PHYSIOLOGY AND LIFE CYCLES SESSIONS

UV-B Inhibition of *Pseudo-nitzschia australis:* Primary Production Is Minimized by UV-A Enhancement Effects

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Abstract

While it is generally accepted that solar UV-B radiation inhibits *in situ* phytoplankton productivity, consequences of elevated UV-B radiation for phytoplankton community dynamics are unknown. UV-B radiation reaching the Earth's surface has been rising slowly for the last 25 years due to atmospheric ozone-depletion. It has been suggested that algal species with some UV tolerance will gain a competitive edge in the elevated UV environment of the upper ocean and perhaps secure a greater dominance within phytoplankton assemblages. Here we report an apparent UV tolerance of *in situ* primary production by the domoic acid-producing diatom *Pseudo-nitzschia australis* compared to the UV sensitivity of phytoplankton in the surrounding waters of the Santa Barbara Channel during June 1998. Primary production of *P. australis* was enhanced 15% by UV-A while that of the surrounding community was inhibited 13% by UV-A at short UVA wavelengths. Although the sensitivity to UV-B was similar in both communities, the UV-A enhancement in the *P. australis* community offsets the inhibition due to UV-B, resulting in minimal net loss of primary production relative to PAR. In the community surrounding the *P. australis* bloom, the UV-B inhibition was far greater than the UV-A enhancement at long UVA wavelengths, ensuing in a significant loss of primary production due to UV radiation. This ability to utilize UV-A radiation to enhance primary production is certainly advantageous in the high light environments of the upper ocean and may be one factor increasing the probability of a successful bloom of *P. australis* in nature.

Introduction

Loss of stratospheric ozone and associated increases in UV-B radiation (280–320 nm) are evident at all latitudes and will persist for decades to come (Madronich *et al.,* 1998). Inhibitory effects of UV (280–400 nm) on phytoplankton primary production are generally greater for shorter UV-B wavelengths than longer UV-A wavelengths (320–400 nm). Unlike UV-B, UV-A radiation has been shown to inhibit and/or enhance net primary production. Different algal species can vary in their sensitivities to UV, which raises concerns that UV climate change might lead to alterations in community assemblages with unknown consequences (Prézelin *et al.,* 1994). Hargraves *et al.* (1993) observed *P. pungens* var *multiseries* growth to be unchanged when exposed to UV and suggested that toxic *P. multiseries* might be one species to benefit from present changes in UV climatology. If the genus *Pseudo-nitzschia* were to become more prevalent as a result of the changing UV climatology, it would negatively impact local fisheries, coastal economies and marine ecosystem dynamics. During our cruises designed to quantify the UV sensitivity of diverse phytoplankton communities, we encountered a *P. australis bloom* and had the opportunity to assess its UV sensitivity in nature and compare it with an adjoining community where *P. australis* was a much smaller component of the phytoplankton assemblage.

Materials and Methods

Field experiments occurred June 8–9, 1998, at the west end of the Santa Barbara Channel. Five-meter samples were collected, spiked with C^{14} labeled HCO^- and deployed back into the ocean, enclosed in specially designed experimental drifters (Prézelin *et al.,* 1994). Replicate samples were simultaneously exposed to 7 different light treatments (5 replicates per light treatment) that progressively excluded shorter wavelengths of UV radiation by use of spectral band-pass filters with 50% transmission at 295, 309, 318, 328, 341, 382, and 403 nm. The >295 nm treatment represents the responses to the total *in situ* light field while the >403 nm treatment represents primary production in response to exposure to photosynthetically available radiation (PAR, 400–700 nm) only. C^{14} incorporation was measured for each replicate at the end of the experiment, whereas pigment concentration was measured by combining the 5 replicate treatments before filtering in order to obtain

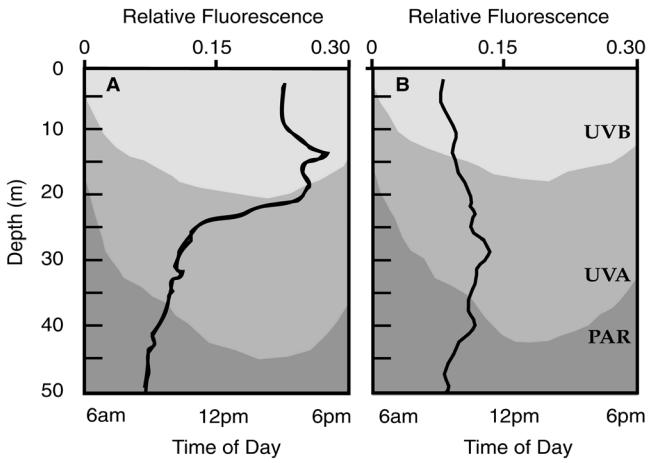

Figure 1 Vertical profile of phytoplankton biomass as indicated by relative fluorescence (solid black line) **A** inside and **B** outside the *P. australis* bloom. Lightest gray tone indicates the depth-penetration of UV-B +UV-A+PAR radiation over the course of the day on **A** June 8 and **B** June 9 (inside and outside the bloom respectively). Middle tone of gray specifies the depth penetration of UV-A+PAR and the darkest gray displays region where only PAR is present.

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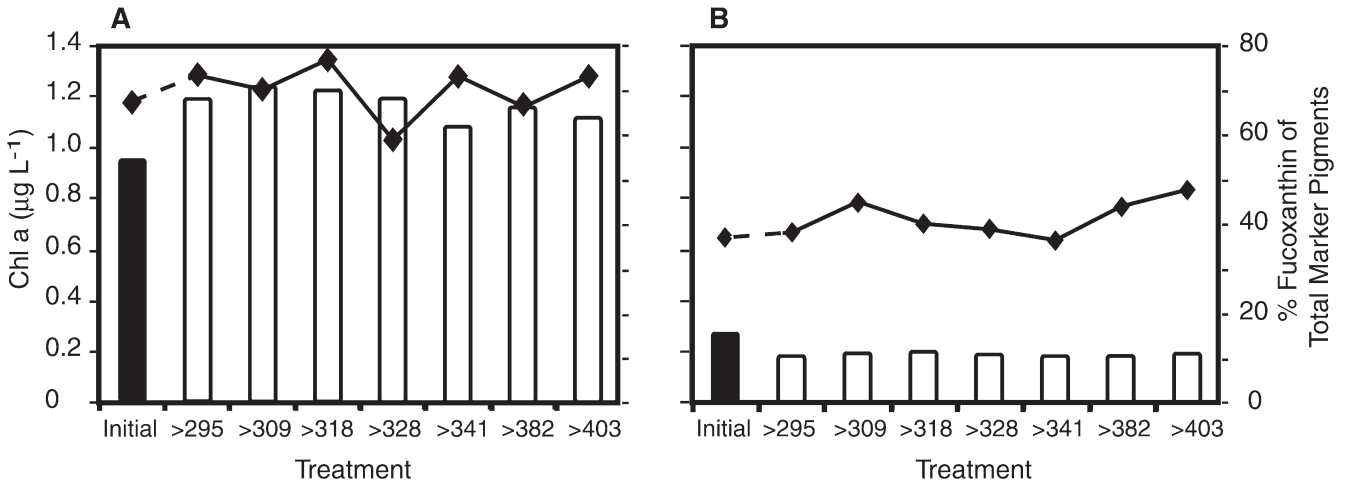

Figure 2 UV impacts on chl *a* biomass (black and white bars) and the percent fucoxanthin of total marker pigments (black diamonds) for the **A** *P. australis* bloom sampled on June 8 and **B** for the mixed phytoplankton population on June 9, 1998.

enough sample for pigment analysis (for details on sample prep and analysis see Prezelin *et al.,* 1994). Total amount of carbon fixed over the course of the day in each light treatment was compared to the carbon fixed in the PAR-only treatment (>403 nm) and the difference normalized to the carbon fixed at >403 nm to calculate the percent enhancement. To calculate the percent inhibition, the carbon fixed in each treatment was compared to the maximum amount of carbon fixed and the difference normalized to the maximum carbon fixed. Absolute exposure to underwater UV and PAR light (UWL) was determined with the aid of a Biospherical PUV-PAR profiling system and a specially designed software code (Hastings *et al.,* 2002, www.lifesci.ucsb.edu/eemb/labs/prezelin/tech_reports/). Community composition was determined using chemotaxonomic marker pigments quantified by HPLC (Wright *et al.,* 1997) and *P. australis* verified microscopically.

Results and Discussion

Figure 1 depicts the chlorophyll *a* (chl *a*) biomass distribution overlaying the under-water light (UWL) fields of the 2 water columns where we compared the UV-sensitivity inside (Fig. 1A) and outside (Fig. 1B) a *P. australis* bloom community. Chl *a* biomass was ca. two- to threefold higher inside the *P. australis* bloom (Fig. 1A) confined to a 20 m deep mixed layer (data not shown) than in surrounding waters where the entire water column was stratified. Maximum PAR irradiance at the surface of the ocean was 25% higher on June 8 (2000 µEin m^{-2} sec^{-1}) than on June 9 (1600 µEin m^{-2} sec^{-1}), however the UWL fields in the upper euphotic zone were nearly identical due to the greater chl-dependent light attenuation inside the *P. australis* bloom.

By the end of the dawn-to-dusk incubation, *P. australis* chl *a* had increased approximately 20% regardless of whether samples had been exposed to UV radiation (Fig. 2A). In

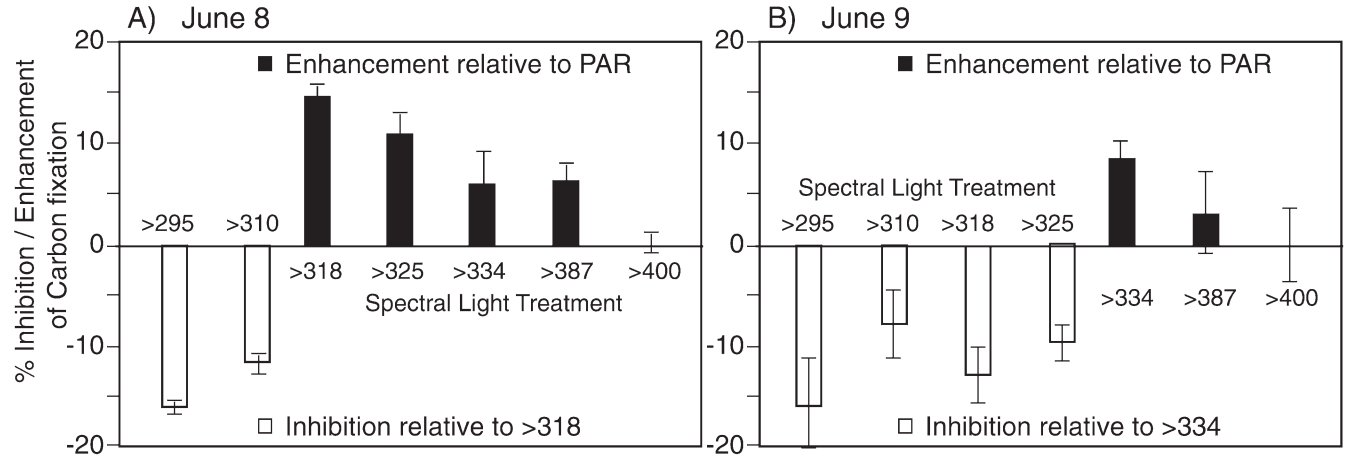

Figure 3 Percent inhibition (white) and percent enhancement (black) of carbon fixation relative to the maximum amount of carbon fixed and relative to PAR treatment, respectively, as a function of light treatment in the **A** *P. australis* sample and **B** the mixed phytoplankton population.

contrast, chl *a* declined 40% in all light treatments of the surrounding phytoplankton community (Fig. 2B). The percent of total marker pigments attributable to fucoxanthin, the taxonomic marker pigment for diatoms, increased from 65% to 75% over the day inside the bloom (Fig. 2A) and increased from 35% to 45% outside the bloom (Fig. 2B). While diatom dominance increased, phytoflagellates (dinoflagellates, prymnesiophytes, cryptophytes, etc.) dominance declined. Primary production by the *P. australis* bloom population was enhanced (max. 15%) relative to PAR in all UV-A treatments (Fig. 3A). In the mixed phytoplankton population, UV-A enhancement (max. 9%) is only observed in the long wavelength portion of the UV-A spectrum (>341 nm), whereas short wavelength UV-A leads to photoinhibition (Fig. 3B). Levels of photoinhibition due to UV-B are comparable for both populations (Fig. 3). This indicates that UV-A enhancement of primary production in bright light environments is an important element of *P. australis* photoecology. The UV-A enhancement almost entirely offsets the UV-B inhibition relative to PAR. In general, UV-A enhancement appears to be a common feature in phytoplankton communities at low UV-A dose rates, but clearly declines as UV-A irradiance increases (data not shown). However, this *P. australis* community displays much higher UV-A enhancement at high UV-A irradiances compared to other algal populations from the Channel. UV-A is known to induce photoprotective and photorepair mechanisms (Karentz *et al.,* 1991), which could be partially responsible for our observations. Field data and additional laboratory studies (data not published) suggest that UV-A enhancement of carbon fixation does not occur as a result of primary photochemistry. Based on our *in situ* observations, we conclude that *P. australis,* although susceptible to UV-B inhibition, is overall more UV-resistant and is particularly well adapted to utilize the UV-A portion of the UV-spectrum to enhance carbon fixation. Consequently, optimal light-growth conditions for *P. australis* would be at depths where UV-A and PAR are high but UV-B is absent.

Acknowledgements

NSF (OCE-9618390) funded the SUPACC field study. The University of California's Marine Council under the Coastal Environmental Quality Initiative Program (02T CEQI 080094) funded a graduate fellowship to C. Mengelt. We thank J. Robidarts for her contribution to data collection and analysis.

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Dark Survival and Subsequent Light Recovery for *Pseudo-nitzschia multiseries*

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Abstract

We conducted experiments to determine whether addition of dissolved organic nitrogen (DON) in the form of glutamate and glutamine would prolong dark survival for *Pseudo-nitzschia multiseries* and/or alter its potential for light recovery. This study represents a first test of our working hypothesis that *P. multiseries* blooms can be initiated by upwelling of a seed population capable of remaining viable heterotrophically in the aphotic zones for prolonged periods of time. In addition to having a dark survival strategy, we hypothesize that successful initiation relies on the microbe's ability to quickly resume maximum growth rates when reintroduced into the light. Log phase photoautotrophic cultures were transferred from the light into the dark with and without glutamine/glutamate additions. Each week for the next 10 weeks, dark samples were retrieved and immediately analyzed for cell numbers and percent viable cells and then kept in the light for several weeks while the photoautotrophic growth potential was determined. Cell density in the dark control remained fairly constant over the course of the experiment. Cell density in the DON culture doubled during week 1 in the dark, coinciding with the disappearance of glutamate/glutamine from the medium, declined quickly in the next 2 weeks and stayed constant for the next 7 weeks. The percent of viable cells decreased from >90% to ca. 60% in both the control and DON populations during the first 3 weeks in the dark. DON populations had a longer delay than control populations before renewed growth in the light occurred. For both cultures, the length of the lag phase during photorecovery increased, the longer the dark incubation. If transferred back to the light within 2 weeks of dark survival, the DON cells divided twice as fast as the control cells during photo-recovery. Findings of this study suggest that *Pseudo-nitzschia* spp. blooms would occur with weeks of delay after a seed population is brought to the euphotic zone due to the prolonged lag phase after more than a month in the dark, particularly in high DON waters. Whether dark survival is prolonged in the presence of DON and the seed population's viability enhanced remains to be seen after the experiment is repeated with weekly spiking of DON.

Introduction

In the past decade, toxic blooms of *Pseudo-nitzschia australis* and *P. multiseries* have caused high mortality among pelicans, sea lions, and dolphins on the west coast of the United States (Shaw *et al.,* 1997; Scholin *et al.,* 2000) and resulted in human illness and deaths on the east coast of Canada (Bates *et al.,* 1989). Accordingly, considerable effort has been made to elucidate the ecology and what triggers such blooms. Trainer *et al.* (2000) suggest that these blooms are initiated via the upwelling of a seed population from below the euphotic zone. However, this mechanism of bloom-initiation requires the presence of a viable seed population at depth, implying the ability to survive extended periods in the dark either by forming resting cells or becoming heterotrophic. Based on previous work that has found *P. multiseries* to grow more rapidly with

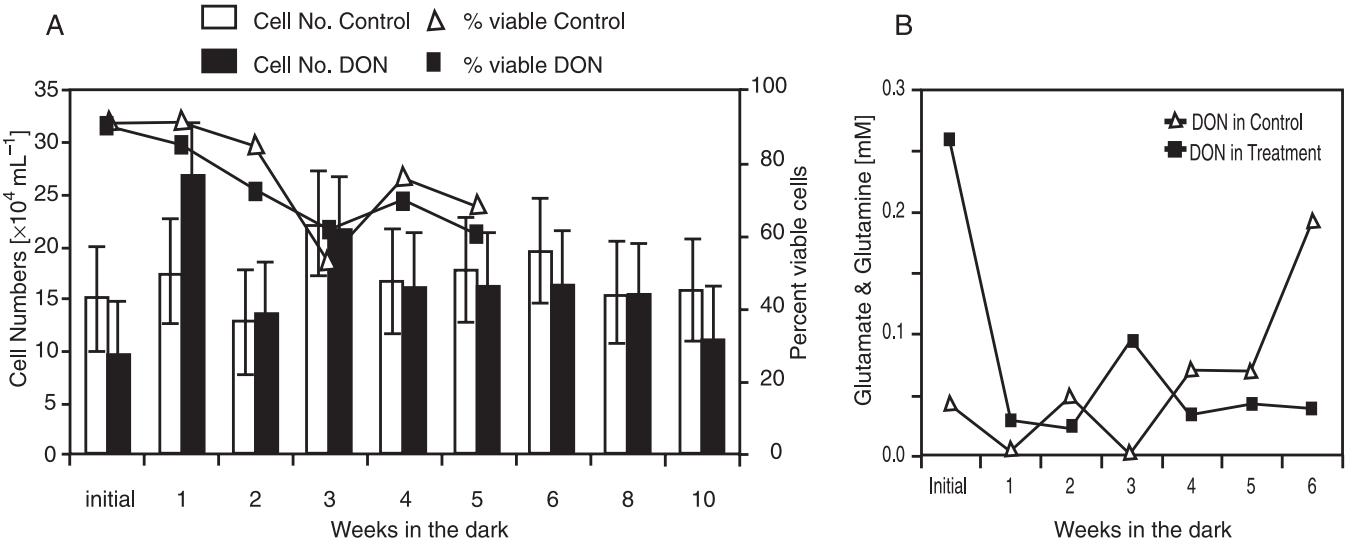

Figure 1 **A** Weekly estimates of cell density and percent viability of *P. multiseries* cultures just prior to and during several weeks in the dark, with and without dissolved organic nitrogen added at the beginning of the experiment (means of 3 cell counts ± SD). **B** Changes in the combined concentration of glutamate and glutamine during dark survival of *P. multiseries* just prior to and during several weeks in the dark, with and without the addition of dissolved organic nitrogen added at the beginning of the experiment.

