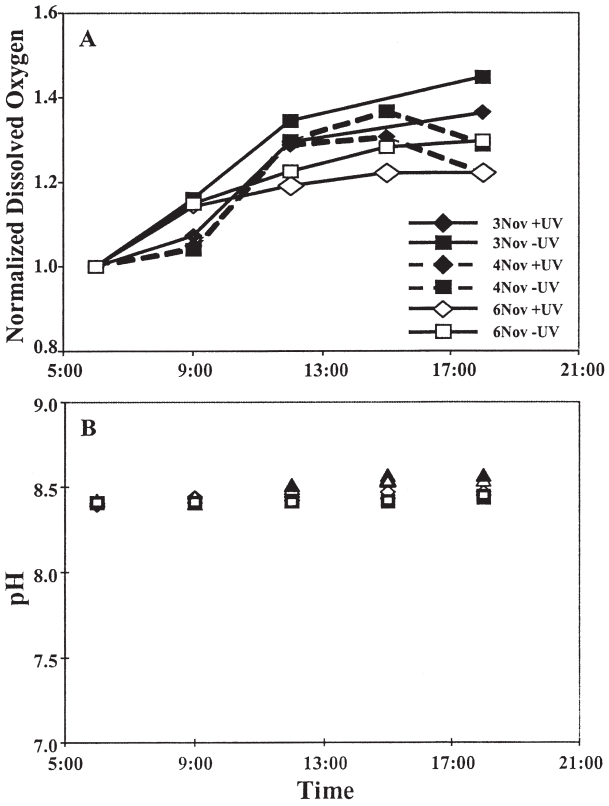


**Fig. 4.** The pattern of dissolved oxygen (**A**) and pH (**B**) over the course of the photoperiod for the PAR-only (denoted as '–UV') and PAR + UV (denoted as '+UV') treatments on 3, 4 and 6 November 1998. Note that there are no DO data at the 15:00 h sampling time for either treatment on 3 November.

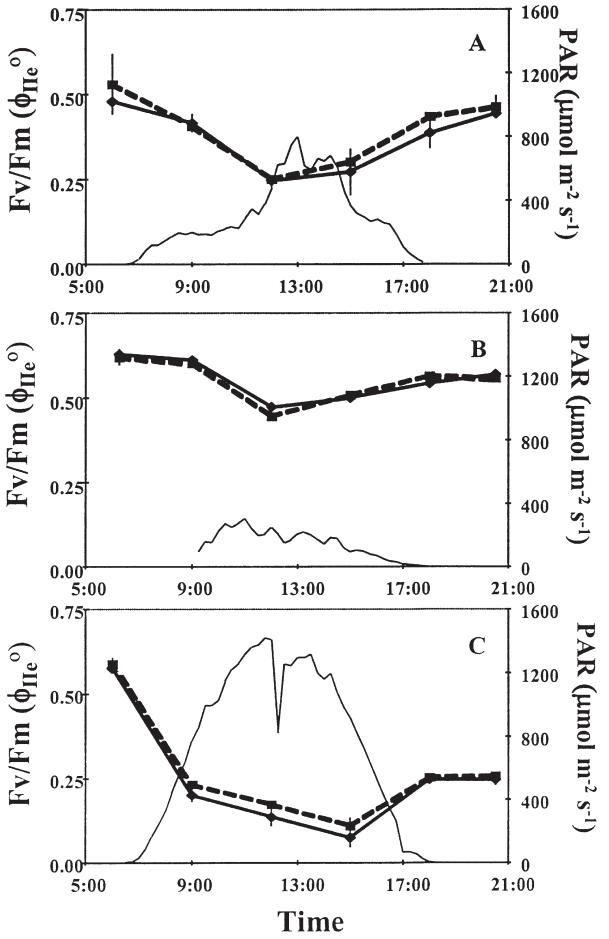


**Fig. 5.** The diurnal pattern of $F_V/F_M$ ($\phi_{IIe}$°) for the PAR-only (squares + dashed line) and PAR + UV (diamonds + solid line) *G. breve* cultures on 3, 4 and 6 November 1998 (**A**, **B** and **C**, respectively). The incident PAR irradiance (µmol quanta m$^{-2}$ s$^{-1}$) is also shown.

There was no significant difference in $F_V/F_M$ at any time between the two treatments (*t*-test, $p \geq 0.05$) for any of the three culture-based experiments.

## Culture photopigments

Chlorophyll *a* (Chl *a*) and fucoxanthin were the major pigments present in cultured *G. breve* at an average of 43.8% (± 3.1%) and 25.0% (± 5.6%), respectively, of the total pigment content for all three experiments combined. Total Chl (*a* and $c_1/c_2$) and carotenoid concentrations on 4 November [90.8 (± 16.3) pg pigment/cell] differed markedly from the concentrations on 3 and 6 November [69.2 (± 12.0) and 65.6 (± 11.7) pg pigment/cell, respectively]. Total pigments on 4 November showed a general upward trend in pigment/cell values, while 3 and 6 November remained relatively steady over the course of the majority of the photoperiod. Most of the variation in total pigment concentrations came from the increases in pigmentation at the end of the photoperiod on all three days.