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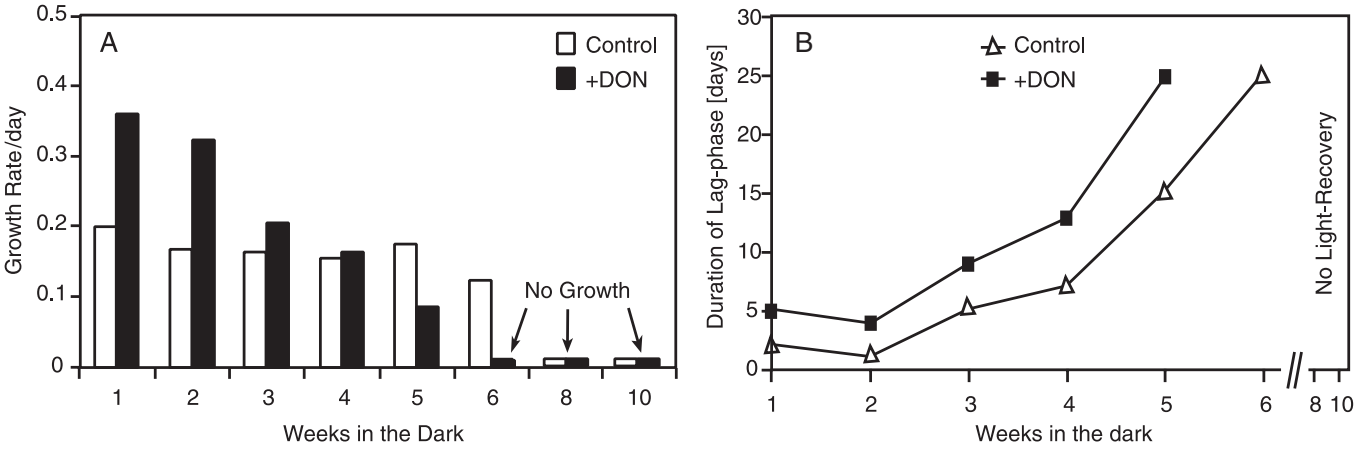

Figure 2 **A** Growth rates during light-recovery after dark incubation for control (□) and DON treatment (■). **B** Duration of the delay before the onset of growth during light recovery for control (△) and DON treatment (■).

glutamate addition (Hillebrand and Sommer, 1996) and outcompete other algal species with the addition of organics from sewage effluent (Pan and Subba Rao, 1997), we hypothesize that survival of *Pseudo-nitzschia* populations in the aphotic zone for a prolonged period in the dark is dependent upon the availability of organic nutrients. As a result, the percent of viable cells returned to the surface during a subsequent upwelling event would be higher, as would be the probability for a successful bloom initiation. Our goal in this study is therefore to test in laboratory experiments whether the dark survival of these algal cells is enhanced with the addition of organic nitrogen and whether growth after an extended period in the dark is enhanced for a population that was supplemented in the dark with organic nutrients.

Materials and Methods

Cultures of *Pseudo-nitzschia multiseries* initially isolated from Monterey Bay were provided courtesy of Ralph Kudela's culture collection at University of California, Santa Cruz. Non-axenic batch cultures of *P. multiseries* were maintained on L1+silicate medium at 18°C ± 2°C on a 12h light : 12h dark cycle at a growth irradiance of 200 µE m^{-2} sec^{-1}. A photoautotrophic, nutrient replete, log phase culture of *P. multiseries* was the inoculum for this study. One of two replicate cultures was supplemented with dissolved organic nitrogen (DON) to yield a final concentration of 100 µM glutamate and 100 µM glutamine. Samples were collected from both the control and DON-supplemented culture for determinations of initial cell densities, cell viability and glutamate/glutamine concentration. Then each treatment was divided into replicate sterile growth flasks (100 mL each) and placed into a dark growth chamber maintained at 17°C ± 1°C. After one week in the dark, one of the 15 replicate flasks for both the control and DON-supplemented cultures was removed from the dark and sampled for cell density, viability and glutamate/glutamine concentration. They were placed into illuminated growth conditions described above to assess the ability to recover in the light. These light recovery control and DON-supplemented cultures were then sampled daily to determine cell numbers, cell morphology, and number of cells in chains. Each week for 10 weeks thereafter, 1 flask from each treatment was recovered, placed back in the light and sampled as described above. Cell concentrations were determined microscopically with a haemacytometer. For each treatment, three replicate cell counts were performed to determine the measurement error. Cell viability was measured by staining live cultures with 5-(and 6)-carboxyfluorescein diacetate (Molecular Probes). Concentrations of glutamate and glutamine were determined spectrophotometrically using a commercial glutamate determination kit (Sigma-Aldrich, GLN-1).

Results and Discussion

Dark Survival When grown as obligate photoautotrophs, *P. multiseries* had a maximum growth rate of 0.4 divisions per day under the established culture conditions. For photoautotrophic populations placed in the dark without organic nutrient supplements of glutamate and glutamine, there was no change in the cell density over the 10 weeks of imposed dark survival (Fig. 1A). Thus cell division ceased quickly in the dark without organic nutrient supplements. For populations placed in the dark with DON, cell density appears to have doubled within the first week (Fig. 1A), coinciding with the disappearance of DON from the growth medium (Fig. 1B). However, the higher cell density could not be maintained and by the end of week 2, *P. multiseries* cell concentration was the same as for the control population. Regardless of whether *P. multiseries* or contaminant bacteria took up the organic nitrogen in the first week, it was clear that DON-treated cultures responded differently from control populations during the first week in the dark. Experiments with the addition of antibiotics are underway to determine if bacteria played a role in the differential growth responses of *P. multiseries* control and DON treatments. After 6 weeks in the dark, there was a rapid accumulation of glutamate and glutamine in the control treatment, likely due to cell lyses, which raises questions about what role it plays in the maintenance of a seed population. However, the elevated DON concentration in week

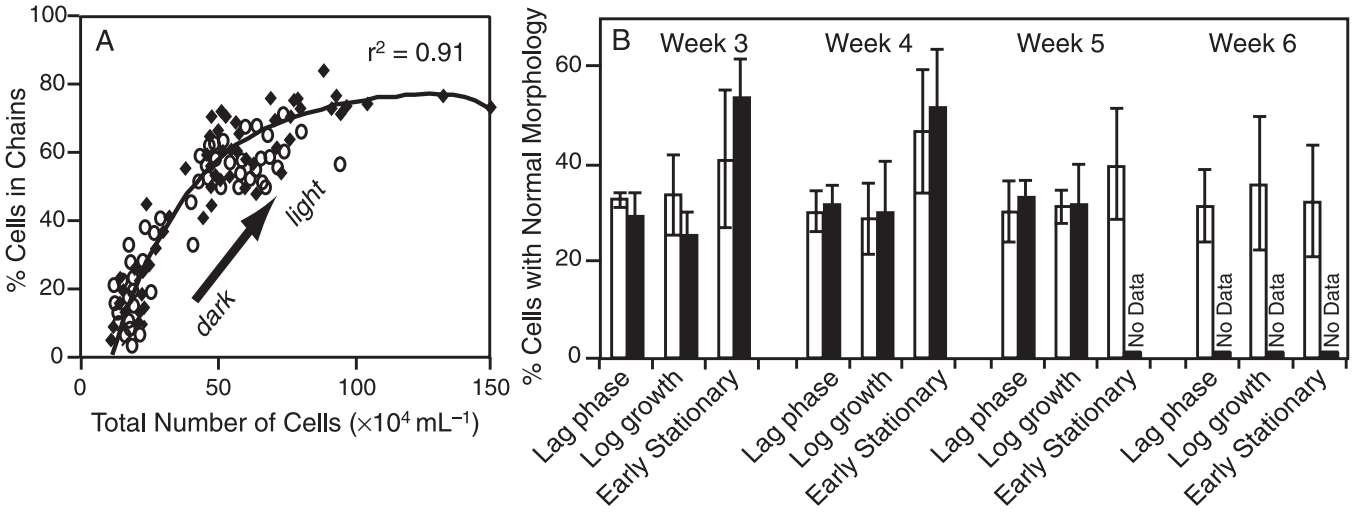

Figure 3 **A** Percent of total cells that formed chains as a function of total cell numbers during light recovery for both the control (♦) and DON treatment (◯). **B** Percent of total cells with normal morphology increased during early stationary growth phase for cells in the DON treatment ($P < 0.01$) but not for cells in the control ($P > 0.01$) (means of 3 cell counts ± SD).

six did not translate into dark growth in control populations nor increased light recovery for the control population during the remaining 4 weeks. Based upon present observations alone, it would appear that any growth benefits from a single addition of DON at the outset of dark survival was short-lived.

Light Recovery The ability for light recovery was altered in response to DON addition at the onset of the dark period (Fig. 2A, B). DON treatments returned to the light after 1–2 weeks in the dark had a 4–5 days longer growth lag than controls but had growth rates twofold higher than controls. This initial lag might indicate that cells had to adjust their metabolism from heterotrophy to photoautotrophy in order to accommodate re-exposure to light. While control populations had light recovery growth rates that were relatively constant if returned to the light within 6 weeks of dark incubation, the much higher light recovery growth rates of DON-treated cells progressively declined and eventually was less than that of control populations by week 6. The decrease in growth rate for the DON cells could be related to the rapid disappearance of DON in the first week. Cultures that were in dark for longer than 6 weeks did not grow when placed back into the light regardless of whether DON was supplemented at the beginning of dark incubation.

During light recovery, the percentage of cells that formed chains increased significantly and reached a maximum value of ca. 80% at the end of light recovery (Fig. 3A). This was true for all cultures capable of photo-recovery no matter what the length of dark incubation. It is unclear whether the observed increase of cells in chains was a consequence of rapid cell division or due to the change from dark to light growth. Additionally, we tracked the numbers of cells with abnormal morphology, where cells grew with two round lobes on one valve (as reported by others, M. Silver, pers. communication). We found the percent of cells with typical morphology to be significantly greater ($P < 0.01$) for populations during late log/early stationary growth phase for the DON treatment during their light recovery (Fig. 3B). Although results are not conclusive, they suggest that availability of DON alters the timing of a *P. multiseries* bloom resulting from an upwelled seed population. Weekly spiking of DON during a replicate experiment will be necessary to draw further conclusions about the prolonged dark survival and enhanced light recovery.

Acknowledgements

We thank R. Kudela for providing cultures, Mary Silver for helpful suggestions and C. Swan for experimental assistance. Funding was provided by University of California Marine Council's Coastal Environmental Quality Initiative Program (02T CEQI 080094) and by NSF (OCE-9618390).

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Effects of Light, Temperature and Salinity on the Growth Rate of *Alexandrium tamarense* from Patagonia (Argentina)

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Abstract

Recurrent blooms of the toxic dinoflagellate *Alexandrium tamarense* occur in the Patagonian gulfs, Argentina, producing high risk to human health and economic losses to the activity of harvesting and culture of shellfish. Laboratory experiments were carried out to elucidate the influence of irradiance, temperature and salinity on the growth rate of the species isolated from Golfo Nuevo (Chubut) in unialgal batch cultures. Exponential growth rates of the species increased with increasing irradiance to a maximum of about 150 μmol m^{-2} s^{-1}. The maximum daily growth rate was 0.45 divisions day^{-1} at 15°C. *A. tamarense* could grow at all the tested salinities from 20 to 35 psu; the optimum growth was attained at 34 psu, and the differences in growth rate between salinities were less than 10%.

Introduction

Blooms of the toxic dinoflagellate *Alexandrium tamarense* usually occur in spring along the north Patagonian coast (Carreto *et al.,* 1981, 1993; Esteves *et al.,* 1992; Gayoso, 2001). High interannual variation in the magnitude and timing of occurrence was observed in a field study carried out in Golfo Nuevo, a semienclosed body of water situated at 43°S (Gayoso, 2001). To elucidate the influence of basic environmental factors (light, temperature and salinity) on the growth rate of *A. tamarense,* laboratory experiments were carried out in batch cultures using a clone of the species isolated from Golfo Nuevo waters.

Materials and Methods

Clonal cultures of *A. tamarense* (clone AT-3D) were established from vegetative cells collected with a 20 μm mesh size plankton net in the Golfo Nuevo. Cultures were maintained in f/2 medium without silicate (Guillard 1963), and triplicate growth experiments were carried out in batch culture. Erlenmeyer flasks of 250 mL filled with 100 mL of f/2 medium and an inoculum of 5000 cells per mL from a culture in exponential growth were used.The cells were counted every two days using a Palmer-Maloney chamber and growth rate estimated during the period of exponential growth by least-squares fit of a straight line of the data logarithmically transformed (Guillard 1973). Experiments sought to establish the growth-irradiance relationship and the temperature-dependence of growth. Cultures were grown at photon flux densities of 30, 75, 100, 150, 200 and 230 μmol $m^{-2}s^{-1}$ under a light cycle 12h light–12h dark and 10, 15 and 20°C, *i.e.,* the temperature range usually observed in Golfo Nuevo. Cells were preconditioned at each experimental condition through at least one transfer. The same batch of filtered, aged Golfo Nuevo water at 34 psu was used to prepare the f/2 medium used in the experiments. For salinity growth assays, filtered sea water was concentrated by evaporation at 60°C or diluted by the addition of distilled water. Salinity was measured with a Beckman salinometer.

The growth rate curves were fitted by the points and explained by the hyperbolic tangent function (Yoder 1979): $\mu = \mu_{max} Tan (\alpha [I-I_c] x\mu_{max}^{-1})$. In this equation, μ is the specific growth rate; α is the initial slope of the curve in the linear, sub-saturated region; μ_{max} is the maximum growth rate; and I_c is the compensation point. The values of μ_{max}, α and I_c were estimated by nonlinear least-squares regression, based on the Levenberg-Marquardt algorithm.

Results

The maximum daily growth rate of *A. tamarense* was 0.44 divisions at 15°C, I_c varied from 0.6 to 49.7 μmol $m^{-2}s^{-1}$, α values were 0.0028–0.0077 division μmol^{-1}, and the calculated I_k for growth (the irradiance at which the initial slope line reaches the maximum rate of growth) varied between 90 and 130 μmol $m^{-2}s^{-1}$ (Fig. 1). *A. tamarense* could grow at all the tested salinities from 20 to 35 psu. The optimum growth was attained at 34 psu and the differences in growth rate between salinities were less than 10%.

Discussion

The maximum growth rate observed was 0.44 divisions day^{-1}. This is comparable with the results from northeastern Canada (Parkhill and Cembella, 1999) and from South Korea (Kim *et al.,* 1996). The optimal experimental temperature for growth (*i.e.,* 15°C) and the range of temperature at which *A. tamarense* can grow agree with the temperature range in the Golfo Nuevo during spring, when *A. tamarense* populations flourish (*i.e.,* 9°–15°C), confirming the characterization of this species as temperate and well adapted to relatively cold waters (Prakash 1967).

High solar radiation during spring was linked to *A. tamarense* blooms in Golfo Nuevo (Gayoso 2001). Cultured cells in the light experiments showed that the mean growth rate of the species increases as light increases up to saturation level at 150 μmol $m^{-2}s^{-1}$. Because diatoms and dinoflagellates respond differently to light intensity (Langdom 1987), *A. tamarense* could be favored during periods of high sunlight and no cloudy days over the diatom assemblage characteristic of the spring bloom in Golfo Nuevo. Salinity also affects growth of *A. tamarense* (Prakash 1967, White 1978, Parkhill and Cembella 1999). The Golfo Nuevo clone grew well at salinities ranging from 20 to 35 psu. Higher growth rates were found between 30–35 psu, show-

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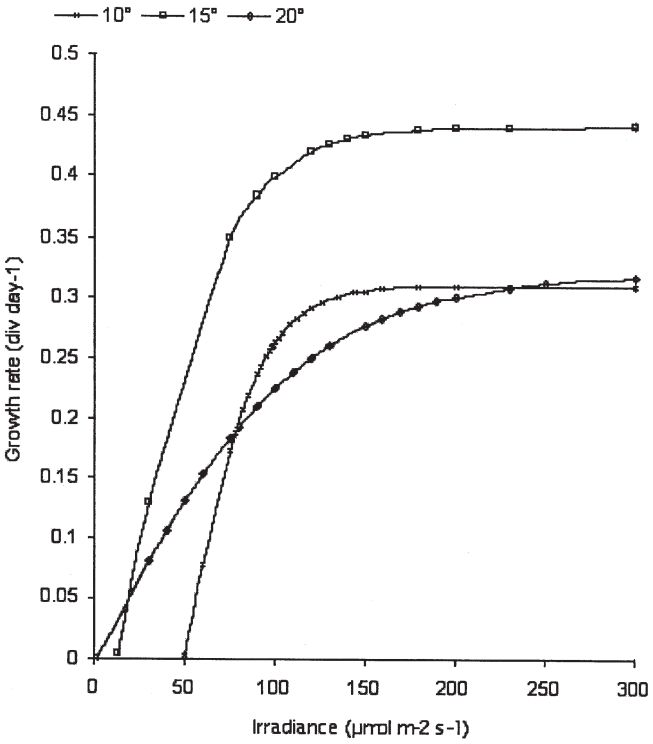

Figure 1 Growth rate of *Alexandrium tamarense* from Golfo Nuevo as a function of photon flux densities at different temperatures.

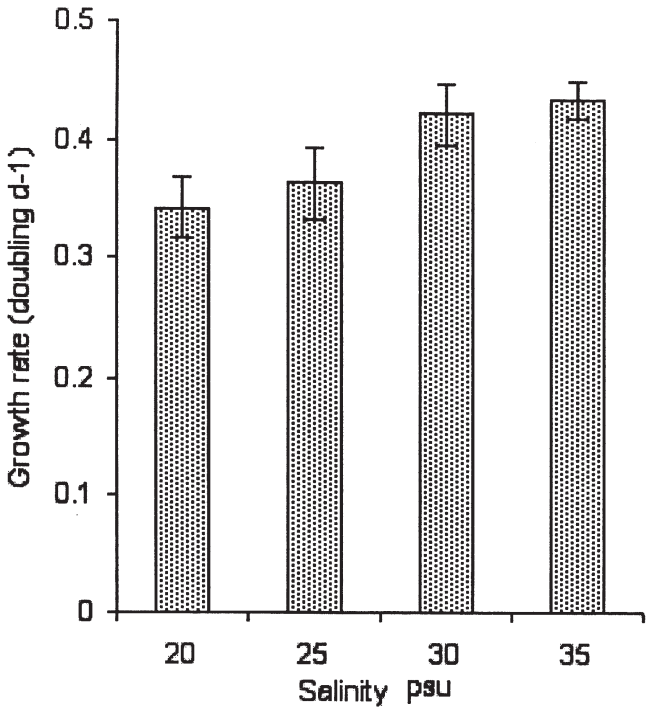

Figure 2 Growth rate of *Alexandrium tamarense* from Golfo Nuevo in relation to salinity.

ing a good adaptation to the high salt content characteristic of the gulf (Rivas and Beier 1990). Salinity has been mentioned as an important biogeographical determinat for *A. tamarense* blooms (Parkhill and Cembella 1999). Most of the *A. tamarense* blooms and shellfish toxicity have been registered from 34°S to 47°S in the Argentine Sea (Carreto *et al.,* 1993). This broad area includes Nuevo, San José and San Matías gulfs, all semienclosed basins with little exchange with the open shelf waters (Rivas and Beier 1990). In a time series over the 1980–1995 period, the northern area of San Matías Gulf (at 41°S) appeared free of toxin with the exception of the 1990 extraordinary outbreak of toxicity when the area was affected (El Busto *et al.,* 1993). The hydrography of the northern San Matías Gulf is characterized by high salinities (close to 35 psu) (Rivas and Beier, 1990). The clone AT-3D isolated from Patagonia is well adapted, however, to high salinities; other factors (*e.g.,* no retention of resting cysts) could probably explain the fact that toxic blooms of the species are not a recurrent phenomenon in the northern San Matías Gulf.

Acknowledgements

This study was supported by funds from ANPCyT, Argentina, PICT/98 0103709.