Chlorophyll *a* values for the three culture-based experiments averaged 25.8 (± 4.6), 34.3 (± 5.3) and 24.3 (± 4.1) pg Chl *a* cell$^{-1}$ on 3, 4 and 6 November, respectively (Figure 6). The concentrations were relatively stable over the course of the photoperiod, with only a slight decrease discernible on 3 and 6 November However, regression slopes of Chl *a* concentrations against time were not significantly different from zero (Student's *t*-test, $p > 0.05$). Both treatments (PAR + UV and PAR-only), on all 3 days, exhibited an increase in Chl *a* concentrations at the final sampling period, which is contrary to the diel pigmentation shifts found in several other algae (Kana and Gilbert, 1987; Henley and Ramus, 1989). Daily averaged fucoxanthin values ranged from ~13–15 pg/cell on 3 and 6 November to >22 pg/cell on 4 November. On 4 November, both treatments displayed an increase in fucoxanthin concentrations at the noon sampling, with a

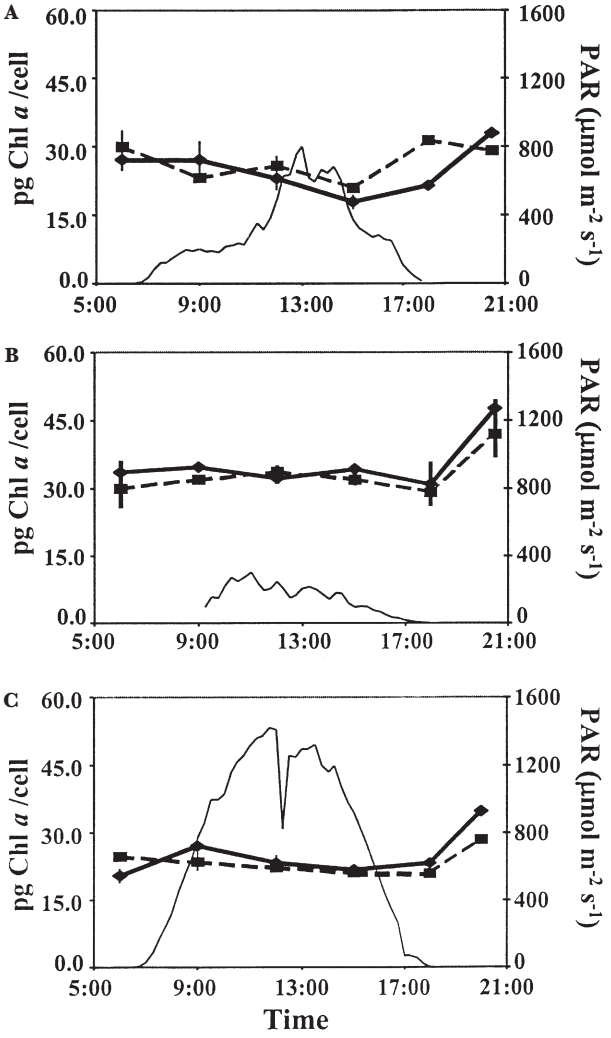


**Fig. 6.** The diurnal pattern of Chl *a* concentrations (pg Chl *a* cell$^{-1}$) for the PAR-only (squares + dashed line) and PAR + UV (diamonds + solid line) *G. breve* cultures on 3, 4 and 6 November 1998 (**A**, **B** and **C**, respectively). The incident PAR irradiance (µmol m$^{-2}$ s$^{-1}$) is also shown.

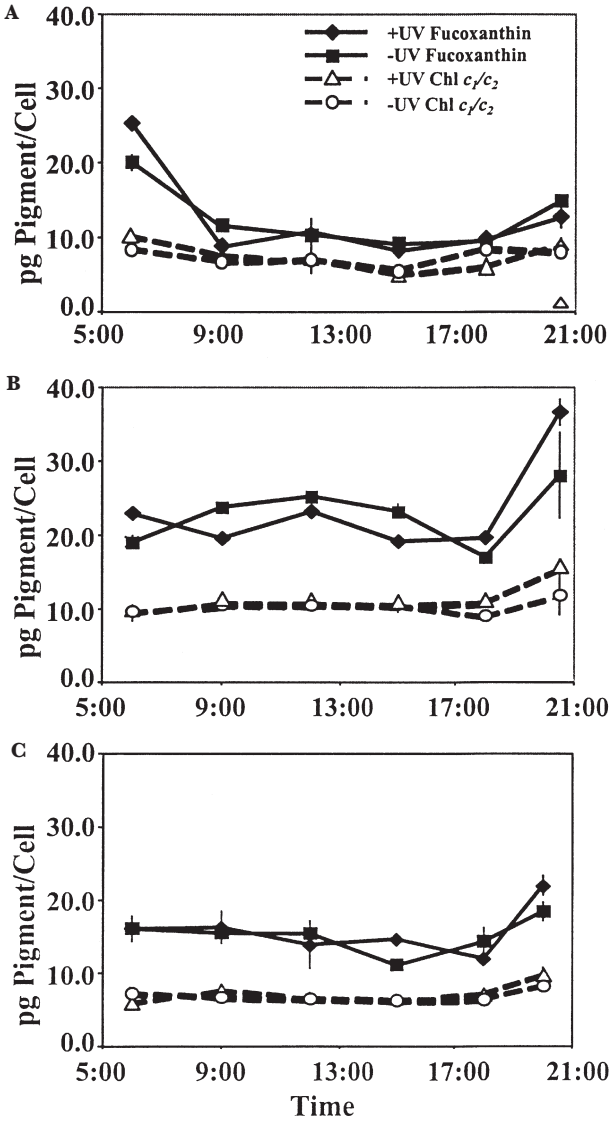


**Fig. 7.** The diurnal pattern of fucoxanthin and chlorophyll $c_1/c_2$ concentrations (pg pigment cell$^{-1}$) for the PAR-only (denoted as '–UV' in the legend) and PAR + UV (denoted as '+UV' in the key) *G. breve* cultures on 3, 4 and 6 November 1998 (**A**, **B** and **C**, respectively).

concurrent decrease to near pre-dawn values at the 18:00 h sampling. Additionally, both treatments displayed a marked increase in pigment concentrations by 20:30 h. On 3 November, fucoxanthin concentrations exhibited a marked fall after the pre-dawn sample, followed by a stable interval over the rest of the photoperiod. The concentrations of Chl $c_1/c_2$ were relatively constant over the course of the photoperiod on all 3 days, with the same pattern of increases in pigment concentrations at the end of the photoperiod (Figure 7).