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Effects of Ultraviolet Radiation on the Toxin Composition of the Dinoflagellate *Alexandrium catenella*

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Abstract

For 12 days, cultures of *Alexandrium catenella* were exposed to two irradiance levels and three different spectral compositions: 1) PAR+UVT, 2) PAR+UVA and 3) PAR. In all conditions after two days of growth, there was a lag period for cell division and toxin production. In the cultures exposed to PAR and PAR+UVA, following the lag phase there was a recovery in the rates of growth and toxin production to levels similar to those initially found. In contrast, long term exposure of cultures to UVB radiation not only blocked toxin production but also arrested growth and cell division. At the highest UVB irradiation, toxins were also catabolized or leaked from the cell. With the exception of interconversion between epimeric pairs (C2: C1, GTX3: GTX2 and GTX4: GTX1), the relative proportions of the main toxins remained essentially unchanged throughout the experiments.

Introduction

Recent data indicate that the springtime decrease in the ozone layer is significant in the southern region of South America. Associated with ozone depletion, there is an increase in the levels of biological harmful ultraviolet-B radiation (UVB) reaching the earth's surface. This situation has promoted some controversy about the ecological effects of this atmospheric phenomenon on southern ecosystems. Although some events of *A. catenella* blooms in the southern region of Argentine and Chile have coincided with high UVB radiation episodes, causal relationships have not been found among these processes (Benavides *et al.,* 1995). These observations and the exceptionally high cell toxicity associated with these blooms of *A. catenella* have induced us to study for the first time the effect of UV radiation on toxicity and toxin composition in a low toxicity clone of the *A. catenella* isolated from the XI Region of Chile.

Materials and Methods.

An *A. catenella* (Weedon and Kofoid) Balech clone CC08 isolated from the XI Region of Chile (IFOP, Chile) was used in our study. Cultures were maintained at 12°C in L1 medium without silicon addition. Before experiments, cultures were allowed to adapt for several days at 100 µmol quanta m^{-2} s^{-1} PAR and 182 µW cm^{-2} UVA irradiance on a 12:12 L:D cycle. Aliquots of the adapted cultures were exposed over 12 days to two higher irradiance levels (Level A: 200 µmol quanta m^{-2} s^{-1} PAR + 363 µW cm^{-2} UVA + 21 µW cm^{-2} UVB and Level B: 800 µmol quanta m^{-2} s^{-1} PAR + 1452 µW cm^{-2} UVA + 86 µW cm^{-2} UVB). At each of these intensities of irradiance, three different spectral compositions were studied: 1) PAR + UVT (280–700 nm), 2) PAR + UVA (320–700 nm), and 3) PAR (400–700 nm). Light was provided by a SS 1000 W solar. To remove the UVB and UVT radiation, the vessels were covered with Mylar or HeyDi foils, respectively. PAR scalar irradiance was measured with a QSL-100 quantum sensor and UVA and UVB radiation with an IL 1400 A radiometer. Subsamples for cell counts and toxin concentrations were always taken at the same time. Subsamples were fixed in Lugol's iodine solution and cell concentrations were determined by counting in a Sedgwick-Rafter counting chamber. Subsamples for toxin analysis were concentrated on Whatman GF/F filters and maintained in liquid nitrogen. Toxins were analysed by the HPLC method of Oshima *et al.* (1995). Results obtained at the different light treatments were compared with the "*u* test" for a linear hypothesis when linear regression was used (Fombay *et al.,* 1984)

Results and Discussion

Toxin composition of this *A. catenella* strain has been reported previously (Carreto *et al.,* 2001). In the culture acclimated to 100 µmol quanta m^{-2} s^{-1} PAR + 182 µW cm^{-2} UVA irradiance, the low potency N-sulfocarbamoyl toxins (C2 + C1) were the largest components (83.9 %). In decreasing order, there were GTX5 and the high-toxicity carbamate derivatives GTX3 + GTX2. NeoSTX, GTX4, GTX1, STX and dc STX were also present in minor amounts.

Among the six different experimental conditions, the net toxin production in the cultures varied over the growth cycle of the cultures. A significant increase in toxin concentrations began after the first day of acclimation to the new experimental conditions and continued up to day 2, after which there was a period of toxin synthesis inhibition evidenced by a flat or decreasing slope in all treatments (Fig. 1). The strength and persistency of toxin synthesis inhibition was strongly dependent on the intensity and spectral light composition to which the cells were exposed. Inhibition of toxin production was higher at the higher light intensities ($u_{par\ 200\ vs\ par\ 800}$ = 16.4; P = 0.01; $u_{par+uvt\ 200\ vs\ par+uvt\ 800}$ = 385.9; P = 0.0003) and was most severe in the cultures exposed to PAR+UVT ($u_{par\ 800\ vs\ par+uvt\ 800}$ = 357.8; P = 0.00005). In the cultures exposed to PAR and PAR+UVA, following the lag phase there was a recovery in the toxin production rate to levels similar or higher to those initially found.

Variations in the toxin cell content are more conservative (40–80 fmol $cell^{-1}$) because changes for both toxin production and growth rate were similar in all tested con-

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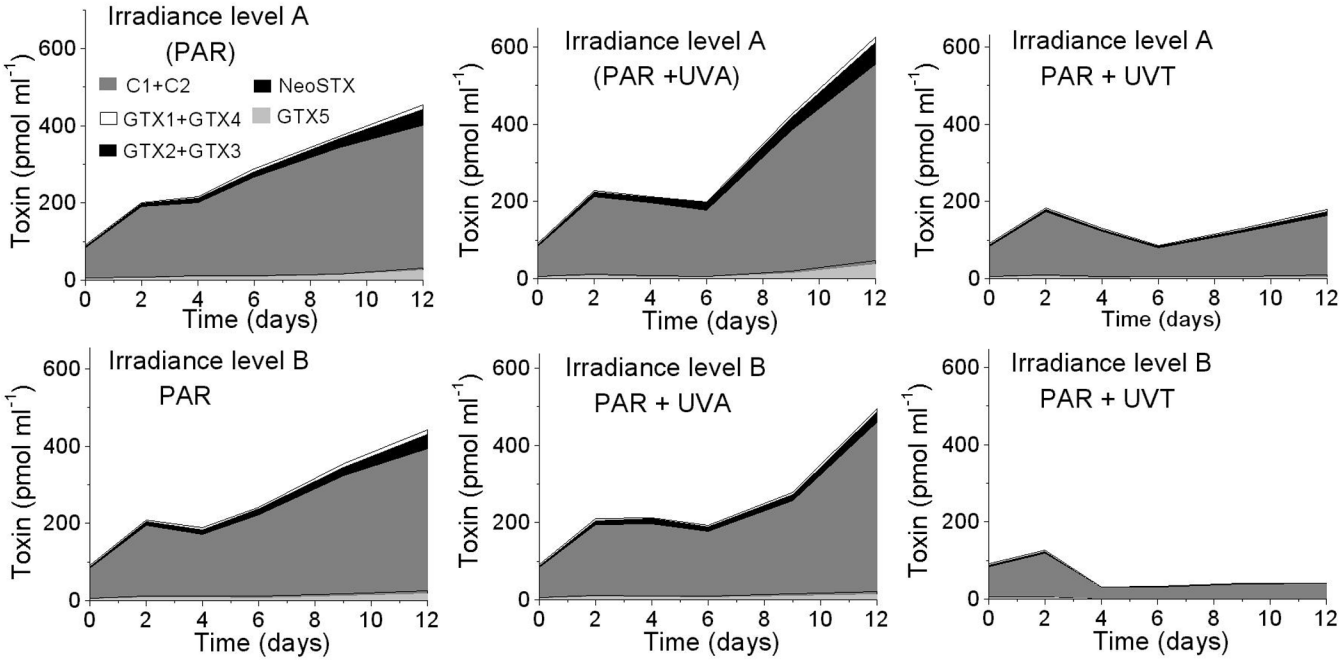

Figure 1 Time variation of the specific toxin concentration (pmol mL^{-1}) in *A. catenella* at different light irradiances and spectral compositions.

ditions, indicating that long-term exposure to UVB radiation completely inhibited toxin production and arrested cell division and caused cell death. A linear relationship between *A. catenella* cell concentration, under varying light conditions, and total toxin concentration in the cultures was observed (r = 0.93) (Fig. 2).

With the exception of interconversion between epimeric pairs (C2:C1, GTX3:GTX2 and GTX4:GTX1), the relative proportions (mole percent of total toxin) of the main toxins remained essentially unchanged throughout the experiments. Epimerization of the major component C2, and of the GTX3 and GTX4 to the more thermodynamically stable α-epimers, C1, GTX2 and GTX1, respectively, occurred over the course of the experiment in all tested light conditions (Fig. 3). The observed changes in the β:α epimer ratios over the time appear to be directly related with the evolution of the toxin production rate. The more stable a-epimers were accumulated during the period of toxin synthesis inhibition. Inversely, after the recovery of the toxin production rate later in the experiment, an increase in the β:α epimeric ratio was observed. As would be expected, the timing and extension of epimerization showed a strong dependence on the intensity and spectral light composition. Inclusion of UVA and UVB enhanced the α-epimer production, especially at the higher UVB irradiances. The underlying assumption was that interconversion among the toxins would be determined in part by the physiological state of the cells. However, in the cells exposed to UVB, the recovery of the initial β:α epimeric ratio at the end of the experiment indicated that other complex mechanisms

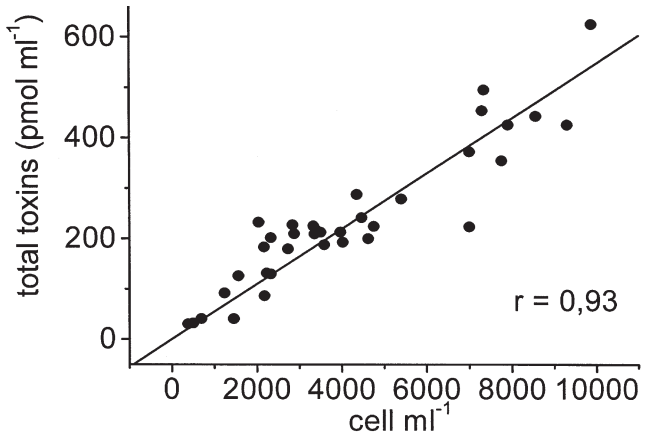

Figure 2 Total toxin concentration (pmol mL^{-1})/cell number (cell mL^{-1}) relationship for *A. catenella.*

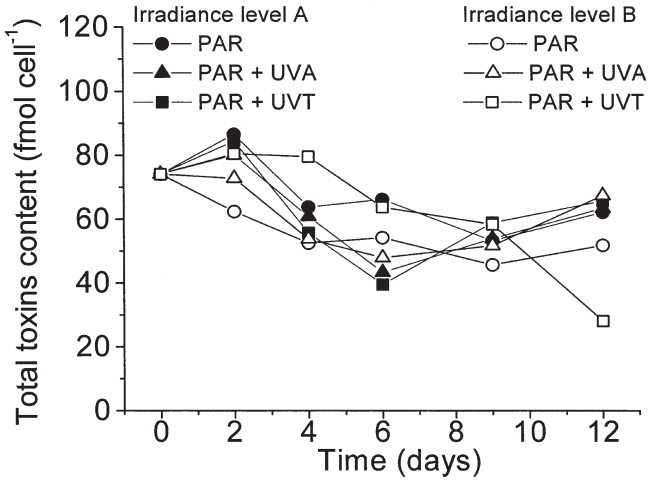

Figure 3 Time course of the cell toxicity (pg STXeq $cell^{-1}$) in *A. catenella.*

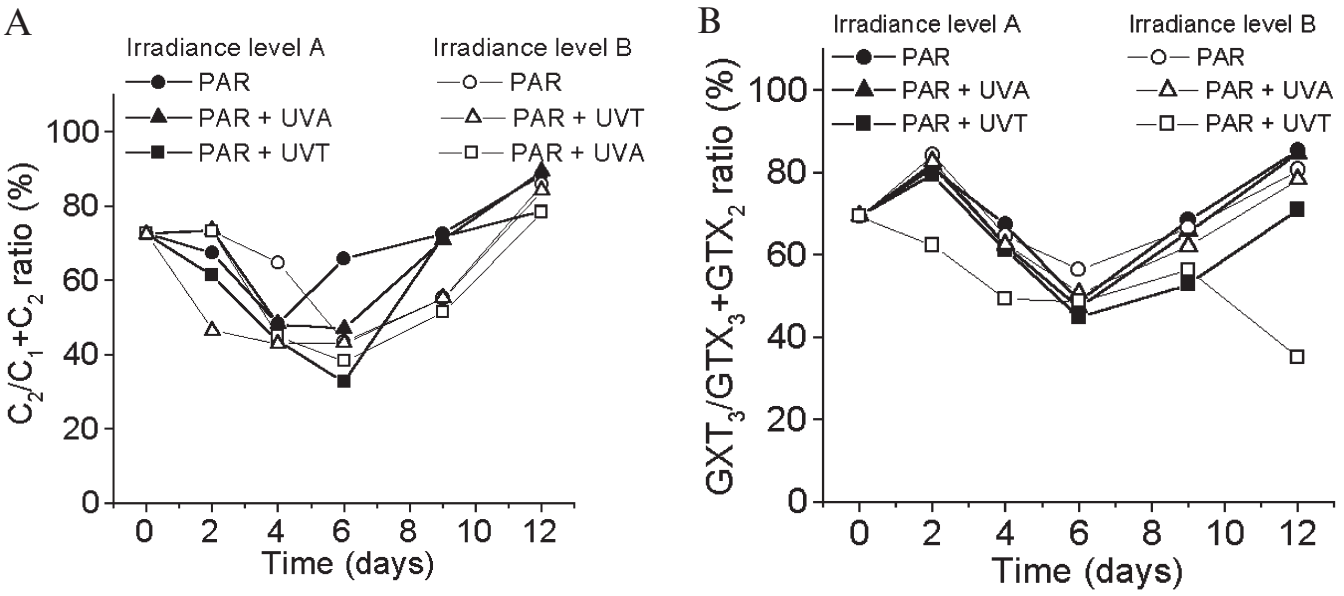

Figure 4 Relative abundance (%) during the course of the experiment for the β-epimers of **A** N-sulfocarbamoil toxins C2+C1 and **B** gonyautoxins GTX3+GTX2.

were operative. The cellular toxicity among the different light treatments varied over the culture cycle (3.0–6.0 pg STXeq $cell^{-1}$) following changes in toxin production and toxin composition (Fig. 4). Generally, toxicity was higher in the early stage of growth, decreasing thereafter as the cells enter the lag phase when the toxin cell content was lower and less toxic epimers C1 and GTX2 predominated. The subsequent increase in toxicity after the lag phase was also coincident with the recovery of toxin production and the increase in the β:α epimeric ratio.

Several studies indicate that a major effect of prolonged exposures to UVB on marine organisms is mediated by direct damage to DNA (Buma *et al.,* 2000). DNA damage causes inhibition of DNA replication, arresting the cell cycle at the end of the G1 phase. Toxin synthesis requires DNA transcription, is induced by light, and occurs during a defined time frame within the G1 phase of the cell cycle (Taroncher-Odelung *et al.,* 1999). On this basis, we can speculate that in *A. catenella* exposed to prolonged periods of high UVB levels, the DNA damage causes inhibition of both processes: DNA replication and DNA transcription. At the highest UVB exposures (BED DNA 305 nm = 2.78 KJ m^{-2} d^{-1}), the production of toxins was not only totally blocked, but toxins were also catabolized or leaked from the cell. However, a significant toxin production and cellular division rate was observed in the first two days of acclimation to UVB radiation, indicating that inhibition of toxin synthesis by UVB radiation was a dose-dependent process. The daily biological effective dose weighted by the DNA action spectrum for the higher exposure (BED DNA 305 nm = 2.78 KJ m^{-2} d^{-1}) was higher than those found in surface temperate waters (BED DNA 305 nm = 1.0 KJ m^{-2} d^{-1}). In addition, our results have described the *A. catenella* response to a sharp environmental shift, assuming that vertical mixing is reduced to the upper few centimeters, yielding a high light surface layer during the 12 days of the experiment. This environmental situation was unrealistic and also incompatible with the migratory behavior of *Alexandrium* species. Buma et al (2000) showed that low UVB doses (up to $BED_{DNA\ 300\ nm}$ = 0.4 KJ m^{-2} d^{-1}) arrest the cell cycle in *Emiliania huxleyi* at the end of the G1 phase, but synthesis of pigments and other cell components, as well as chloroplast division, still seem to take place. It is unknown if this mechanism can take place for cell toxin accumulations in natural populations of *A. catenella.*

Acknowledgements

We thank Lic. A. Aubone for their assistance in statistical treatment of the data and 2 anonymous reviewers who provided helpful comments and suggestions. INIDEP contribution N° 1269.

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Toxic *Phaeocystis globosa* Strains from China Grow at Remarkably High Temperatures

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Abstract

Toxic blooms of *Phaeocystis globosa* occurred in China in 1997 and 1999 at remarkably high sea surface temperatures. Two isolates originating from Hong Kong and Shantou were examined for their actual growth performance at high temperatures. The results exclude the possibility that hydrodynamic events concentrated colonies that had been growing in cold open water into warm coastal waters. High temperature requirements, very large colonies and ichthyotoxicity make the Chinese strains special members of the *Phaeocystis globosa* species cluster.

Introduction

Although anecdotal information claims that *Phaeocystis* colonies have been observed yearly in autumn by fishermen around the Nanao Islands (Huang *et al.,* 1999), the first real harmful *Phaeocystis* blooms in China Sea were recorded in 1997 (Fig. 1) in the nearby Zhelin Bay, Guangdong, at a period when southeast coast winds instead of northwest winds were blowing, resulting in warm water conditions (17°–20°C), unusually high for that time of the year (November–December). Three years before, *Phaeocystis* was observed in the Zhelin Bay, but not in densities that caused harmful effects. In 1997, however, caged fish having bloody gills died within a day. Haemolytic compounds identified as glycolipids were extracted from filtered cells with 1'-O-heptadecadienoyl-3'-O-(6-O-α-D-galactopyranosyl-β-D-galactopyranosyl)-glycerol as the major constituent (He *et al.,* 1999).

Also in 1999, farmed fish suffered from *Phaeocystis* blooms along the China coast, with ambient temperatures up to 30°C during the August bloom. (Qi, personal communication, Chen *et al.,* 2002). Sequence data from a strain isolated near Shantou from the Zhelin Bay clustered with the *Phaeocystis globosa* species complex that comprises both temperate and warm water strains (Chen *et al.,* 2002, Lange *et al.,* 2002). A special feature of the Chinese strains isolated from Shantou and Hong Kong is their occurrence at high ambient temperatures and the relatively big size of the colonies, a combination reported earlier for *Phaeocystis* sp. from the Arabian Gulf only (Al-Hasan *et al.,* 1990).

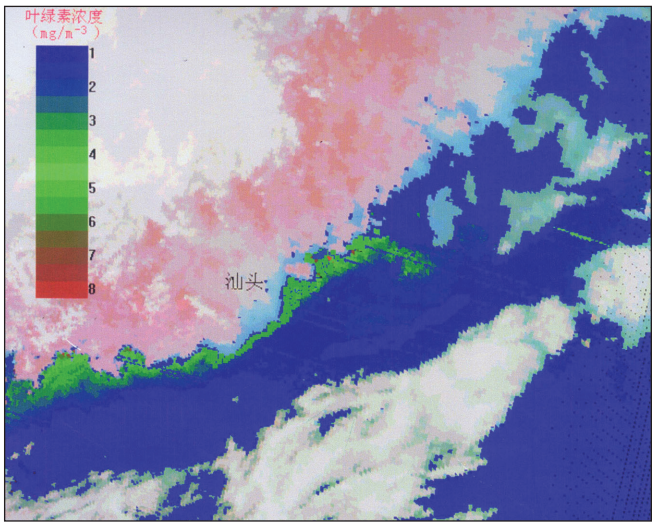

Figure 1 Satellite image of the coastal *Phaeocystis* bloom in the Chinese Sea, 1997.