DD and DT showed marked variability over the course of the photoperiod on 3 and 6 November (Figure 8D–F) due to the induction and relaxation of the xanthophyll cycle. The increase in DT was stoichiometrically inverse to the loss of DD. The epoxidation state [EPS; (Demmig *et al.*, 1987)], defined as the ratio [DD]:[DD + DT], varied inversely with the irradiance on 3 and 4 November (Figure 8A–C), and reached a maximum of ~0.72 on 3 November and ~0.07 on 4 November. EPS did not vary inversely with irradiance on 6 November, reaching a maximum of ~0.68, and exhibiting a response similar to the $F_V/F_M$ irradiance dynamics for this day. Total concentrations of DD + DT were constant over the course of the photoperiod on 3 and 6 November, indicating no net *de novo* synthesis of these pigments. In contrast, total

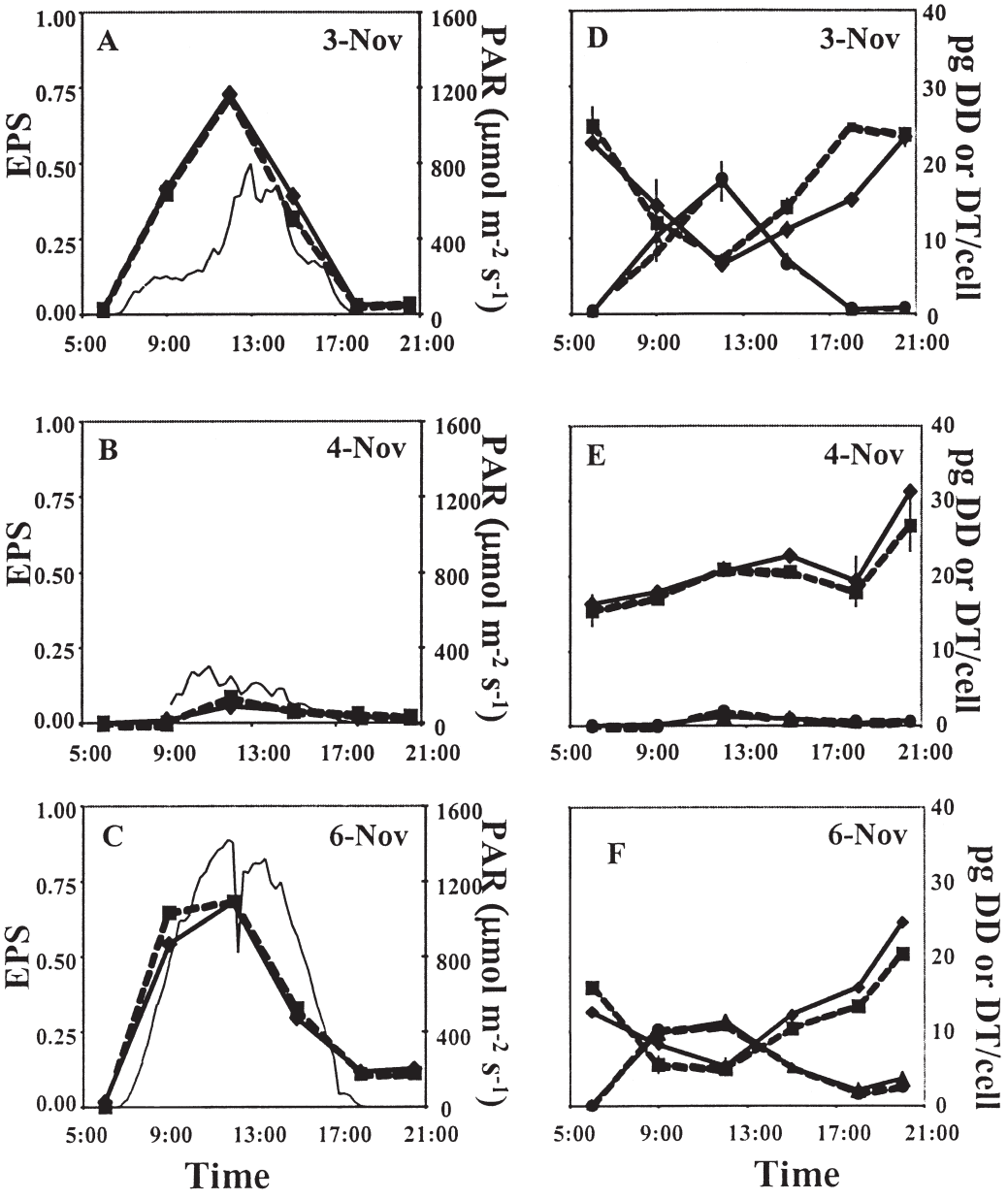


**Fig. 8.** The epoxidation state (EPS = [DT]/[DT + DD]) (**A–C**) of the xanthophyll cycle and the DD and DT concentrations (**D–F**; pg pigment cell$^{-1}$) for the PAR-only (squares + dashed line) and PAR + UV (diamonds + solid line) *G. breve* cultures on 3, 4 and 6 November 1998. The incident PAR irradiance (µmol m$^{-2}$ s$^{-1}$) is also shown in (A–C).

DD + DT concentrations (driven primarily by DD) exhibited a general upward trend on 4 November.

## Dithiothreitol incubations

The total amount of DD and DT did not change by more than a few percent (the error level of the HPLC technique) between the dark-adapted and irradiated samples, thus indicating that a significant amount of net DT synthesis did not occur during these supplementary incubations. The subsamples incubated without DTT had a greater EPS in all cases (Figure 9). During times of high irradiance (e.g. at 12:00 h on 3 November, and 09:00, 12:00 and 15:00 h on 6 November), the presence of DTT did not prevent some conversion of DD to DT, suggesting that there is possibly a second, DTT-insensitive pool of DD that is de-epoxidized at high irradiances [see (Olaizola *et al.*, 1994)].

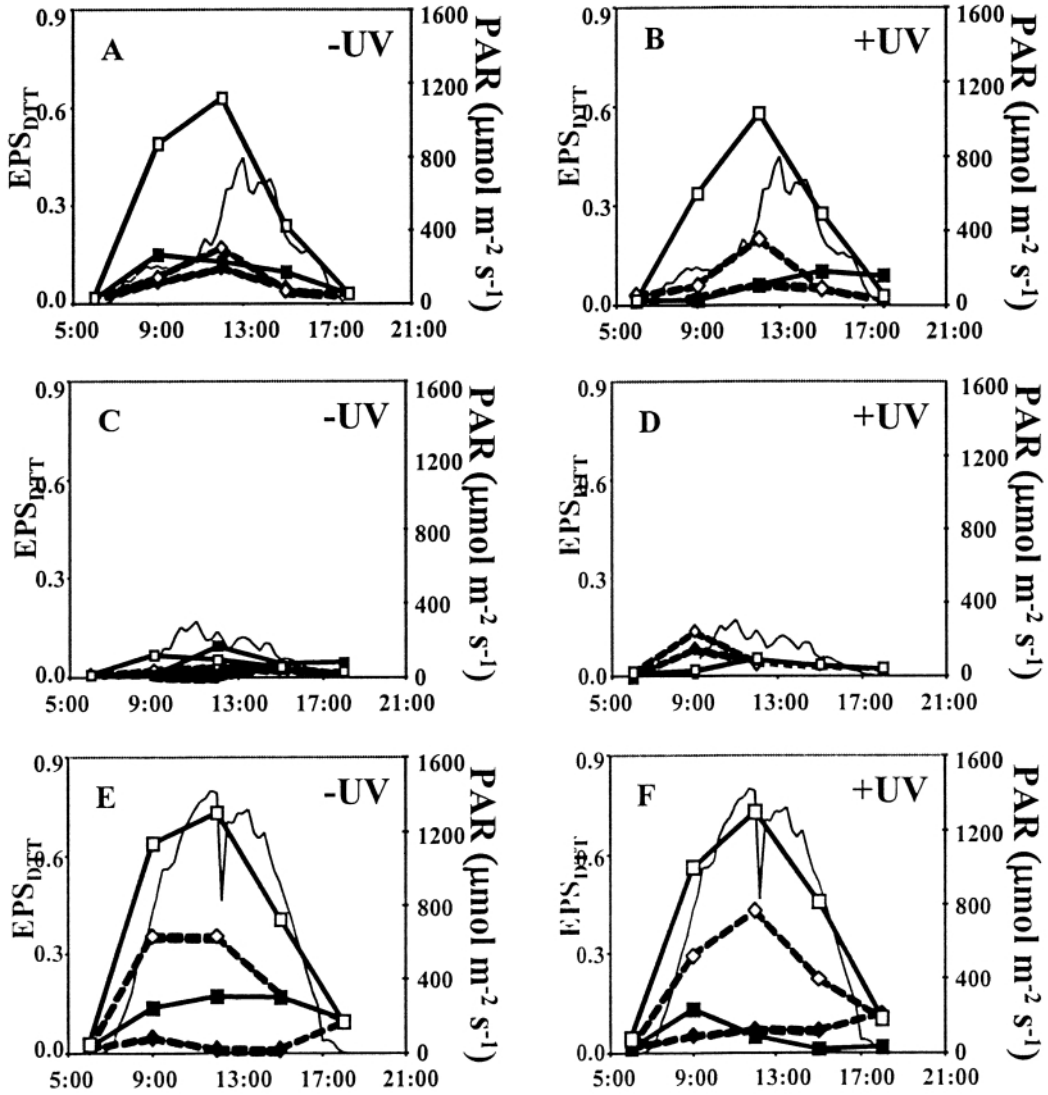


**Fig. 9.** The instantaneous irradiance (PAR; µmol m$^{-2}$ s$^{-1}$) and epoxidation state (EPS; [DT]/[DT + DD]) of the DD/DT xanthophyll cycle for the DTT experiments (see the text for details) on 3 (**A** and **B**), 4 (**C** and **D**) and 6 (**E** and **F**) November 1998. PAR-only treatments are denoted as '–UV' and PAR + UV treatments are denoted as '+UV'. DTT-treated samples (diamonds) and non-DTT-treated samples (squares) are identified as either dark adapted (filled symbols) or exposed (open symbols). It should be noted that the final removal of these supplemental incubations from the irradiance was ~1 h later than the samples shown in Figures 2–6.

The magnitude of $F_V/F_{M\text{-}D}$ was highest for the PAR-only (–DTT) samples in all three experiments (Figure 10). On 4 November, when the xanthophyll cycle was not significantly induced, both of the PAR-only samples exhibited a higher $F_V/F_{M\text{-}D}$ for most of the day than either of the PAR + UV (with and without DTT) samples. However, on 3 and 6 November, when the xanthophyll cycle was induced for a majority of the photoperiod, the DTT-treated samples exhibited a depression in $F_V/F_{M\text{-}D}$ values below either of the samples without DTT. An analysis of the $FI_D$ (Figure 11) reveals that the cultures on 3 and 6 November were being simultaneously heavily impacted by UV and appreciably protected by the xanthophyll cycle.