The occurrence of species at a certain sea surface temperature does not guarantee that it is active at that temperature. Therefore, we studied two Chinese stains for their growth performance at high temperatures.

Materials and Methods

Two unialgal cultures, *Phaeocystis globosa* strain HK and strain ST (red tide algal culture collections of Jinan University), were isolated from Jiangjunao seawaters, Hong Kong and Raoping seawaters, Shantou, respectively. Routinely, cultures were kept in seawater enriched with f/2 nutrients (Guillard, 1975) at 20°C under 12h light:12h dark cycle at 120 µmol photons · m^{-2} · s^{-1}. Growth was monitored using the optical density measured at 680 nm (OD_{680}) after homogenizing the subsamples with a clean syringe. OD_{680} correlated with the amount of cells counted by microscopy using a blood counting slide.

The experiments for each strain in triplicate were initiated by transferring 1.0 mL culture algae to 80 mL of fresh f/2 medium (in 150 mL bottle) and were kept at different temperatures (20°, 25°, 30°C). Every 24 hours, 3.0 mL samples were withdrawn from each treatment to measure the OD_{680}. The maximal growth rate was calculated from the exponential.

Results and Discussion

For the first time, laboratory growth experiments confirm that the *Phaeocystis* species in Chinese waters do not only occur at high temperatures but that they are growing at these temperatures as well (Fig. 2). The Hong Kong (HK) strain has an optimal temperature of 25°C, while the Shantou strain (ST) is not even at its optimum at 30°C. Jaqueline Stefels (pers. comm.) also observed that the ST strain could barely survive at 16°C and still grew well at 32°C. The maximal specific growth rates measured were 0.38 d^{-1} and

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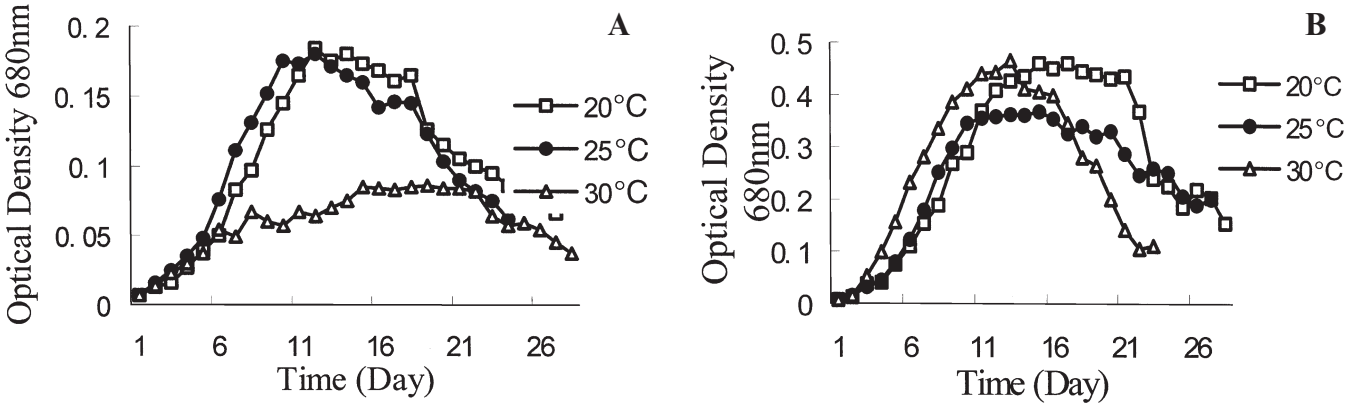

Figure 2 Development of *Phaeocystis* biomass in batch cultures grown at different temperatures. **A** strain Hong Kong, **B** strain Shantou.

0.48 d^{-1} for the HK and ST strains, respectively.

Taxonomic criteria to distinguish colony-forming *Phaeocystis* involve the size and morphology of the colonies (palmeloid stage), as well as temperature range for growth (Baumann *et al.,* 1994). Our observations, both in laboratory and the field, showed that there were two different morphological forms: free-living cells (motile and non-motile) and colonies. The free-living cells were spherical, about 6–9 µm in diameter with two flagellae visible by light microscopy. The round colonies were different in size; some had to be observed through the microscope while some could be seen by the naked eye (Fig. 3). The colonies of the Shantou strain, which had a diameter up to a maximum of 3.0 cm or even bigger in field (Fig. 3), were much larger than those of Hong Kong strain (max 1.0 cm in diameter). As to both strains, the free-living cells reproduced by repeated bipartition, and these cells aggregated when forming a colony. In these colonies, cell division and subsequent enlargement resulted in growth. When the nutrition in the culture was depleted, the colony integrity was lost and free-living cells were released.

All Chinese strains, including ours (He *et al.,* 1999), had their cells evenly distributed over their spherical colonies,

Figure 3 Huge *Phaeocystis* colonies were observed during the 1999 bloom near Shantou.

a characteristic for *P. globosa* and *P. antarctica* (Baumann *et al.,* 1994). In addition, *P. globosa* should have bigger colonies (8–9 mm versus 2 mm) than that of *P. pouchetii* and higher temperature requirements (4°–22°C) compared to the range –2°–14°C for both *P. pouchetii* and *P. antarctica.* Both our strains more than meet the requirements to distinguish them from *P. pouchetii* and *P. antarctica* but set a record for *P. globosa* as well. Another characteristic feature of the Chinese strains is their toxicity towards fish (He *et al.,* 1999, Huang *et al.,* 1999). Although negative effects on fish (larvae) have been reported in European waters for *P. pouchetii* (Stabell *et al.,* 1999), the toxic principle was anaesthetic rather than the haemolytic effect displayed in the toxic Chinese blooms.

Molecular studies on the Chinese strains isolated in 1997 have shown that the 18S genes have remarkable similarity with *P. globosa* from the German Bight and Mexico (Chen *et al.,* 2002), suggesting that these strains belong to the *Phaeocystis globosa* species complex (Lange *et al.,* 2002, Vaulot *et al.,* 1994). Sequence data of 18S rDNA obtained by Wang (2000) in our project proved that the 1999 isolates were similar to other *P. globosa* sequences as well.

Nevertheless, colony size, high temperature requirements, and hemolytic properties are different for the Chinese strains compared to characteristics described for *P. globosa* so far. These characteristics should be taken into account when trying to understand their role in the Chinese coastal ecosystem.

One of the most intriguing questions right now is why there seems to be a recent increase in blooms recorded along the East and South China Sea. Blooms occurred from the Quanzhou Bay, Xiamen (East China Sea), to Shantou, Guangdong, into the Hong Kong area (South China Sea), and even in the tropical Hainan coastal waters. Since the ancestors of modern *Phaeocystis* were probably cosmopolitan warm water species (Medlin *et al.,* 1994), it seems reasonable to assume that warm water *Phaeocystis* might have been in the Chinese area already for a long time. It is tempting to speculate that the increase in blooms is a result of an altered climate more favorable for endemic

Phaeocystis species. Alternatively, recent blooms could be induced by antropogenic nutrient enrichments similar to the increase in bloom formation in the continental zones of the North Sea in the eighties (Lancelot et al). Physiological studies to support either hypothesis are currently underway.

Acknowledgements

This work was supported by the National Science Foundation of China. We are grateful to many other team members for their help in preparing the manuscript.

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Continuous-Flow and Batch Cultures of Toxic *Alexandrium catenella* and *A. minutum* in Stirred Tank Bioreactors

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Abstract

Extrinsic environmental factors are known to exert profound effects on the general physiological status of toxicogenic microalgae. In order to study this relation and minimize natural variability, toxic *Alexandrium* strains were cultured in 2.3-L stirred-tank bioreactors equipped with various probes to measure light absorption, pH, temperature and gas flow. Specific supervisor software managed the calibration of these probes and the on-line recording of culture parameters. Chemostat cultivation of *Alexandrium minutum* originating from the N. Atlantic and *Alexandrium catenella* from the Thau Lagoon (W. Mediterranean coast) produced cultures with a relatively high division rate (0.5 d^{-1}) and maximum cell concentrations of 80,000 and 60,000 cells/mL, respectively. Growth kinetics showed considerable variations relative to available nitrogen and phosphorus sources. Analysis of cell extracts by liquid chromatography/fluorescence detection (LC/FD) gave remarkably consistent toxin profiles, but the cell toxin quota exhibited substantial variations in relation to the physiological stage of the culture. As continuous-flow cultures could be maintained for several months in our experimental system, it was possible to investigate the capacity of these organisms to adapt to exogenous parameters such as salinity, temperature, nitrogen and phosphorus. The combination of all probes (light absorption, pH, temperature and gas flow) and the real-time monitoring and storage of data provided a historical record of each culture set, which was of considerable value for understanding growth kinetics and toxicogenesis.

Introduction

The potential of some microalgal species to produce toxins is probably genetically determined, but both the phytoplankton toxin content and composition are influenced by environmental growth conditions such as light, nutrient levels and temperature (Plumley, 1997). Reviews of autecological studies on the relationship between cellular toxicity and environmental factors indicate that the relationship between growth rate and the cell toxin quota and ambient nutrient levels is complex. Moreover, this relationship is not entirely consistent among (or even within) species (Parkhill and Cembella, 1999). To avoid the influence of variations due to growth rate, a continuous-flow culture system was developed in stirred-tank bioreactors to test two native microalgal species responsible for paralytic poisoning (PSP) events on French coasts. *Alexandrium minutum* produces recurrent summer blooms in embayments of the Brittany coast (North Atlantic) during the period of maximum sea surface temperature (16°–20°C) and relatively low salinity, whereas *Alexandrium catenella* blooms in the Thau Lagoon (W. Mediterranean coast) occur during November and December at relatively high salinity (39–40‰). A*lexandrium minutum* (originating from the North Atlantic) and *Alexandrium catenella* (from the W. Mediterranean coast) were cultured in modified K and F/2 seawater media within 2.3-L stirred-tank bioreactors equipped with various probes to measure light absorption, pH, temperature and gas flow. Specific software managed probe calibration, ensured pH

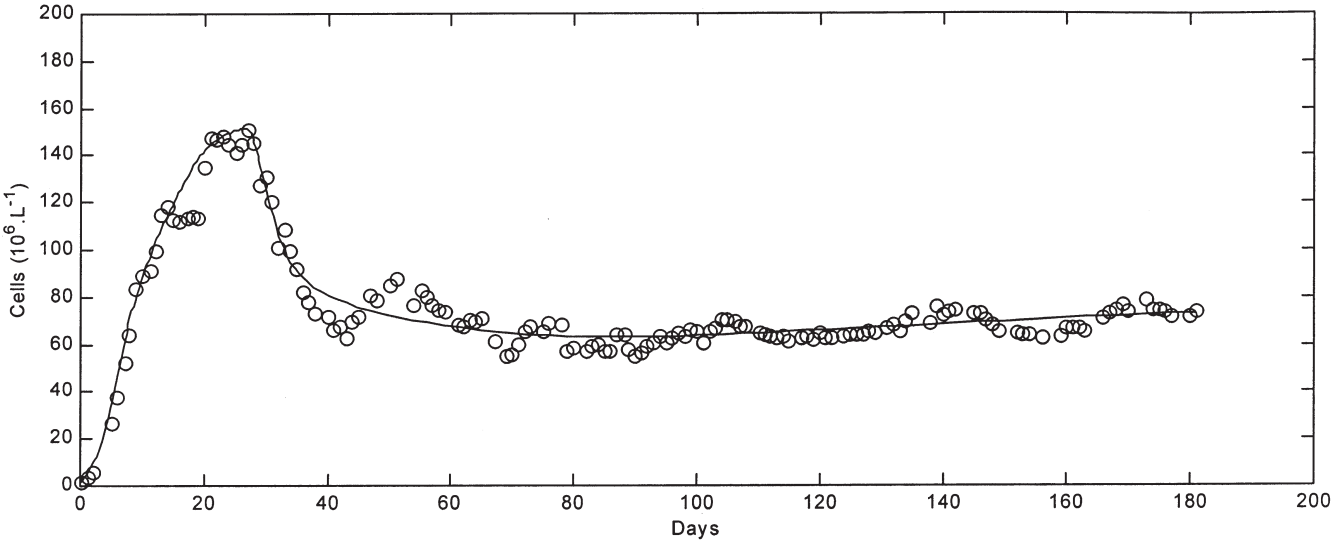

Figure 1 *Alexandrium minutum.* Continuous culture experiment.

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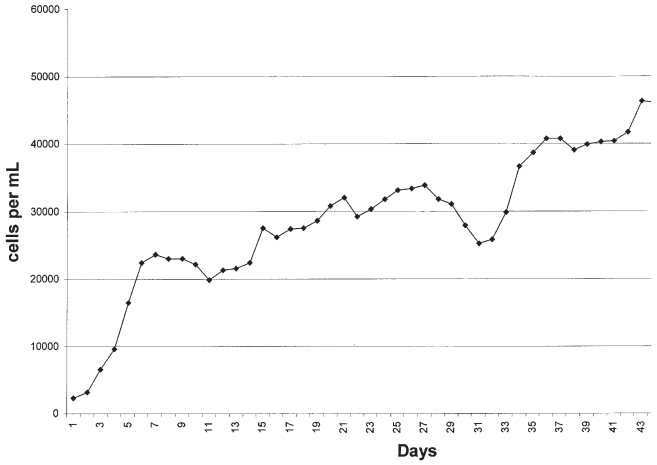

Figure 2 *Alexandrium catenella.* Continuous culture experiment.

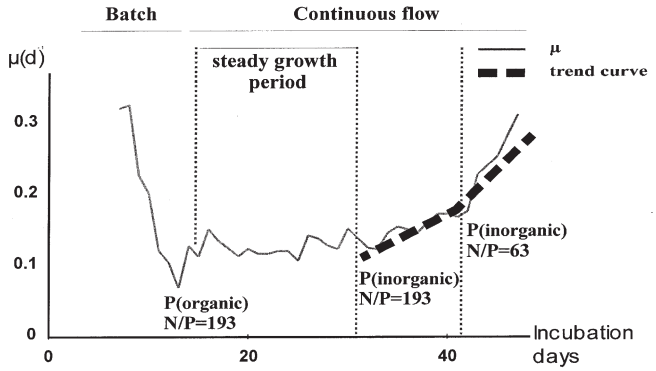

Figure 3 *Alexandrium minutum.* Changes in cell-specific growth rate versus available N and P.

stability, and provided on-line recording of culture parameters. This study focused on the growth and toxicity kinetics of the microalgae in batch or continuous-flow cultures.

Materials and Methods

The toxic strain of *Alexandrium minutum* (AMBM89) originated from North Brittany, and the *Alexandrium catenella* strain (ATTL01) was obtained from the Thau Lagoon (Lilly *et al.,* 2002) during a 1998 bloom. Both were cultured in modified K or F/2 medium (Keller *et al.,* 1987) in 2.3-L stirred-tank polymethyl methacrylate bioreactors. Cultures were stirred by a 4-paddle impeller turbine at a rate of 80 rpm. The upper headplate of the bioreactor was designed to support all probes and tubing. The probes were connected to a computer data-acquisition system equipped with a microprocessor interface board with 32 analogue channels. Each bioreactor used 4 channels to collect data on light absorption, temperature, pH and gas flow. A probe based on the Beer-Lambert law was designed to measure light absorption of microalgal cells and provide on-line estimation of biomass after standardization with a Coulter-counter multisizer® and counts by inverted microscopy according to the Utermöhl method. An on-line pH-meter transmitted measurements to a central computer that controlled pH value by injecting CO_2 into the bioreactors. The entire system was controlled by a computer equipped with specific software developed in Visual Basic, which managed probe calibration and on-line recording of culture parameters. The reactors were placed in a heat-regulated culture facility, with light supplied on a 16 h/8 h light/dark cycle. The system was adapted to continuous-flow culture by adding a diaphragm pump to supply nutrients and a peristaltic pump to collect overflow fractions (Bougaran, 1997). Analysis of the main PSP toxins was performed using the post-column oxidative fluorescence method of Oshima (1995). Toxic compounds were separated by reversed-phase chromatography using a C8-silica column (5 μm Develosil, i.d. 4.6 × 250 mm) with a flow rate of 0.8 mL/min. Some derivatives of gonyautoxins (GTX) B and C were assayed after acid hydrolysis (HCl 0.4 N, 97°C, 5 min), as indicated by Franco and Fernandez-Vila (1993). Ion chromatography with chemical suppression preceding conductance detection (Dionex 120) was applied to measure anion and cation concentrations in the culture media. Separation columns for this procedure included Dionex IonPac AS-9Hc® for anion determinations and Dionex IonPac CS 12® for cation separations.

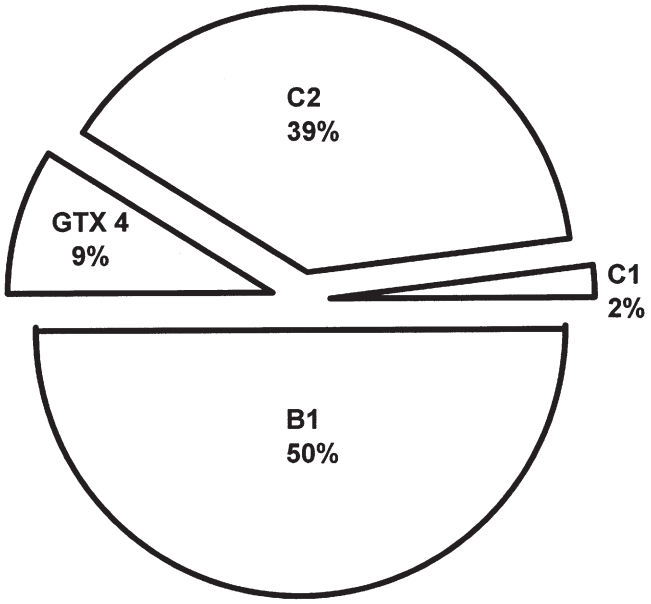

Figure 4 Mole percentage of toxin composition in culture extract.

Results and Discussion

Growth Chemostat cultivation of *Alexandrium minutum* and *Alexandrium catenella* produced cultures with a high division rate (0.5 d^{-1}) and a maximum cell concentration of 80 and 60 million cells per liter, respectively, in the batch experiment. The system was then adapted to continuous-flow cultures to mitigate the effects of unbalanced growth in batch cultures. Sustained steady-state cultures were main-

tained for more than six months at a high density of 70–80 millions cells of *A. minutum* per liter (Fig. 1). *Alexandrium minutum* cells proved less fragile in this type of culture than *A. catenella* cells, which required more than a month to reach steady state (Fig. 2). Large variations were observed relative to ratios of available nitrogen and phosphorus (Fig. 3).

Toxin content In the batch experiment, all *Alexandrium minutum* and *Alexandrium catenella* cultures showed a characteristic convex pattern of toxin content versus time, with a peak in mid-exponential growth and a decline as cells entered steady state. In continuous-flow cultures, cell toxin content decreased after cells reached steady state. Cell toxin dynamics were complex, but the molar ratio of the various PSP derivatives remained stable. Both strains lacked saxitoxin (STX). Extracts of *A. catenella* ranged from 5 to 15 pg STX equivalent/cell, with N-sulfocarmabamoyl toxins (B1, C1 and C2) constituting a major proportion (up to 90%) of the molar fraction (Fig. 4), whereas highly toxic carbamate toxins (GTX4) represented only 10–12%. For *Alexandrium minutum,* only GTX 3 and GTX2 were detected. The lower toxin content values obtained in continuous-flow cultures may reflect either a balanced growth, in which the toxin biosynthetic complex and the necessary precursors were in equilibrium and in competing pathways for cell division, or a greater, progressive release of toxins into the medium.