## Natural population parameters

Chlorophyll *a* values for the natural populations were 8.1 ± 3.1 and 8.5 ± 3.5 pg cell$^{-1}$ for 2 and 3 October, respectively. Cell counts ranged from 2.7 × 10$^6$ cells l$^{-1}$ (± 1.8 × 10$^6$) to 4.1 × 10$^6$ cells l$^{-1}$ (± 1.7 × 10$^6$). The domination of *G. breve* within the population was confirmed through microscopic analysis of whole-water samples and by the linear regression of Chl *a* and the *G. breve* signature pigment, gyroxanthin diester [$r^2$ = 0.95; data not shown;

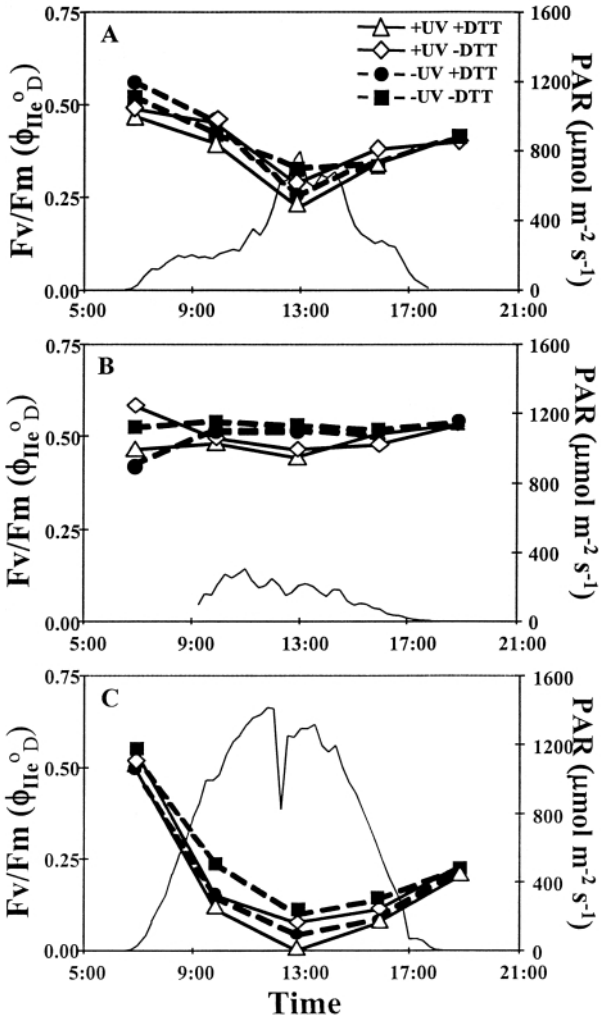


**Fig. 10.** Values for $F_V/F_M$ ($\phi_{IIe}{}^{o}{}_{D}$) for the DTT experiments (see the text for details) on 3, 4 and 6 November 1998 (**A**, **B** and **C**, respectively). The incident PAR irradiance (µmol quanta m$^{-2}$ s$^{-1}$) is also shown. PAR-only treatments are denoted as '–UV' and PAR + UV treatments are denoted as '+UV' in the key. It should be noted that the final removal of these samples from the irradiance was ~1 h later than the samples shown in Figures 2–6.

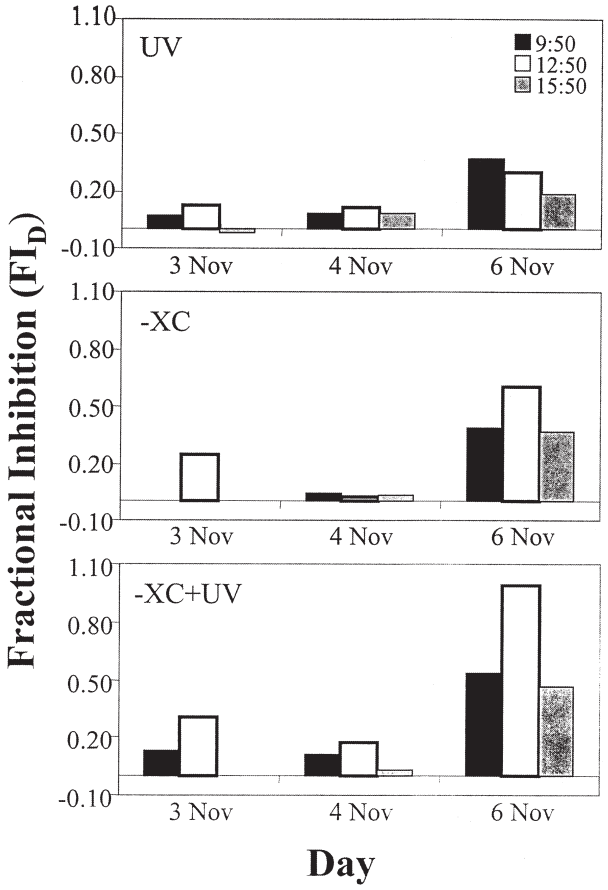


**Fig. 11.** The fractional inhibition ($FI_D$) due to UV radiation (UV), the loss of the xanthophyll cycle (–XC) and the combination of these (–XC+UV) within the *G. breve* cultures.

(Millie *et al.*, 1995)]. Pre-dawn samples yielded $F_V/F_M$ values of 0.6 and 0.5 on 2 and 3 October, respectively (Figure 12). In a pattern very similar to the high-light cultures, the time of minimal $F_V/F_M$ for the natural population occurred near the end of the photoperiod, and fell to ~37 and 31% of the maximum pre-dawn values for each day.

## DISCUSSION

Cultures and natural populations of *G. breve* exhibited distinct diurnal patterns in $F_V/F_M$ and accumulation of photoprotective, xanthophyll cycle pigments corresponding to varying surface irradiance, as reported earlier in macroalgae (Henly and Ramus, 1989; Schofield *et al.*, 1998), microalgae (Platt *et al.*, 1980; Falkowski *et al.*, 1994; Behrenfeld *et al.*, 1998) and terrestrial plants (Adams *et al.*, 1989; Demmig-Adams and Adams, 1992; Murchie *et al.*, 1999). During overcast conditions ($PAR_{MAX}$ of ~200 µmol quanta m$^{-2}$ s$^{-1}$), the *G. breve* cultures exhibited minimal alterations in both $F_V/F_M$ and in xanthophyll cycle pigment concentrations. During partly cloudy conditions ($PAR_{MAX}$ of ~800 µmol quanta m$^{-2}$ s$^{-1}$), the cultures exhibited a decrease in midday $F_V/F_M$ to ~50% of pre-dawn values and a xanthophyll cycle $EPS_{MAX}$ of 0.72. During sunny conditions ($PAR_{MAX}$ of ~1500 µmol quanta m$^{-2}$ s$^{-1}$), *G. breve* cultures exhibited an immediate and distinct decline in $F_V/F_M$ values early in the morning, which continued to decrease to ~15% of pre-dawn values by 15:00 h. EPS approached maximum values (0.68) by 09:00 h. An extremely similar $F_V/F_M$ response pattern was evinced by the *G. breve*-dominated natural

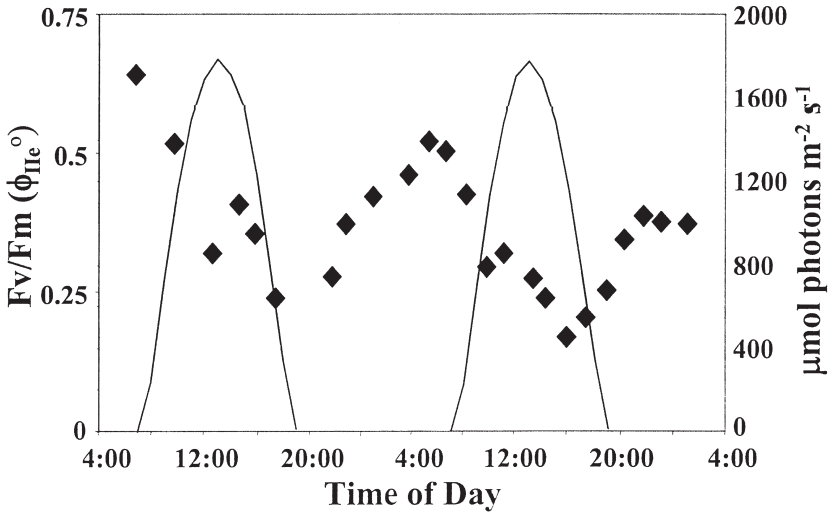


**Fig. 12.** The diurnal pattern of $F_V/F_M$ ($\phi_{IIe}$°) for the *G. breve*-dominated natural population on 3 and 4 October 2000. The incident PAR (μmol quanta m$^{-2}$ s$^{-1}$) is also shown.

population (Figure 11) when weather conditions were very sunny ($PAR_{MAX}$ of ~1800 μmol quanta m$^{-2}$ s$^{-1}$); however, minimum $F_V/F_M$ values were higher in these natural populations, decreasing to ~50% of pre-dawn values by midday, with further depressions reaching a minimum $F_V/F_M$ of ~35% in the latter part of the photoperiod.

The maximum quantum yield for stable charge separations at PSII is determined by alterations in $F_0$ and/or $F_M$. Depression of $F_M$ may be related to the induction of rapidly reversible photoprotective mechanisms such as PSII down-regulation (PSIIα–PSIIβ heterogeneity) and/or to photodamaging effects [see (Heil and Senger, 1987; Kroon, 1994)]. Increases and decreases in $F_0$ may result from photoinhibition and photoprotection, respectively; the antagonism of the combined effects makes them difficult to distinguish without corroborating evidence (Krause, 1988). Photoprotective responses in microalgae are generally rapidly reversible (Osmond, 1994), and are conventionally expressed as depressions in quantum yields that are (roughly) symmetrical about local noon. Chronic photoinhibitory responses usually involve some type of repair/replacement mechanism; thus, their effects are reversed on much longer time scales. During the morning hours (sunrise to midday), depression of $F_V/F_M$ in all of the experiments, both cultured and natural populations, was driven by concurrent decreases in $F_0$ and $F_M$ (Figure 13). During partly cloudy and overcast conditions (i.e. 3 and 4 November), both $F_0$ and $F_M$ increased to pre-dawn values by the end of the photoperiod, indicating that the changes in $F_V/F_M$ on these days were driven primarily by photoprotective responses. During sunny conditions (i.e. 6 November and 2 and 3 October), both $F_0$ and $F_M$ remained depressed well into the evening hours, signifying that a significant amount of chronic photoinhibitory damage had probably occurred.