Future investigations will study the multidimensional relationships among light, salinity, nutrient concentrations, and toxin production and excretion in continuous-flow cultures of *Alexandrium* species. This new continuous-flow bioreactor system will allow the standardization and real-time monitoring of various experimental factors and the determination of their influence on the toxin production process for a population in steady state.

Acknowledgements

This work was supported by EU Contract QLRT-1999-30778.

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Does Urea-Carbon Contribute Significantly to *Aureococcus anophagefferens* Carbon Nutrition?

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Abstract

It has been suggested that the utilization of dissolved organic matter (DOM) by *Aureococcus anophagefferens* subsidizes a strict autotrophic nutritional mode during periods of intense brown tide blooms. Of the diverse array of chemical compounds in DOM, urea is arguably the most studied and well-known, and therefore has received significant attention as a potential nutritional supplement. Although rates of urea-C uptake were measurable, relative to carbon fixation by photosynthesis, the uptake of urea-C contributed only ~1% in short-term physiological measurements at light levels >200 µmol photons m^{-2} s^{-1}. At very low levels of incident irradiance, urea-C uptake could be as high as 40% of photosynthetic carbon fixation. Contrary to what would be expected if urea-carbon were serving as a nutritional supplement, urea-carbon uptake is negatively related to *A. anophagefferens* densities in mesocosm and field populations.

Introduction:

Since 1985, blooms of the pelagophyte algae *Aureococcus anophagefferens* have sporadically plagued a number of shallow, turbid bays along the U.S. east coast. In the coastal bays of New York's Long Island, a once-prosperous scallop fishery as well as the seagrass nursery habitats for a variety of other species have been devastated (Cosper *et al.,* 1987). Perhaps not unexpectedly, both top-down and bottom-up controls have been proposed over the past 17 years as crucial links during the bloom initiation phase, but this paper will focus on bottom-up controls. Bottom-up controls have centered on the enhanced utilization of dissolved organic nutrients by *A. anophagefferens* (Berg *et al.,* 1997, LaRoche *et al.,* 1997, Gobler and Sanudo-Wilhelmy 2001, Lomas *et al.,* 2001). In particular, dissolved organic nitrogen (DON) has received special attention, given the generally low levels of dissolved inorganic nitrogen available during brown tide blooms in Long Island embayments. LaRoche *et al.* (1997) suggested that differences in rainfall might lead to differences in the amount of nitrate input to these systems, resulting in the increased importance of DON compounds that then favored the growth of *A. anophagefferens.* This "Rainwater" hypothesis, however, has not held for more recent years or other Long Island embayments (Lomas unpubl. data). However, field data still suggests that brown tides are correlated with periods of organic nutrient enrichment (Gobler and Sanudo-Wilhelmy 2001, Lomas *et al.,* 2001, Mulholland *et al.,* 2002), although it is no longer restricted to just DON; organic carbon (DOC) and phosphorus (DOP) are increasingly recognized as important. This correlative field data is supported by a number of physiological studies in culture and in near mono-specific blooms in the field that show very high rates of DON and DOP uptake (*e.g.,* Dzurica *et al.,* 1989) and enhanced growth rates when presented with organic nitrogen sources (Berg *et al.,* 1997; Gobler and Sanudo-Wilhelmy 2001). This is consistent with the recent findings of Kana and colleagues (2002), where even in the presence of a complete grazer community, selective growth of the brown tide could be achieved in a variety of organic nutrient treatments in mesocosms. This study examines the use of carbon from urea, a simple organic molecule where the carbon atom is readily available for assimilation following hydrolysis. These studies were conducted in near mono-specific blooms in the field, mesocosms with mixed assemblages and in *A. anophagefferens* cultures.

Materials and Methods

Field samples were collected in 1999 and 2000 from Quantuck Bay, Long Island, which has a mean depth of ~3m. Using an acid cleaned 10-L bucket, surface samples were collected, stored in a cooler at ambient temperature, and transferred to Southampton College Marine Science Station, where isotopic manipulations (see below) were initiated within 1.5 h. In June 2001, mesocosm (~200 L) manipulation experiments were conducted as part of a larger brown tide research program. Five experimental treatments were designed to test the hypothesis that organic enrichment would favor the growth of *Aureococcus anophagefferens,* even in the presence of an active grazer community. Treatments were a control, a nitrate addition, a urea + glucose addition, a macroalgae addition and an intact sediment core addition, and all were conducted in triplicate. Surface samples for urea-C utilization were collected on days 1, 5 and 9 of the 10-day experiment from each mesocosm. Lastly, utilization of urea-C was investigated using exponentially growing *A. anophagefferens* culture isolate CCMP1794 grown at 50 µmol photons m^{-2} s^{-1} on urea- and nitrate-based f/20 media. These cultures were not axenic, but bacterial numbers were low (<10^4 cells mL^{-1}) during the exponential phase when the cultures were harvested.

From all field and mesocosm samples, 15 mL subsamples were fixed in 1% glutaraldehyde (final concentration), stored at 4°C and *A. anophagefferens* counts conducted within 2 months using the polyclonal anti-body labeling protocol (Anderson *et al.,* 1993). Cell densities in culture were determined under light microscopy using a haemocytometer. Urea concentrations in all cases were determined on filtered water using the urease method of McCarthy (1970) followed by colorimetric determination of ammonium.

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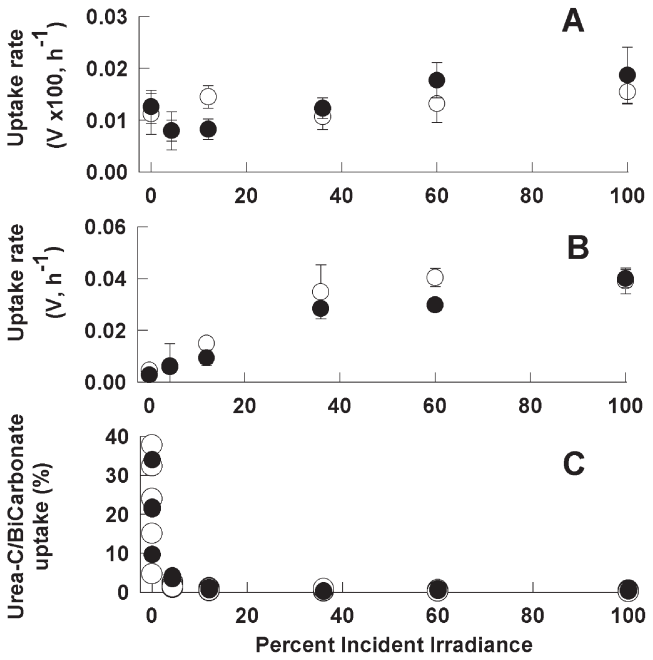

Figure 1 Mean values for ^{13}C-urea uptake **A**, ^{13}C-bicarbonate uptake **B**,and the ratio of the two **C** when Brown Tide densities were >100,000 cells mL^{-1} (filled symbols) and <100,000 cells mL^{-1}. Note that ^{13}C-urea uptake rates are multiplied by 100.

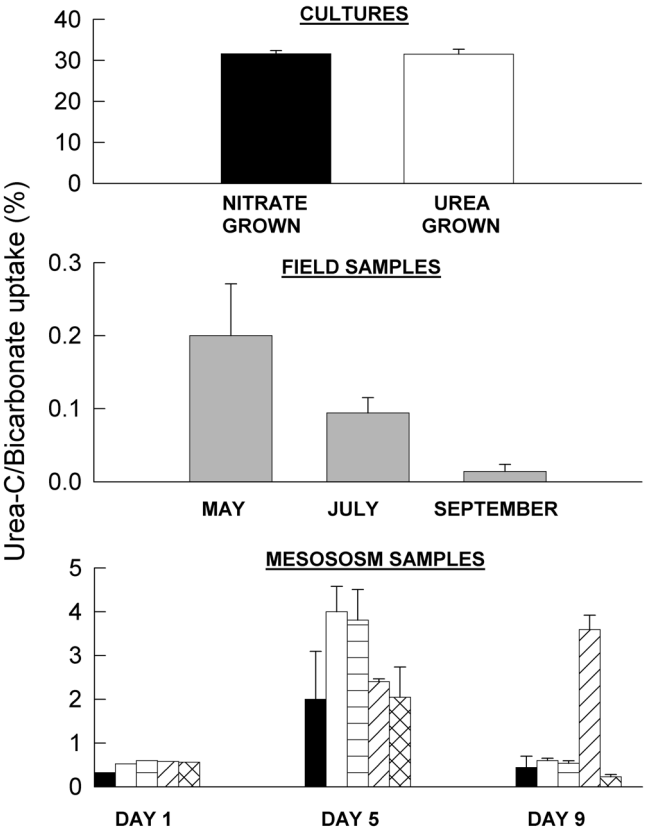

Figure 2 ^{13}C-urea to ^{13}C-bicarbonate uptake ratios for a variety of plankton assemblages containing *A. anophagefferens* at varying densities and experimental conditions. In the mesocosm samples, the different bars represent the following treatments (left to right): control, nitrate addition, urea+glucose addition, macroalgal addition, and intact sediment core addition. See text for further details.

Rates of urea-C and photosynthetic C utilization were determined by the addition of ^{13}C-urea and ^{13}C-HCO_3^- (both 98% isotope enriched) at ~10% of ambient concentrations to parallel incubation flasks (0.5L). Field and mesocosm samples were incubated at 60% of incident irradiance (range in irradiance during incubations was 500–1000 µmol photons m^{-2} s^{-1}) with the exception of samples collected in 1999 that were incubated across a light gradient of 0–100% incident irradiance. Samples from the brown tide culture experiments were incubated at the growth irradiance of 50 µmol photons m^{-2} s^{-1}. All incubations were short, ~1h, and terminated by gentle (<100 mm Hg) filtration onto GF/F filters, previously precombusted at 450°C for 5 hours. Samples were dried at 60°C overnight and stored in a desiccator until analysis by continuous-flow isotope ratio mass spectrometry. Photosynthetic C and urea-C uptake rates were calculated using the equations given by Collos and Slawyk (1984).

Results and Discussion:

Aureococcus anophagefferens densities in the field and mesocosm samples ranged from <5,000 to 500,000 cells mL^{-1} and at times represented up to 80% of the chlorophyll biomass. Urea concentrations were consistently low (<1 µmol-N L^{-1}) and did not exhibit any correlation to changes in brown tide density.

A total of 7 weekly samples were collected in 1999 before, during, and after a brown tide bloom where cell densities reached 500,000 cells mL^{-1}. To facilitate an assessment of the role of the brown tide on urea-C uptake dynamics, relative to natural assemblages that are also known to assimilate urea (*e.g.,* Kaufman *et al.,* 1983; Lively *et al.,* 1983), these sampling dates were divided into two categories based upon brown tide cell density: greater than and less than 100,000 cells mL^{-1}. This is a density previously shown to inhibit feeding in shellfish (Bricelj *et al.,* 2001) and therefore an ecologically relevant threshold for comparing and contrasting different levels of brown tide populations. Between these two cell density categories, urea-C uptake rates were 100-fold lower than photosynthetic C uptake rates (Fig. 1). Urea-C uptake rates were independent of irradiance (Fig. 1A), while photosynthetic C uptake rates followed the anticipated hyperbolic response to irradiance (Fig. 1B). Expressed as a fraction of photosynthetic C uptake, urea-C uptake rates were ~10–40% in the dark or very low light but rapidly decreased to <1% at irradiances greater than ~5% of incident irradiance, which is consistent with work done by Mitsamura (Mitamura and Saijo 1975, Mitamura and Saijo 1980) where urea-C contributed ~3% to cellular carbon nutrition. Laboratory studies have shown that *A. anophagefferens* growth rates, at 50 µmol photons m^{-2} s^{-1}, are in fact slightly higher with urea as the nitrogen source than

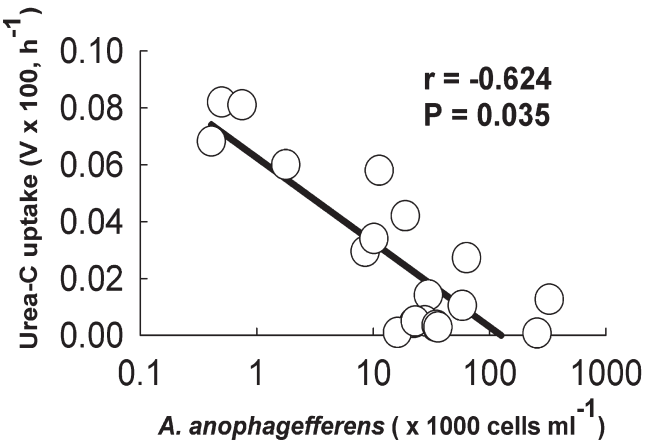

Figure 3 ^{13}C-urea uptake plotted as a function of *Aureococcus anophagefferens* cell density. Data are for field and mesocosm samples only.

with nitrate (Alexander *et al.,* unpubl.). Bacterial uptake of urea-C, or remineralization of urea with subsequent release of carbon dioxide, is possible in these samples, however, size fractionation studies (Lomas unpubl. data) suggest that utilization of urea by the <1.2 µm size-fraction is very low relative to total urea uptake.

More extensive studies in culture, field and manipulated mesocosm populations (Fig. 2) further support the observations presented above. Culture experiments, conducted at a low growth irradiance of 50 µmol photons m^{-2} s^{-1}, show that urea-C uptake rates were ~30% of photosynthetic C uptake rates. Interestingly, there was no difference between cultures grown on nitrate or urea (Fig. 2, top), supporting previous work suggesting that urease activity in *A. anophagefferens* is constitutively expressed (Fan *et al.,* 2003). Field samples (Fig. 2, middle) displayed a seasonal decrease in relative urea-C uptake rates that were consistent with higher brown tide densities in July and September than in May and overall were the lowest relative rates found in this study. Mesocosm manipulations of natural field assemblages found slightly higher rates than the non-amended field samples, although there were no consistent differences between experimental treatments (Fig. 2, bottom).

These data suggest that over a broad range of experimental and natural conditions, urea-C is not likely to provide a substantial carbon supplement to *A. anophagefferens,* in direct contrast to uptake rates of urea-N that clearly do contribute significantly to the overall nitrogen nutrition of the brown tide (Lomas *et al.,* 1996; Berg *et al.,* 1997). Indeed, rates of urea-C uptake were negatively related to densities of *A. anophagefferens* (Fig. 3). Recent work by Mulholland *et al.* (2002) suggests that both culture and field populations of *A. anophagefferens* do, however, take up significant amounts of carbon from amino acids, supporting the hypothesis that the brown tide can get a significant carbon supplement from the DOC pool, just not from urea.

Acknowledgements

Many people have contributed to the data presented in this manuscript. The author thanks (in alphabetical order) Jeff Alexander, Deb Clougherty, Allison Coe, Jeff Cornwell, Jeremey Jones, Todd Kana, Hugh MacIntyre, and Dave Weiss who all assisted in the collection and analysis of the data presented herein. New York Sea Grant through the Brown Tide Research Initiative Phases I and II supported this work.

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Comparison of Regional Clones of the Genera *Chattonella* and *Fibrocapsa* for Growth Characteristics and Potential Toxin Production

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Abstract

Clonal cultures of *Chattonella* (*C. marina, C. subsalsa*) and *Fibrocapsa japonica* isolated from Japan, New Zealand, South Carolina, Texas, Delaware, and Salton Sea, U.S.A., were grown under constant temperature (24°C), salinities of 20 and 36 psu, and 100 µmol photons m^{-2} s^{-1} cool white light in a seawater-enriched Erdscreiber medium. Both *Chattonella* and *Fibrocapsa* clones were capable of growth exceeding 1 div·d^{-1} on successive days and averaged between 0.25 and 0.5 div·d^{-1}, respectively, for the 36-day growth period. Maximum cell densities occurred after day 25 and varied from 6.0 and 11.8 × 10^4 cell · mL^{-1}. Initial screening for brevetoxins of each clone gave positive results using the sensitive ELISA assay only for certain growth phases. Positive ELISAs for brevetoxins were found in late log to stationary phase nearing maximum cell densities. Both cell pellets and extracts for *Chattonella marina* and the two *Fibrocapsa* clones were found to be toxic using the fish bioassay. The cell pellets from all the *Chattonella subsalsa* clones (Salton Sea, Texas, and Delaware) were positive by ELISA and resulted in fish mortality during the bioassay.

Introduction

Marine toxic events caused by species of the Raphidophyceae are now known from a variety of areas around the world. Mortality of fish from Japan (Okaichi, 1983), British Columbia (Taylor, 1993; Taylor *et al.,* 1993), Tasmania (Hallegraeff *et al.,* 1998) and most recently Norway and Delaware, USA (Bourdelais *et al.,* 2002) during blooms confirm the destructive role of these species. Toxicity appears to be a complex interaction of brevetoxin-like compounds (Onoue, 1989, Chang *et al.,* 1990, Ahmed *et al.,* 1995, Kahn *et al.,* 1995, 1996) that can be affected by salinity (Ono *et al.,* 2000), reactive oxygen species (ROS) (Yang *et al.,* 1995; Kim *et al.,* 1999) and possibly other cellular consitituents. Bourdelais *et al.* (2002) formally confirmed the presence of brevetoxins in a natural raphidophyte bloom and along with a highly specific ELISA for brevetoxins (Naar *et al.,* 2002), gave definitive confirmation of these toxins that were purified from natural populations. The role of reactive oxygen species, principally H_2O_2, ^{-}OH, and O_2^{-} (Oda *et al.,* 1997) is less clear in causing mortality of fish. Twiner *et al.* (2001) consider ROS species alone unable to kill fish despite the fact that levels of H_2O_2 were comparable to those found in natural waters (Skeen *et al.,* this Proceedings). The potential role of single and complex compounds working synergistically in causing mortalities was offered as another possible means of causing fish mortality (Marshall, this Proceedings). While the action of ROS is still unresolved, the production of brevetoxins is confirmed and can be detected using the sensitive ELISA and fish bioassay. The question remains if brevetoxins are produced by all raphidophytes species. This study examined the production of these toxins from a known brevetoxins producer (*C. marina*), two clones of *Fibrocapsa* isolated from natural blooms and from *C. subsalsa* clones isolated from blooms associated with fish mortalities in U.S.A.

Materials and Methods

Two clonal cultures of *Fibrocapsa japonica* and four clones of *Chattonella* species were used. The *F. japonica* cultures consisted of a New Zealand clone (NZ) and one recently isolated from a bloom in South Carolina (HH). The *Chattonella* cultures were *C. marina* (Akashiwo Institute, Kagawa Japan) and three *C. subsalsa* clones isolated from blooms in Texas (TX), Delaware (DE) and California (Salton Sea, SS). Each culture was grown in 3-L Fernbach flasks with Erdschreiber media at 20 psu for *F. japonica* and 36 psu for *Chattonella* spp., respectively. Cultures were grown in continuous light and constant temperatures of 24 ± 0.5°C. Daily cell counts were made using a Beckman Coulter Multisizer IIe particle counter. The cell densities for each day were used to calculate daily and average growth rates. A volume of 45 mL from each clone was taken during the growth period and frozen at –30°C for subsequent ELISA assays for brevetoxins. After 35 days, aliquots of cultures were harvested with a continuous flow RC2B Sorvall centrifuge at 5000 rpm. Subsamples of the whole culture, supernatant, and pellet were collected for testing with the ELISA assay and for the standard fish bioassay using *Gambusia affinis.* The cell pellet was sonicated prior to analysis. Concentrations of toxin were calculated from the ELISA results and normalized on a per cell basis. Twenty-six fish were used for the assay. The fish assay results were recorded, noting the time and number of dead fish as compared to controls consisting of fish in toxin-free water.