The difference in the magnitude of $F_V/F_M$ depression between the cultured and natural populations is probably a function of both pre-acclimation and mixing/taxis effects. The pigment data from the natural *G. breve* population indicate that these cells were probably relatively high light acclimated (~8 pg Chl *a* cell$^{-1}$ for the natural population versus ~30 pg Chl *a* cell$^{-1}$ for the cultures). Additionally, the natural populations were not constrained from mixing/taxis effects that could have brought them deeper into the water column during the day, thus reducing the rate of photoinhibition/-damage. However, given the above caveats, the similarity in the $F_V/F_M$ response pattern between the cultured and natural populations exposed to high irradiance is encouraging. Both experiments exhibited a hysteresis in $F_V/F_M$ values in relation to incident light levels, reaching minimum values well after irradiance levels had fallen considerably. Additionally, from the data presented in Figure 12, it appears that the mechanisms driving $F_V/F_M$ were also similar between the cultured and field populations.

The apparent insensitivity of *G. breve* to UVR within these experiments is noteworthy. The cultures had been shielded from UVR prior to the experiments, yet the UV effects were secondary to PAR-related photoinhibition at all values of PAR. This is supported by the similarity in the rates of inhibition and recovery of $F_V/F_M$ in both the PAR-only and PAR + UV treatments. Additionally, it should be emphasized that due to the effects of the high cut-off wavelength of the UV-screening plastic (Figure 1),

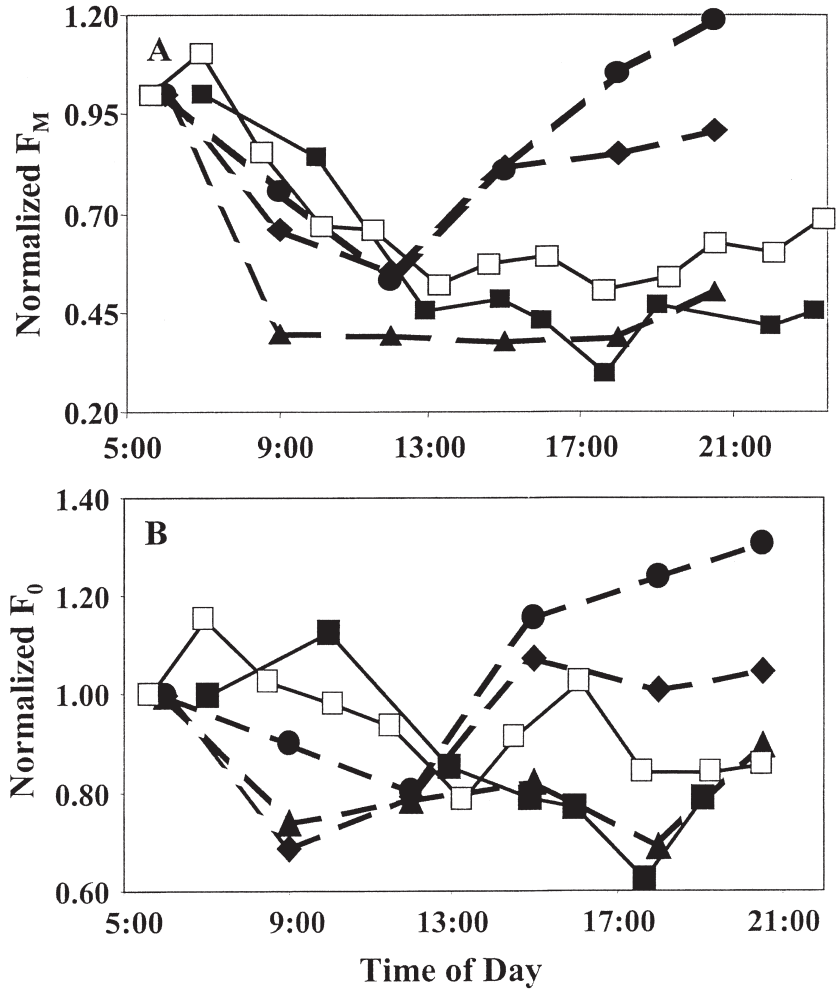


**Fig. 13.** The diurnal pattern of normalized maximum (**A**) and minimum (**B**) fluorescence values for the culture-based and field populations of *G. breve*. Dashed lines indicate culture-based experiments on 3 (circles), 4 (diamonds) and 6 (triangles) November 1998. Solid lines indicate field-based natural populations on 3 (filled squares) and 4 (open squares) October 2000.

the PAR + UV treatment was receiving ~8% more PAR and, most likely, some PAR radiation in the near UV than the PAR-only treatment. Consequently, the PAR + UV treatments reached supersaturating levels of PAR earlier and longer than the PAR-only treatments. Thus, even the minor differences in $F_V/F_M$ and EPS between these two treatments cannot be reliably attributed to UV-induced effects. We did not examine any of the *G. breve* samples for UV shading compounds, such as mycosporine-like amino acids.

This disparity in PAR between +UV and –UV treatments may partly reconcile the depressed values of DO in the PAR + UV treatments for all three culture-based experiments (Figure 4A). Additionally, it may be that UVR (directly or indirectly) inhibits $O_2$ production, stimulates $O_2$-scavenging mechanisms, stimulates respiration and/or photorespiration, or a combination of any of these. However, the average difference in DO (at 18:00 h) between the PAR + UV and the PAR-only treatments for all three culture-based experiments varied by only 10% (0.076 ± 0.0074).

Regression analyses of lipid and $F_V/F_M$ values indicate that significant correlations exist for both treatments on 3 and 6 November ($r^2 > 0.80$, $p \leq 0.01$). Crawford and Dodge (Crawford and Dodge, 1974) have reported that lipids can be a primary reserve for dinoflagellates, and Kamykowski *et al.* (Kamykowski *et al.*, 1998) further postulated that *G. breve* lipid status might be inversely correlated to the strength of the negative geotaxis response exhibited by swimming cells. The apparent inhibition of lipid synthesis on the 2 days when $F_V/F_M$ was also heavily impacted suggests that these two culture populations may

not have stayed in the upper water column during the middle of the day if not restrained by the cubitainers. This would most likely have decreased the amount of photodamage suffered by these cultures.