Results and Discussion

Culture growth, shown in Fig. 1, demonstrated how rapidly densities exceeding 10^4 cells·L^{-1} were attained. The clone *Fibrocapsa* HH grew slower than the others. The maximum densities of 6.0 to 11.5 × 10^4 cells·L^{-1} were attained during the growth period. While terminal densities of these

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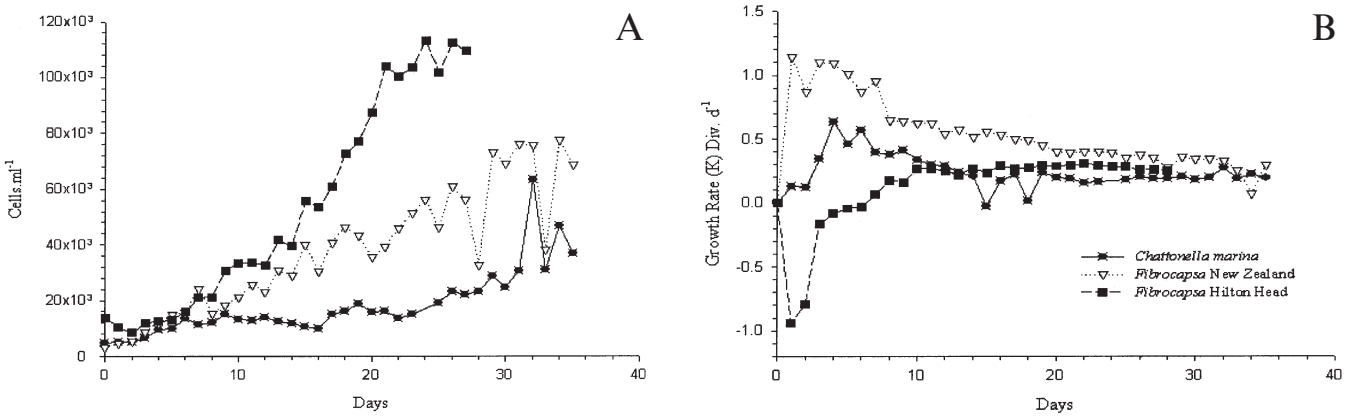

Figure 1 Growth of the *Chattonella marina, Fibrocapsa* New Zealand, and *Fibrocapsa* Hilton Head cultures. **A** Cell density during the 35-day period; **B** Mean and daily growth rate for over a 35-day period.

cultures varied, 25 to 30 days were required for them to reach their maximum, indicating an estimate of time needed for bloom formation of these species if losses were kept to a minimum. Average growth rate (K) was modest, however the specific growth rate between successive days indicated growth potential in excess of 1.0 div·d^{-1}. The clone *F. japonica* NZ was particularly notable in being able to maintain average growth rates around 1.0 div·d^{-1} for the first 10 days of culture (Fig. 1B). Blooms of these species are often episodic, and the potential for explosive growth suggested in these experiments may reflect a growth strategy of these required for developing bloom populations.

The ELISA assays of cultures during different growth stages (Table 1) suggest that toxin production is not constant over time. *Fibrocapsa* NZ showed a decrease in production over time, while *Fibrocapsa* HH and *Chattonella marina* showed an increase. The overall toxin normalized per gram of cell pellet had the following pattern in descending order of toxin content: 1) *Chattonella marina* at 22,006 ng/g, 2) *Fibrocapsa* HH at 3,663 ng/g, 3) *Fibrocapsa* NZ at 2,189 ng/g, 4) *Chattonella* TX at 1709 ng/g, 5) *Chattonella* SS at 939 ng/g, and 6) *Chattonella* DE at 737 ng/g. There is a tenfold decrease of toxin content between *Chattonella marina* and *Fibrocapsa* HH, and the toxicity rapidly decreased thereafter. Toxin content can be variable and at this point it is uncertain what these differences mean. However, the data does show that toxin content can be variable. The factors governing this variability are unknown.

All the clones tested killed fish and gave positive ELISA results for this highly selective assay for brevetoxins (Table 1). Cell pellets, supernatant and whole cell cultures were positive, with the ELISA assay suggesting that the toxins produced by these organisms may readily be released into the environment to cause toxic episodes. A summary of the fish bioassay indicates time of death of each fish tested (Table 1). All fish exposed to toxic cultures died during a period from 30 minutes to 6 hours. There were some physical changes observed in the fish bioassay. During the fish bioassay, the gills and the surrounding tissue on some of the fish turned red after being placed in toxic cultures. Behavioral characteristics, such as swimming erratically when placed in toxic culture, were also observed. These observations are common in fish exposed to brevetoxin. No deaths were

Table 1 ELISA, toxin content and fish bioassay results for clones of *F. japonica* NZ, *F.japonica* HH, *C. marina, C. subsalsa* SS, *C.subsalsa* TX, *C. subsalsa* DE and control.

Species	Fibro NZ	Fibro HH	*C. marina*	Cs. SS	Cs.TX	Cs. DE	Control (neg)
ELISA	+	+	+	+	+	+	+
Supernatant ng/mL	35.33	40.06	18.44	—	—	—	
Whole Culture pg/cell							
Day 16	5.11	0.38	—	—	—	—	
Day 23	2.27	1.92	1.37	—	—	—	
Day 30	1.05	—	5.30	—	—	—	
Cell Pellet ng/g	21.89×10^2	36.62×10^2	220.06×10^2	9.39×10^2	17.08×10^2	7.37×10^2	
Fish Bioassay							
Supernatant	3/0	4/4	2/2	—	—	—	0/0
Whole culture	3/3	4/3	2/0	—	—	—	0/0
Cell Pellet	4/4	4/4	2/2	4/3	4/4	4/3	0/0

Fish bioassay code: 0 = no deaths; 1 = death after 6 hrs; 2 = death in 4–6 hrs; 3 = death in 2–4 hrs; and 4 = death in <2 hrs.

recorded in the control tests of fish exposed to media in which no cells had been inoculated. These data confirm the previous observations on *C. marina* and *F. japonica* from Japan and further strengthens the notion that brevetoxins are the major toxin component of more than just one species of raphidophytes. Similar results were found with the various clones of *C. subsalsa,* suggesting that these organisms may have caused fish kills in areas where their blooms occurred. *Chattonella antiqua* and *C. marina* were reported as having brevetoxin-like fractions (Onoue, 1989, Kahn *et al.,* 1996). *Chattonella verruculosa* associated with fish kills in Japan had no defined toxin, although a *C.* aff. *verruculosa* from the North Sea was implicated in salmon deaths, and *C.* cf. *verruculosa* collected where menhaden died during a Delaware bloom had PbTx-2, -3 and -9, as confirmed by ELISA, HPLC, NMR spectrometry and mass spectroscopy (Bourdelais *et al.,* 2002). *Fibrocapsa japonica,* similarly implicated in intoxication of seals and sea birds in the North Sea coasts, also had a brevetoxin-like compound called Fibrocapsin. These disparate observations suggest that these neurotoxins may be common to most, if not all, species of this class. The relative cellular toxicity of these raphidophytes is 0.1 to 0.5 that of a *Karenia brevis* cell. This would suggest the blooms of the raphidophytes would have to be of higher densities to have the same effect as that of a *K. brevis* bloom. Further studies of the raphidophytes, their toxins and mechanisms regulating them, such as those proposed by Bourdelais *et al.* (this Proceedings) are needed to fully define and evaluate their harmful impact on coastal environments.

Acknowledgements

We thank Dr. F.H. Chang, NIWA New Zealand for the *Fibrocapsa japonica* clone and Dr. Sadaaki Yoshimatsu, Akashiwo Research Institute of Kagawa Japan for the *Chattonella marina* clone and Dr. Alan Lewitus for bloom samples containing *F. japonica* from Hilton Head. This research was supported by grant NCWRRI NCSU 2001-0095-02 awarded to C. Tomas.

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Phylogenetic and Functional Diversity of the Bacteria Associated with *Gymnodinium catenatum:* Evidence from the SSU rDNA of Cultivable and Non-cultivable Bacteria

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Abstract

Previous studies indicate that the bacteria associated with algal cells can influence the production of paralytic shellfish toxins (PST) by *Gymnodinium catenatum* (Dinophyceae). As a first step to understanding the key algal-bacterial interactions, we have focused on characterising the cultivable and non-cultivable bacteria associated with toxic (GCDE08) and low-toxicity (GCHU11) *G. catenatum* cultures. The data derived from the cultivable bacteria show that members of the Alphaproteobacteria dominate in number and diversity. Secondary in dominance and diversity are members of several groups within the Gammaproteobacteria that show a strong affiliation to bacteria capable of hydrocarbon utilisation. The bacterial community associated with *G. catenatum* was comprised of several notable phenotypic groupings: the aerobic anoxygenic photoheterotrophic Alphaproteobacteria, and hydrocarbon-degrading Gammaproteobacteria.

Introduction

The bacteria associated with toxic algae have been implicated directly or indirectly with the production of phycotoxins such as the paralytic shellfish toxins (PST). There is no doubt that bacteria and algae have an integral relationship, but the question is how this relationship is manifested in terms of bloom population dynamics and toxin production (Doucette *et al.,* 1998). Previous evidence examining PST production by *G. catenatum* suggested that the bacterial flora were having an effect on toxin production (Bolch *et al.,* 2001). As an initial step toward examining the involvement of bacteria in the lifecycle and PST production by *G. catenatum*, we cultured bacteria from two *G. catenatum* laboratory strains that produce high (GCDE08 PST = 189 fmol cell^{-1}) and low amounts of PST (GCHU11 PST = 4 fmol cell^{-1}). We used molecular methodology to examine the total bacterial community–*i.e.,* the cultivable and non-cultivable bacteria. The primary aim was to identify the bacteria present, deduce what their function is based on their phylogenetic identity, and extend this hypothesis to how specific bacteria may be important in the lifecycle of *G. catenatum* and the production of PST.

Materials and Methods

G. catenatum strains GCDE08 and GCHU11 were grown in GSe medium at 18°C (14:10; L:D) (Blackburn *et al.,* 1989) and harvested for bacterial culturing and DNA extraction in late log growth phase. Bacteria were cultured from algal cells on a modified marine agar (1.5%) prepared with 75% seawater, peptone (0.05%), yeast extract (0.01%), and GSe medium (Blackburn *et al.,* 1989) trace elements and vitamins, and incubated in the dark at 18°C for 3 weeks. Total genomic DNA was extracted using a method based on CTAB purification (Ausubel *et al.,* 1999). Total bacterial diversity was examined by the method of amplified rDNA restriction analysis (ARDRA) according to the method of Moyer *et al.* (1995). Representatives of each RFLP-type was sequenced and compared with sequences available via the Ribosomal Database Project II (Maidak *et al.,* 2001). The photosynthetic reaction centre genes *pufLM* were amplified using the methods of Béja *et al.* (2002) and their identity confirmed by DNA sequencing. Utilisation of *n*-hexadecane as the sole carbon source was demonstrated by growth on agar after 3 weeks incubation at 25°C (Yakimov *et al.,* 1998). Saxitoxins (STX) and sodium channel blockers (SCB) were detected in bacterial cell pellets harvested from 100 mL of Zobell's 2216 marine broth. Cell pellets were resuspended in 0.05 N acetic acid, sonicated, and cell debris removed by centrifugation and 0.2 µm membrane filtration prior to being assayed with the saxiphilin and mouse neuroblastoma assays (Llewellyn *et al.,* 1998).

Results

Cultivable Bacteria A total of 10 distinct bacterial types were cultured from strain GCDE08 compared to only 4 types from strain GCHU11 (Table 1). Only three bacteria (DG876, 880 and 881) were closely affiliated with characterised bacterial species. Most other strains could be assigned to extant genera within the *Cytophaga-Flavobacteria-Bacteroides* (CFB), Alpha- and Gamma-proteobacteria or Gram-positive divisions. Based on the phenotypes of the nearest SSU-rDNA neighbours, it was hypothesised that several bacterial strains would be capable of hydrocarbon-degradation or photosynthesis. In each case,this was confirmed by demonstrating growth on *n*-hexadecane as the sole carbon and successful PCR amplification and sequencing of *pufLM* genes from the Alphaproteobacteria (Table 1). None of the strains examined appeared to be capable of autonomous PST production or sodium channel blocker production.

Total Bacterial Diversity Analysis of bacterial SSU-rDNA clone libraries from GCDE08 and GCHU11 cultures revealed a greater number of bacterial genotypes than we

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Table 1 Cultivable bacteria isolated from GCDE08 and GCHU11.

Strain	Origin	Nearest Species Affiliation[a]	SSU % ID[b]	*pufLM*[c]	*n*-Hexadecane Utilisation	STX/ SCB
DG868	GCHU11	(CFB) *Zobellia*) sp.	96.3	ND	ND	ND
DG869	GCHU11	(α) *Roseobacter gallaeciensis*	93.8	+	–	–
DG870	GCHU11	(γ) *Marinobacter hydrocarbonoclasticus*	95.5	–	+	–
DG873	GCHU11	(CFB) *Microscilla furvescens*	91.3	ND	ND	ND
DG874	GCDE08	(α) *Ruegeria algicola*	92.4	+	–	–
DG876	GCDE08	(G+) *Micrococcus luteus*	99.2	ND	+	ND
DG877	GCDE08	(α) *Roseobacter* sp.	94.9	+	–	–
DG878	GCDE08	(α) *Ruegeria algicola*	92.4	+	–	–
DG879	GCDE08	(γ) *Marinobacter hydrocarbonoclasticus*	95.5	–	+	–
DG880	GCDE08	(γ) *Acinetobacter lwoffii*	99.3	ND	–	–
DG881	GCDE08	(γ) *Alcanivorax borkumensis*	99.4	–	+	–
DG882	GCDE08	(α) *Ruegeria gelatinovorans*	95.0	+	–	–
DG885	GCDE08	(α) *Sulfitobacter mediterraneus*	94.5	–	+	–
DG886	GCDE08	(CFB) *Zobellia* sp.	96.3	ND	ND	ND

[a]Named species based on the most similar characterized SSU rDNA sequence listed on the RDP II. CFB = *Cytophaga-Flavobacteria-Bacteroides;* α / γ = Alpha- or Gamma-proteobacteria; G+ = Gram positive. [b]Percentage SSU rDNA sequence identity to the named species. [c]*pufLM* photosynthetic reaction centre genes are indicative of the capability of photoheterotrophic growth. ND = not determined.

were able to cultivate (Table 2). DNA sequence analysis of representative RFLP-types from the library showed that the majority of the additional bacterial genotypes were phylogenetically closely related to the cultivable strains of *Roseobacter* and *Marinobacter* listed in Table 1, while the others belonged to the Alphaproteobacteria genera *Stappia, Rhodobacter, Caulobacter* and to unidentified *Pseudomonas*-like species and *Oleiphilus messinensis* in the Gammaproteobacteria (data not shown).

Discussion

Using molecular approaches combined with traditional bacteriology, we have characterised the total cultivable and non-cultivable bacterial flora associated with two *G. catenatum* cultures. At the gross flora level, our results are similar to previous reports indicating that members of the Alphaproteobacteria typically dominate the bacterial flora of toxic dinoflagellates (Alavi *et al.,* 2001; Hold *et al.,* 2001; Prokic *et al.,* 1998). The majority of these belong to one family, the Rhodobacteraceae. A key feature of members of the Rhodobacteracea is that they are capable of a mode of phototrophic growth termed aerobic anoxygenic photosynthesis (AAP) (Yurkov and Beatty, 1998). All except one of the cultivable Alphaproteobacteria (Table 1) were confirmed as being capable of AAP. The role of these AAP bacteria in the marine ecosystem is not understood (Kolber *et al.,* 2001), however, their association with dinoflagellates may yet provide a clue to their potential function.

The balance of the cultivable and non-cultivable bacteria belonged to the Gammaproteobacteria or CFB. Members of the CFB are well-recognised as important in degrading particulate and high molecular weight organic matter (Kirchman, 2002), and frequently associated with algicidal activity. Gammaproteobacteria in *G. catenatum* were dominated by *Marinobacter* spp., which accounted for 72–76% of the Gammaproteobacteria identified in the clone libraries (data not shown). We have also cultured *Marinobacter* sp. from 6 of 7 *G. catenatum* cultures (data not shown). The consistent finding of *Marinobacter* in *G. catenatum* cultures and its association with other dinoflagellates (Alavi *et al.,* 2001; Hold *et al.,* 2001; Seibold *et al.,*

Table 2 Total bacterial diversity in SSU-rDNA clone libraries from strains GCDE08 and GCHU11.

Culture	Clones examined	Unique Profiles[a]	Total and Cultivable Bacterial Genotypes			
			Alphaproteobacteria	Gammaproteobacteria	CFB	Gram positive
GCDE08	94	28	15 (47)	6 (20)	6 (32)	1 (1)
GCHU11	94	11	4 (19)	3 (19)	4 (62)	–

[a]Both the cultivable and non-cultivable bacteria detected in the clone libraries. Figures in parentheses indicate the percentage of clones analysed that were classified as belonging to that division.

2001) suggest that *Marinobacter* may be of general importance to dinoflagellate growth and physiology. *Alcanivorax* sp. (Table 1) and *O. messinensis* identified in the DE08 clone library, both of which, like our *Marinobacter* isolates, are recognised to be or have been shown to be (Table 1) hydrocarbon-degrading bacteria. We speculate, therefore, that hydrocarbon-degrading bacteria may be an important defining characteristic of the bacterial flora of *G. catenatum* and a number of other dinoflagellates.

The total bacterial diversity of the strain of low PST-producing *G. catenatum* GCHU11 is considerably reduced compared to a typically toxic strain (GCDE08). A similar phenomenon has been noted in other low-PST producing *G. catenatum* cultures (Bolch *et al.,* 2001), implying that the bacterial community is directly or indirectly influencing PST production in some way. All low PST-producing *G. catenatum* cultures have been derived from cysts germinated in the laboratory (Bolch and Negri, unpubl. data). The process of isolation, sonication and washing inevitably would remove much of the surface-associated bacterial flora on cysts. Therefore, it is possible that the low per-cell toxicity of GCHU11 (and other atypical *G. catenatum* cultures) may be related to the loss of specific bacteria that directly or indirectly induce toxicity. Alternatively, reduced bacterial diversity in cultures may physiologically compromise *G. catenatum* in some way that leads to a reduction in PST content. However, strain GCHU11 grows well in laboratory culture, and any potential physiological effect does not appear to be manifested as a reduced growth rate.

In summary, a number of bacteria were identified that may be important in the lifecycle of *G. catenatum*. In both *G. catenatum* cultures, there was a greater diversity of non-cultivable bacteria than were cultivable. It was observed that low toxin production by *G. catenatum* correlated with a reduced bacterial diversity, but why this is so is not yet clear. Our current research is aimed at understanding what biological roles the identified bacteria may have in the lifecycle of *G. catenatum* and how and why they might modulate PST production by *G. catenatum*.

Acknowledgements

DHG is very grateful to the New Zealand Foundation for Research, Science and Technology for funding for this research.

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Photosynthetic Variation Among Eight Different *Karenia brevis* Clones

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Abstract

Laboratory characterization of the red tide dinoflagellate species *Karenia brevis* has primarily focused on the Wilson clone (CCMP719) isolated in 1953. Previous studies with other species showed that analysis of one clone may lead to misinterpretations when extrapolating to field populations. The photosynthetic capabilities of eight different *K. brevis* clones were compared using a PAM-FL. Standard laboratory techniques including ^{14}C primary productivity, DCMU-FL and chlorophyll extraction were compared against the PAM-FL measurements in four of these clones. All experiments were conducted on a modified radial photosynthetron. Interclonal differences occurred at intermediate and high light intensities based on both the PAM-FL and the three standard laboratory techniques.

Introduction

Physiological research with clones of marine phytoplankton species provides useful information on the physiology of field populations (Brand, 1991; Wood and Leatham, 1992). Though it has been shown that clones of the same species can vary in physiological responses when grown under the same conditions (Gallagher, 1980; Wood and Leatham, 1992), laboratory characterization of the red tide dinoflagellate species *K. brevis,* however, has focused primarily on the Wilson clone isolated in 1953. Laboratory experiments reported here investigated the photosynthetic capabilities of eight different *K. brevis* clones. Piney Island, Mexico Beach, and Apalachicola clones geographically represented populations from the northwest region of Florida. Charlotte Harbor, New Pass, and Manasota clones geographically represented the west coast of Florida. The Jacksonville clone geographically represented the east coast of Florida. Our experiments investigated whether or not all *K. brevis* clones have the same photosynthetic capabilities. If all clones have the same capabilities, then any clone, including Wilson, is representative of the *K. brevis* species. If not, can a few functional groups be identified?

Materials and Methods

Jacksonville (C3), Charlotte Harbor (C2), Apalachicola (C6), Mexico Beach (C5) and Piney Island (C4) clones were obtained from the Florida Marine Research Institute, St. Petersburg, Florida. Manasota (CCMP2229) and New Pass (CCMP2228) clones were obtained from Mote Marine Laboratories, Tampa, Florida. The Wilson clone (CCMP718) was obtained from Bigelow Center for Culture of Marine Phytoplankton, McKown Point, Maine.

Pre-experiment cultures were grown in 9-L containers under 100 µmol quanta $m^{-2}s^{-1}$ under 12-hour light/12-hour dark cycles at 22°C and were allowed to photoadapt for one week. All clone cultures were adjusted to equivalent cell concentrations for the experiments. Cultures were thoroughly mixed and all subsamples were drawn from the container before lights on. An initial dark sample was measured prior to lights on; subsequent incubation times for the remaining subsamples were 4 hours and 8 hours. Only 8-hour incubation results are shown here. Experiments were conducted in a modified radial photosynthetron (Babin *et al.,* 1994), which provided controlled conditions of light and temperature. The photosynthetron included 12 black boxes extending as spokes around a 250 watt metal halide lamp (OSRAM HQISE250DX). Each black box held ten 25 mL or 50 mL Falcon tissue culture flasks. A light gradient was formed by the distance of each bottle within a box from the light source. Typically, light intensity decreased 15% between bottles. Light levels ranged from a maximum of ~2,000 µmol quanta $m^{-2}s^{-1}$ to 55 µmol quanta $m^{-2}s^{-1}$. One darkened bottle was included per set of 20 incubated bottles to complete the irradiance range.

Calibration of a Walz Water-Pulsed Amplitude Modulated Chlorophyll Fluorometer (PAM-FL) was conducted against standard laboratory techniques including DCMU fluorescence (DCMU-FL) (Vincent *et al.,* 1984), chlorophyll extraction and ^{14}C primary productivity (Parsons *et al.,* 1984). Experiments incorporating DCMU-FL, chlorophyll extraction, ^{14}C primary production and PAM-FL measurements were conducted on the Jacksonville, Manasota, Wilson, and Piney Island clones. Only the PAM-FL was used to assess the photosynthetic capabilities of the remaining clones.

Results and Discussion

Initial dark samples for all clones showed similar photosynthetic capabilities at all light intensities at the start of each day. In 4-hour (not shown) and 8-hour (Fig. 1) incubations, low-light responses were similar (*i.e.,* ETR alpha $P = 1.00$) among all clones while intermediate and high light responses showed differences among all clones. As light intensity increased, DCMU-FL yield generally decreased ~30% and PAM-FL yield decreased ~78%. PAM-FL yields showed a weak correlation with DCMU-FL (Rsq = 0.50). PAM-FL yield was determined with an actinic light pulse while DCMU-FL was an induced chemical fluorescence which was responsible for the low correlation. Extracted chl *a*/cell concentration decreased 50% for the Wilson clone and

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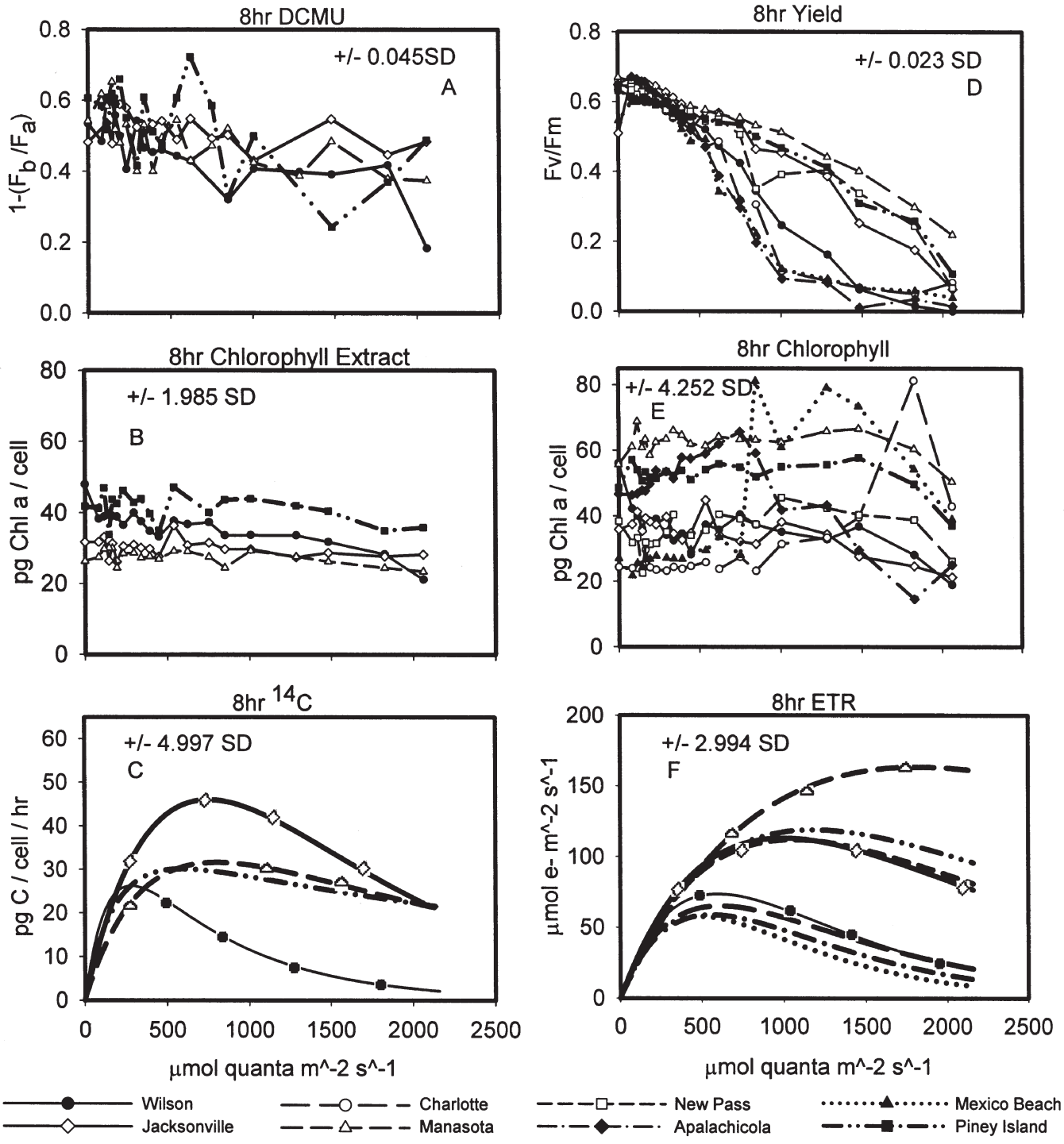

Figure 1 Only 8-hour incubations are shown. Standard laboratory techniques including **A** DCMU-FL, **B** chlorophyll extraction and **C** ^{14}C primary productivity curves (symbols selectively placed for clarity) were used on Jacksonville, Manasota, Wilson and Piney Island clones. PAM-FL measured **D** yield, **E** chl *a*/cell and **F** ETR (symbols selectively placed for clarity) on all clones. The average standard deviation (SD) around the data points is reported for each technique.

10–15% for the three other clones as light intensity increased. PAM-FL chl *a*/cell concentrations showed complex patterns depending on the clone. PAM-FL chl *a*/cell values showed weak correlation with extracted chl *a*/cell values (Rsq = 0.40). PAM-FL chl *a*/cell was based on theoretical calculations while extracted chl *a*/cell was based on fluorescence measurements. ^{14}C primary productivity curves showed intermediate ($P < 0.001$) and high light ($P = 0.012$) responses that were different among the four clones as fit by a three parameter Platt curve (Zimmerman *et al.,* 1987). Though

interclonal variability existed, Wilson (Rsq = 0.878), Jacksonville (Rsq = 0.899), Manasota (Rsq = 0.893) and Piney Island (Rsq = 0.823) PAM-FL electron transport rates (ETR) showed strong intraclonal correlation with ^{14}C primary productivity when fit to a hyperbolic curve (Platt and Gallegos, 1980). PAM-FL ETR provided only a relative measurement for ^{14}C primary productivity. PAM-FL ETR measured electron potential with theoretical calculations while the ^{14}C measured actual carbon uptake. Though the general trends in traditional techniques were similar to related measurements based on the PAM-FL, the parallel patterns differed in specifics. The PAM-FL does not replace the traditional measurements. However, the PAM-FL results did complement the trends seen in all three of the more traditional standard laboratory techniques.

Interclonal differences were exhibited in DCMU-FL ($P = 0.022$), extracted chl *a*/cell ($P < 0.001$), ^{14}C primary productivity, PAM-FL derived yield ($P = 0.005$), chl *a*/cell ($P < 0.001$) and ETR (P_{max} $P < 0.001$; beta $P < 0.001$). Based on ^{14}C primary productivity and on PAM-FL yield and ETR, the clones separated into two functional groups. Light-sensitive clones were Wilson, Mexico Beach, Apalachicola, and Charlotte Harbor. Light-capable clones were Jacksonville, Piney Island, Manasota, and New Pass. Therefore, the Wilson clone only partially represented *K. brevis* as a species. This study showed that some clones, in contrast to the Wilson clone, were very capable of maintaining their photosynthetic capabilities at natural surface light intensities. Variations among the photosynthetic capabilities of the eight *K. brevis* clones suggested different clones may represent different functional groups. Based on the eight clones, the subpopulations did not geographically segregate along the Florida coast.

Acknowledgements

Karen Steidinger and Bill Richardson provided five *K. brevis* clone cultures from FMRI. Gary Kirkpatrick provided two *K. brevis* clone cultures from Mote Marine Laboratories. Physical and Mathematical Sciences Instrument Shop at North Carolina State University constructed the radial photosynthetron. The Environmental Health and Safety Center at North Carolina State University provided the scintillation counter. This work was supported by Florida Fish and Wildlife Conservation Commission grant S7701618301, EPA grant R-82937001 and NOAA grant NA160P1440.

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Photophysiology of the Florida Red Tide Dinoflagellate, *Karenia brevis:* Modifications in Thylakoid Lipid Composition in Response to Environmental Conditions

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Abstract

Many algae respond to stressful environmental conditions, such as extremes in temperature, by modulating the fatty acid composition of their lipid membranes in order to maintain the optimal fluidity/permeability necessary for membrane function. Of particular importance to autotrophs are the modifications in thylakoid membrane fluidity as this affects virtually all aspects of the light reactions of photosynthesis: the electron transport chain, the intra- and inter-orientations of the photosystems, and the maintenance of the pH gradient across the thylakoids. However, it is not presently known how *Karenia brevis* modifies its thylakoid membrane composition in response to high fluxes of irradiance, temperature and/or nutrient stresses. The results presented here are from a preliminary study designed to gather information on the magnitude of differentiation in lipids that can be expected from a given clonal culture at various light levels. These initial experiments indicate that the differences in fatty acids (as methyl esters [FAMEs]) between LL- and ML-grown cultures are quantifiable and significant. The results will provide a better understanding of the biochemistry and physiology of this important toxic dinoflagellate.

Introduction

The harmful marine dinoflagellate, *K. brevis,* frequently forms large toxic blooms in the waters off the west coast of Florida (USA), and is responsible for massive fish kills and public health concerns. Despite decades of field studies on this organism, very few investigations have examined the physiology of *K. brevis* lipid biosynthesis and physiology. Consequently, a great deal is known about the biogeography of harmful algal bloom (HAB) species, but our ability to describe the factors controlling the community dynamics that lead to a HAB is limited by critical gaps in knowledge of the physiological and behavioral characteristics of algae in relation to environmental parameters. Without this knowledge, we have only a limited basis for monitoring or predicting the occurrence and impacts of harmful algal blooms.

One of the largest of these knowledge gaps is in our understanding of the biochemistry and physiology of algal lipids, particularly in dinoflagellates and especially with regard to the relationship of fatty acid content to cellular processes such as photosynthesis and reproduction. The information presented here is from a recently initiated preliminary study in which we are attempting to examine the impacts of light upon the lipid composition and the resulting functional capabilities (*e.g.,* thylakoid membrane permeability and/or fluidity) resulting from these changes. In addition, we have examined how different light levels affect the fatty acid composition of membrane phospholipids and storage triglycerides.

Materials and Methods

Growth Conditions *K. brevis* FMRI ("Piney Island Isolate"; K. Steidinger, Florida Marine Research Institute, St. Petersburg, FL) was grown in triplicate in 2.5 L of L1 medium at 21°C and a light/dark cycle of 14/10 hrs. Two irradiance levels were examined: low light (LL; 15 µmol photons m^{-2} s^{-1}) and medium light (ML; 50 µmol photons m^{-2} s^{-1}). Cells were harvested after approximately 30 days of growth.

Lipid Extraction, Fractionation, Derivatization, and Gas Chromatography/Mass Spectrometry Analysis Cells were harvested via filtration (precombusted Whatman 934-AH GF filters). Extraction of lipids was performed according to a modified Bligh and Dyer extraction (Guckert *et al.,*1991). The total lipid extracts were separated into five component lipid fractions on columns of activated Unisil silica (1.0 g, 100–200 mesh, activated at 120°C, Clarkson Chromatography, South Williamsport, PA, USA) according to the solvent regime of Leblond and Chapman (2000): 1) 12 mL methylene chloride (sterol esters), 2) 15 mL 5% acetone in methylene chloride with 0.05% acetic acid (free sterols, tri- and diacylglycerols, and free fatty acids), 3) 10 mL 20% acetone in methylene chloride (monoacylglycerols), 4) 45 mL acetone [monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG)], and 5) 15 mL methanol with 0.1% acetic acid (phospholipids). Derivatization and gas chromatography/mass spectrometry (GC/MS) analyses of FAMEs associated with triglycerides, glycolipids (MGDG, DGDG, and SQDG; Fig. 1), and phospholipids were performed on a Finnigan Magnum ion trap GC/MS system according to the methodology utilized by Leblond and Chapman (2000). Methyl tricosanoate (23:0) was added as an internal standard. Dinoflagellates often have abundant amounts of triglycerides (Parrish *et al.,* 1994), but diglycerides are either absent or are present in amounts below detection limits. Therefore, fraction 2 is referred to in the text as the triglyceride fraction. A students t-test was used to determine significant differences (to 0.05) in FAME percentages between the two light levels.

Results

The fatty acid compositions of glycolipids (derived from the

Steidinger, K. A., J. H. Landsberg, C. R. Tomas, and G. A. Vargo (Eds.). 2004. Harmful Algae 2002. Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO.

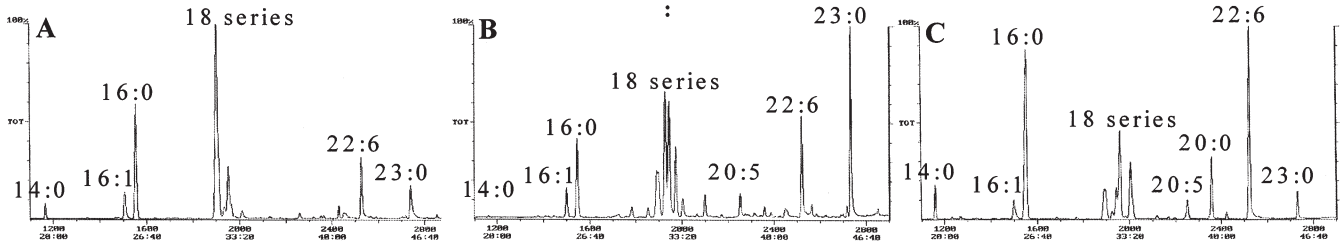

Figure 1 Partial GC/MS traces of FAMEs derived from the glycolipid (**A**), triglyceride (**B**) and phospholipid (**C**) fractions of a representative ML-grown culture.

chloroplast membranes) were similar for both the ML and LL cultures. The LL cultures did have a greater relative percentage of the highly unsaturated fatty acids, octadecatetraenoic acid [18:4(n-3)] and octadecapentaenoic acid [18:5(n-3)]. In the ML cultures these two compounds, which coeluted, were 49.4 (±3.3)% of the relative abundance of total peak area, whereas in the LL cultures they comprised 68.8 (±4.8)% (Tables 1 and 2).The greatest difference in fatty acid composition between the ML and LL cultures was found with fatty acids associated with triglycerides, which are considered storage lipids. The LL cultures had significantly greater relative percentages of the saturated fatty acids, hexadecanoic acid (16:0) and octadecanoic acid (18:0) than the ML cultures. In the LL cultures, these two fatty acids were found at 30.7 (±3.2)% and 15.1 (±4.1)%, respectively, whereas in the ML cultures they were present at 14.5 (±2.9)% and 3.2 (±0.9)%, respectively (Tables 1, 2).

The ML cultures also had significantly greater relative percentages of certain unsaturated fatty acids associated with triglycerides. For example, octadecatrienoic acid [18:3(undetermined)] was found at 7.9 (±2.9)% in the ML cultures; it was not present at all in the LL cultures. In addition, octadecadienoic acid [18:2(n-6)] was present at 20.2 (±2.7)% in the ML cultures, and only 4.2 (±2.2)% in the LL cultures.

The fatty acid compositions of the phospholipid fractions (representative of cellular membrane composition) of the ML and LL cultures were also similar. The ML cultures did have a slightly greater relative percentage of octadecenoic acid [18:1(n-7)] than the LL cultures, 13.9 (±1.0)% and 9.1 (±0.5)%, respectively. The ML cultures did have a significantly lesser relative percentage of eicosanoic acid (20:0) than the LL cultures, 15.4 (±2.3)% and 7.0 (±1.5)%, respectively.

An interfraction comparison of total fatty acid content (examined as total peak area for all fatty acids within that fraction) revealed that the LL cultures had a greater relative percentage of fatty acids associated with thylakoid-derived glycolipids than the ML cultures at 52.9 ± 13.2% and 32.8 7.2%, respectively. The opposite was true of fatty acids associated with phospholipids (LL: 40.8 15.1%; ML: 62.2 6.9%). However, both light conditions led to approximately the same relative percentage of fatty acids associated with triglycerides (LL: 5.5 ±2.3%; ML: 5.1 1.8%).

Discussion

Toxic blooms formed by dinoflagellates, particularly *K. brevis,* have received scant attention with respect to their lipid composition (Leblond *et al.,* 2003). This is in stark contrast to the amount of information available concerning *K. brevis'* toxins and pigments in culture and during blooms (cf. Millie *et al.,* 1997, Steidinger *et al.,* 1998; Daranas *et al.,* 2001). In order to predict the dynamics of bloom initiation, maintenance, and decline, it is necessary to understand the

Table 1 Relative abundance (in % of total peak area) of selected fatty acid methyl esters (FAMEs) derived from *Karenia brevis* glycolipids, triglycerides and phospholipids for ML-grown cultures.