## Xanthophyll cycle photoprotection

The supplemental DTT incubations were undertaken in order to explore the relationship(s) between the photoprotection offered by the xanthophyll cycle and/or the inhibitory effects of UVR. On the overcast day (4 November) when the xanthophyll cycle was not appreciably induced ($EPS_{MAX} = 0.07$), there was no significant difference within $F_V/F_{M\text{-}D}$ between the DTT treatments (*t*-test, $p > 0.5$), signifying that the xanthophyll cycle was not providing any protection. However, there was a significant difference within $F_V/F_{M\text{-}D}$ between +UV and –UV treatments (*t*-test, $p < 0.001$), indicating that UVR was affecting these cultures even during extremely overcast conditions (Figure 10). On the partly cloudy and sunny days when the xanthophyll cycle was appreciably induced for a majority of the photoperiod ($EPS_{MAX} \sim 0.7$), significant differences existed within $F_V/F_{M\text{-}D}$ between the DTT treatments (*t*-test, $p < 0.005$), and did not exist between +UV and –UV treatments (*t*-test, $p > 0.25$). While the magnitude of the effects of high irradiance and UV were quite different between 3 and 6 November, the response patterns for $F_V/F_{M\text{-}D}$ at midday were very similar (i.e. –UV – DTT > + UV – DTT > – UV + DTT > + UV + DTT). Figure 11 provides an estimate of both the level of protection offered by the xanthophyll cycle, and of the relative magnitude of inhibition attributable to UVR. The analysis of $FI_D$ indicates that, without the xanthophyll cycle, the magnitude of photoinhibition due to high PAR irradiance would exceed the level of inhibition due to UV (Figure 11). Thus, the level of protection offered by the xanthophyll cycle appears to be quite significant. It should be emphasized that due to the different exposure conditions, the results from the cubitainers and the supplemental DTT incubations should be compared with caution.

The concentrations of DT found after a 30 min dark acclimation on the partly cloudy and sunny days are consistent with the apparent inhibition of epoxidase activity found by Olaizola *et al.* (Olaizola *et al.*, 1994) in the diatom *Phaeodactylum tricornutum*. Similarly, we observed an apparent DTT-insensitive pool of DD within *G. breve* during these experiments, which resulted in appreciable levels of DT accumulation in the presence of DTT and high light. However, it should be emphasized that the observed responses for *G. breve* were derived from solitary experiments and need to be examined further before determining whether this DTT-insensitive DT is actively involved in fluorescence quenching.

The capabilities of xanthophyll cycle-mediated photoprotection are dependent not upon the absolute concentrations of DD and DT, but upon the proportion of pigment sequestered in a functional role (Peter and Thornber, 1991; Arsalane *et al.*, 1994). During the extremely sunny conditions of 6 November, both the PAR-only and PAR + UV treatments exhibited an ~50% decrease in EPS values at 15:00 h, even though the incident irradiance was still quite high (Figure 9). This fall in EPS could represent a restructuring of the thylakoid architecture to facilitate a more efficient dissipation of excess excitons, thus raising lumen pH and lowering EPS. It may also indicate the photodegradation of DT, explaining the continued fall in $F_V/F_M$ after the 15:00 h sampling time when the irradiance had declined appreciably from the noontime high. However, these hypotheses are not mutually exclusive, and the sampling protocol used here cannot distinguish between DT in a photoprotective role and newly synthesized pigment. Thus, there could have been an increase in non-photochemical quenching efficiency and *de novo* synthesis occurring simultaneously.

The increase and decrease in the cell fucoxanthin content appeared to result in favorable photoacclimatory responses to the incident, instantaneous irradiance occurring on the cloudy and partly cloudy days, respectively. During times of greatest irradiance, no alterations in cell fucoxanthin content occurred, with cellular contents remaining relatively stable over the course of the photoperiod. The observed increases in pigment concentrations within populations towards the end of each day may have resulted from mechanisms to provide the chloroplasts with a large pigment pool to be adjusted at the beginning of the next photoperiod. Although the decrease in cell fucoxanthin content within the early morning hours on the initial day of experiments cannot be immediately explained, Swift and Milborrow (Swift and Milborrow, 1981) have postulated that allenic carotenoids (such as fucoxanthin) may be precursors to acetylenic carotenoids (such as DD and DT). However, no apparent simultaneous (stoichiometric) increase in DD or DT was observed within *G. breve*.

Kamykowski *et al.* have shown that *G. breve* tends to accumulate into two subpopulations ('surface' and 'mid-column') that exhibit markedly different physiologies (Kamykowski *et al.*, 1998). However, it appears that this accumulation has a definite diel cycle, wherein the subpopulations disperse during the night and accumulate during the daylight hours. This trend was seen during the R/V 'Pelican' cruise (data not shown), but the surface accumulation became noticeable near midday and persisted until ~24:00 h. The cultures used in these experiments were homogenized before being placed in the cubitainers; if subpopulations existed in the cultures, they

would have had very little impediment to positioning into 'surface' and 'mid-column' populations during the course of sampling. This was not addressed during the culture-based experiments, and may have contributed to some of the variability in the data.

The experiments presented here are primarily concerned with photophysiological response patterns. The broader implications of these results indicate that while *G. breve* does tend to concentrate into surface blooms, it exhibits a 'typical', photosynthetic down-regulation response to sinusoidal, high-irradiance conditions. However, the apparent UV insensitivity of *G. breve* is anything but typical. Further work relating the behavioral responses of this species to irradiance, nutrients and biochemical parameters should remain a high research priority.

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