	Glycolipids		Triglycerides		Phospholipids	
FAME	Avg.	SD	Avg.	SD	Avg.	SD
14:0	3.0	2.6[3]	tr[1]		2.2	1.1[3]
16:1 (n-7)	4.7	0.2[3]	4.9	1.6	2.9	0.7[3]
16:0	18.6	1.2	14.5	2.9[3]	26.6	2.7
18:5 (n-3) and18:4 (n-3)	49.4	3.3[3]	11.2	2.6	6.0	1.3
18:2 (n-6)	2.2	0.5	20.2	2.7[3]	1.3	0.0
18:1 (n-9)	9.3	0.3	21.7	2.9	5.1	0.5
18:3 (undetermined)	N/D[2]		7.9	2.9[3]	N/D[2]	
18:1 (n-7)	1.8	0.3[3]	N/D[2]		13.9	1.0[3]
18:0	1.4	0.1[3]	3.2	0.9[3]	10.1	1.3[3]
20:5 (n-3)	0.9	0.1[3]	2.3	0.7[3]	2.5	0.1
20:0	N/D[2]		2.1	1.8[3]	7.0	1.5[3]
22:6 (n-3)	9.2	0.3[3]	11.7	4.6	22.9	3.9

[1]"trace" amount; [2]not detected; [3]significantly different from LL culture

Table 2 Relative abundance (in % of total peak area) of selected fatty acid methyl esters (FAMEs) derived from *Karenia brevis* glycolipids, triglycerides and phospholipids for LL-grown cultures.

	Glycolipids		Triglycerides		Phospholipids	
FAME	Avg.	SD	Avg.	SD	Avg.	SD
14:0	tr[1]	[3]	tr[1]		tr[1]	[3]
16:1 (n-7)	2.5	1.1[3]	4.4	0.2	tr[1]	[3]
16:0	14.3	3.2	30.7	3.2[3]	21.9	4.5
18:5 (n-3) and 18:4 (n-3)	68.8	4.8[3]	7.8	3.6	6.0	1.7
18:2 (n-6)	1.4	1.3	4.2	2.2[3]	tr[1]	
18:1 (n-9)	7.5	3.1	27.7	12.8	5.2	0.9
18:3 (undetermined)	N/D[2]		N/D[2]	[3]*	N/D[2]	
18:1 (n-7)	tr[1]	[3]	N/D		9.1	0.5[3]
18:0	tr[1]	[3]	15.1	4.1[3]	13.8	1.4[3]
20:5 (n-3)	N/D[2]	[3]	tr[1]	[3]	2.8	0.2
20:0	N/D[2]		tr[1]	[3]	15.4	2.3[3]
22:6 (n-3)	5.4	0.6[3]	9.2	3.0	24.5	3.7

[1]"trace" amount; [2]not detected; [3]significantly different from ML culture.

physiological autecology of this organism. Assessment of the fatty acid and sterol composition of intracellular lipid classes within a bloom is a relatively facile process that nicely complements measurements of pigment composition to give a more holistic picture of the nutritional status of the community.

It is clearly not known how *K. brevis* modifies its thylakoid membrane composition as a means of coping with high irradiance. Although modifications in both the ratio of glycolipid molecular species (MGDG, DGDG, and SQDG) and the degree of fatty acid unsaturation have been observed as a general response to forms of environmental change, such as light (Renaud *et al.,* 1991; Sukenik *et al.,* 1993), limitations in the availability of key nutrients such as phosphate (Benning *et al.,* 1993; Benning *et al.,* 1995) and nitrate (Ben-Amotz *et al.,* 1985), it is difficult to use such studies to predict both changes in lipid composition and photosynthetic activity in *K. brevis,* even though photosynthetic activity and photo-protection have been characterized in *K. brevis* (Evens *et al.,* 2001).

Thus, this effort presents preliminary data from a study that was initiated in order to characterize the effects of light upon the lipid composition of *K. brevis.* We have begun by examining the effects of irradiance at a whole cell level in order to gather information on the magnitude of differentiation that can be expected from a given clonal culture at various light levels. These initial experiments indicate that the differences in FAMEs between LL- and ML-grown cultures are quantifiable and significant. These differences suggest that the ML thylakoid membranes may be more fluid than the LL cultures, but this is by no means conclusive. Future experiments will examine the response of *K. brevis* lipids to a greater range of light conditions and will involve organelle isolations, so that we may begin to target the locations of lipid adjustments. Final experiments will involve the isolation and characterization of the functionality and lipid complement of thylakoid membranes.

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Photopigment Content of Three *Karenia brevis* Clones in Response to Varying Light Levels

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Abstract

The photopigment content of 3 clones of the Florida (USA) red tide marine dinoflagellate *Karenia brevis* (Davis) G. Hansen and Moestrup (Daugbjerg *et al.,* 2000), isolated from natural bloom events in 1953, 1996, and 2001, were analyzed after acclimating cultures to high and low irradiance treatments. Photosynthetic pigment and carotenoid concentrations in each clone decreased with increasing irradiance. Multiple carotenoid/chlorophyll (chl) ratios differed within and between clones and irradiance treatments. However, the gyroxanthin-diester/chl *a* ratios were not significantly different within each clone exposed to high and low light.

Introduction

Karenia brevis is the most prevalent harmful algae in the coastal waters of the southeastern United States and throughout the Gulf of Mexico (Steidinger *et al.,* 1997). This unarmored marine dinoflagellate produces potent neurotoxins (brevetoxins) that contaminate shellfish, resulting in neurotoxic shellfish poisoning, massive fish kills, and severe respiratory irritation in marine mammals and humans exposed to airborne neurotoxins in association with marine aerosol (Bossart *et al.,* 1998). Large blooms of *K. brevis* frequently concentrate at the water's surface for long periods and are exposed to strong and highly variable amounts of photosynthetically active radiation (PAR). Previous studies have shown photopigment content of *K. brevis* varies depending on growth stage and light history; however, the ratio of gyroxanthin-diester/chl *a* remains constant compared to other carotenoid/chl ratios (Evens *et al.,* 2001; Millie *et al.,* 1995). The gyroxanthin-diester/chl *a* ratio may be useful as a "biomarker" by incorporating it into pigment-based monitoring applications to allow for the detection of *K. brevis* in a mixed phytoplankton assemblage.

Most laboratory culture studies of *K. brevis* conducted within the last 50 years utilized a single clone isolated by W.B. Wilson from a water sample obtained in the vicinity of Johns Pass, Florida, in 1953. The photopigment content and composition of the Wilson clone may markedly differ from what would be found in a more recently isolated clone from a different region. Örnólfsdóttir *et al.* (2003) indicates pigment content varies between blooms in different regions, yet little is known whether pigment content and composition significantly varies between clonal cultures depending on long-term acclimation or adaptation to culture maintenance conditions. Accurate determination of intraspecific variability in pigments, both in natural blooms and in cultures, may further validate the application of certain pigments as "biomarkers" and is critical to our understanding of the physiological and biochemical strategies that underlie bloom dynamics of this organism. This study focuses on comparing culture pigment content and composition between the Wilson clone and 2 more recently isolated clones from water samples obtained in the vicinity of Piney Island (1996) and New Pass (2001), Florida. The intent was to determine the pigment content and composition of the 3 clones in response to high and low irradiance treatments representative of what cells might naturally experience throughout the water column.

Materials and Methods

Culture techniques Non-axenic, unialgal batch cultures of 3 *K. brevis* clones from New Pass (CCMP2228), Piney Island (FMRIB4), and Wilson (CCMP718) were maintained in L1 medium (Guillard and Hargraves, 1993) within 12-L Pyrex bottles at 25 ± 1°C and 33 ppt. Wide spectrum fluorescent lamps provided the cultures with 80 µmol photons m^{-2} s^{-1} PAR in a 12 h light: 12 h dark cycle (lights on at 06:30 h). All PAR measurements were made with a Biospherical Instruments, Inc., model QSL-100 quantum scalar irradiance sensor placed in culture flasks filled with medium.

Experimental design Eighteen 250 mL polystyrene cell culture flasks containing 150 mL L1 were divided into 3 groups of 6 flasks. Each group's flasks were inoculated with 100 mL of 1 of the 3 clonal batch cultures. The cultures were exposed to maintenance light conditions for 5 days. Each clonal group was randomly divided into 2 groups of 3 cultures and exposed to irradiance treatments of 60 or 165 µmol photons m^{-2} s^{-1} PAR for 10 days. Aliquots were withdrawn from each culture for pigment analyses and cell enumeration. Ten mL subsamples were filtered under low vacuum (<75 mm Hg) onto GF/F glass-fiber filters. Filters were immediately frozen and stored in liquid nitrogen until extraction. Pigment analyses and identification using HPLC were performed following the procedures in Wright *et al.* (1991). Cell enumeration was determined using a Beckman-Coulter Multisizer IIe utilizing AccuComp software (Version 1.19). All sampling and procedures were conducted between 1100 and 1300 hrs.

Results and Discussion

Beckman-Coulter Multisizer cell counts indicated biomass was similar within and between clones exposed to the irradiance treatments (Table 1). The photopigments analyzed included chls *a*, c_1/c_2, and c_3 and the carotenoids fucoxanthin, 19'-acylofucoxanthins, diadinoxanthin, diatoxanthin, gyroxanthin-diester, and b-carotene. Chlorophyll *a*, fucoxanthin and 19'-acylofucoxanthins were the major

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Table 1 Biomass (cells × 10^7 L^{-1}) and chl content (pg $cell^{-1}$) of 3 *K. brevis* clones exposed to irradiances of 60 and 165 µmol photons m^{-2} s^{-1} PAR. Data are means ± standard errors (n = 3). Clone (C) and irradiance (I) effects (determined by ANOVA) are significant ($P \leq 0.05$) if designated. Significantly different variable means ($P \leq 0.05$) within and between clones and irradiances (determined by LSD analyses based on 17 degrees of freedom) are identified with arrow bars (vertical arrow bar = within clone; horizontal arrow bar = between clones).

Variable	Effects	Irradiance	New Pass	Piney Island	Wilson
Biomass		60	4.64 ± 1.25	4.59 ± 0.40	5.09 ± 0.20
		165	3.53 ± 0.77	4.25 ± 0.77	5.09 ± 0.54
Chl a	I	60	14.07 ± 0.31	3.90 ± 1.29	14.42 ± 2.05
		165	8.66 ± 1.30	7.79 ± 0.65	8.23 ± 1.28
Chl c_1 and c_2	I	60	2.15 ± 0.01	2.34 ± 0.20	2.37 ± 0.29
		165	1.26 ± 0.20	1.05 ± 0.07	1.17 ± 0.18
Chl c_3	I, C	60	0.0104 ± 0.0025	0.045 ± 0.021	0.046 ± 0.007
		165	0.0089 ± 0.0047	0.017 ± 0.003	0.019 ± 0.004
Total chl	I	60	16.24 ± 0.30	16.28 ± 1.50	16.84 ± 2.34
		165	9.93 ± 1.51	8.85 ± 0.72	9.42 ± 1.46

photopigments present in the cultures. The combined photopigment content of the 3 clones averaged: 14.13 ± 1.22 pg $cell^{-1}$ chl *a* and 4.21 ± 0.38 pg $cell^{-1}$ fucoxanthin and 19'-acylofucoxanthins in cultures exposed to the low irradiance treatment; and 8.23 ± 1.08 pg $cell^{-1}$ chl *a* and 1.97 ± 0.26 pg $cell^{-1}$ fucoxanthin and 19'-acylofucoxanthins in cultures exposed to the high irradiance treatment. Significant decreases in total chl content in each clone, attributable to decreases in chl *a*, Chls c_1/c_2, and chl c_3 content, coincided with increasing irradiance (Table 1). The chl c_3 content in the New Pass clone exposed to high irradiance significantly differed from the Piney Island and Wilson clones (Table 1).

Significant decreases in total carotenoid content in each clone, attributable to decreases in total fucoxanthin and 19'-

Table 2 Carotenoid content (pg $cell^{-1}$) and chl/carotenoid ratios of 3 *K. brevis* clones exposed to irradiances of 60 and 165 µmol photons m^{-2} s^{-1} PAR. Data are means ± standard errors (n = 3). Clone (C) and irradiance (I) effects (determined by ANOVA) are significant ($P \leq 0.05$) if designated. Significantly different variable means ($P \leq 0.05$) within and between clones and irradiances (determined by LSD analyses based on 17 degrees of freedom) are identified with arrow bars (vertical arrow bar = within clone; horizontal arrow bar = between clones).

Pigment	Effects	Irradiance	New Pass	Piney Island	Wilson
Fucoxanthin + 19'-acylofucoxanthins	I	60	4.01 ± 0.06	4.29 ± 0.41	4.33 ± 0.68
		165	2.03 ± 0.30	1.74 ± 0.12	2.15 ± 0.36
Diadinoxanthin + diatoxanthin	I, C	60	1.42 ± 0.25	1.48 ± 0.17	1.51 ± 0.13
		165	1.50 ± 0.18	1.48 ± 0.10	1.96 ± 0.32
Gyroxanthin-diester	I	60	0.58 ± 0.05	0.55 ± 0.05	0.63 ± 0.11
		165	0.35 ± 0.06	0.33 ± 0.02	0.36 ± 0.05
β-carotene	I	60	0.037 ± 0.006	0.027 ± 0.005	0.036 ± 0.011
		165	0.051 ± 0.011	0.038 ± 0.008	0.036 ± 0.003
Total carotenoids	I	60	6.05 ± 0.35	6.35 ± 0.63	6.50 ± 0.91
		165	3.94 ± 0.56	3.59 ± 0.25	4.50 ± 0.73
Total chl/total carotenoid	I, C	60	2.68 ± 0.10	2.56 ± 0.02	2.59 ± 0.06
		165	2.52 ± 0.05	2.47 ± 0.05	2.09 ± 0.05
Gyroxanthin-diester/chl a	C	60	0.041 ± 0.002	0.041 ± 0.000	0.044 ± 0.002
		165	0.041 ± 0.001	0.042 ± 0.000	0.043 ± 0.001
Fucoxanthin + 19'-acylofucoxanthins/chl a	I, C	60	0.29 ± 0.00	0.31 ± 0.00	0.30 ± 0.01
		165	0.23 ± 0.00	0.22 ± 0.00	0.26 ± 0.00

acylofucoxanthins, and gyroxanthin-diester content, coincided with increasing irradiance (Table 2). The fucoxanthin and 19'-acylofucoxanthins/chl *a* ratios were significantly higher at low light within each clone, and significantly varied between clones; however, the gyroxanthin-diester/chl *a* ratios were not significantly different at low and high irradiance within each clone (Table 2). The gyroxanthin-diester/chl *a* ratios were significantly higher in the Wilson clone compared to the other clones at high and low light (Table 2). Diadinoxanthin and diatoxanthin were significantly higher at high light within the Wilson clone, and also significantly higher in the Wilson clone at both light treatments when compared to the other clones (Table 2).

Results indicate that the photosynthetically active pigments and the total carotenoids increased when exposed to the low irradiance treatment. These short-term acclimation responses to highly variable irradiance are well documented (Evens *et al.,* 2001; Millie *et al.,* 1995), yet little is known about differences in acclimation response between cultures isolated from different regions and exposed to varied periods of maintenance conditions. When comparing 3 clonal cultures exposed to maintenance conditions for approximately 6 months, 5 and 50 years, it was presumed there may be differences in physiology between the clones due to pigment acclimation or adaptation. The results show significant differences in certain pigments and pigment ratios between clones in response to varying irradiance, indicating further research relating to the pigment content and photophysiological responses of different *K. brevis* clones is needed. However, the ratio of gyroxanthin-diester and chl *a* remains constant within clones when exposed to varying irradiance. Even though certain pigment contents and ratios differ between clones, the gyroxanthin-diester/chl *a* ratios may be unchanged within and between more recently isolated clonal cultures and natural blooms when exposed to varying irradiance. These findings support the consideration that the gyroxanthin-diester/chl *a* ratio is a unique "biomarker" and can be incorporated into future pigment-based monitoring of natural *K. brevis* blooms.

Acknowledgements

The Florida Fish and Wildlife Conservation Commission (FMRI) and ECOHAB-FL through the NOAA Coastal Oceans Program and the U.S. EPA jointly funded this study.

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A Cell Cycle Synchronization and Culture Purification Technique for Heterotrophic *Pfiesteria* and Cryptoperidiniopsoid Dinoflagellates Analyzed by Flow Cytometry

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Abstract

A method was described for obtaining highly synchronous flagellated cell populations of phagotrophic *Pfiesteria* spp. and cryptoperidiniopsoid dinoflagellates. Cyst populations were held in deionized water for 2–5 days, and flagellated cells excysted upon resumption of previous culture salinity. Encysted cells appeared undamaged by deionized water treatments, and a concurrent sodium hypochlorite purification treatment was used on *P. shumwayae* cysts to reduce or eliminate contaminants. Vacuolar DNA from previous phagotrophy was minimized by digestion during the arrest period, improving flow cytometric nuclear DNA measurements compared to previous analyses of untreated populations. Initial synchrony of flagellated cell populations typically was greater than 90% 1C DNA; 2C DNA subpopulations likely consisted of diploid cells and/or haploid G2 phase cells. The techniques described may be useful in ecological, biochemical, and molecular investigations of these and similar heterotrophic dinoflagellates.

Introduction

Progression through the cell cycle is defined by the physiological events of genome replication, nuclear division, and cytokinesis. In protists, cell cycle events are closely linked to behavior, biochemistry, and life cycle differentiation (Wong, 1996). Study of synchronous populations can provide insights into the development, ultrastructure, and mean duration of specific stages in heteromorphic dinoflagellates (Kubai and Ris, 1969; Bhaud *et al.,* 2000), and effective investigation of regulatory and cyclic biochemical and molecular events in proliferating cells often requires synchronized populations. Many photosynthetic dinoflagellates exhibit natural diel division phasing, which has allowed study of intrinsic and extrinsic factors influencing cell cycle-related physiology (Gerath and Chisholm, 1989; Van Dolah and Leighfield, 1999). Heterotrophic species rarely have been described with diel synchrony (*e.g.,* Ucko *et al.,* 1997), and few have been manipulated into cell cycle synchrony. *Crypthecodinium* species are a notable exception: nonmotile cells (cysts) can be propagated on solid media, and synchronously excysted flagellated cells can be collected after addition of liquid media (Bhaud *et al.,* 1991, 1994; Wong and Whiteley, 1996).

For some dinoflagellate species, reproductive cyst adherence has been used to isolate synchronous populations (Kubai and Ris, 1969; Franker *et al.,* 1973), and resistance of encysted cells to lysis has been used to obtain roughly synchronized populations by selective lysis of less-resistant flagellated cells (Franker, 1971; Parrow *et al.,* 2002). *Pfiesteria* spp. and cryptoperidiniopsoids are estuarine phagotrophic dinoflagellates that undergo cell division in nonmotile cysts that usually occur attached to surfaces in cultures. Submersion of these cysts in deionized (DI) water does not halt cell division, but arrests excystment of flagellated offspring (Parrow *et al.,* 2002; Parrow and Burkholder, 2003a,b). Here, a method was described for obtaining highly synchronized flagellated cell populations of these dinoflagellates with minimal ingested prey DNA, and a simple method was tested for culture purification. The degree of synchrony and reduction of vacuolar DNA were determined by flow cytometric DNA measurements.

Materials and Methods

Dinoflagellates in culture at the CAAE ($n \geq 3$ clones per taxon) were grown in flasks with cryptomonads or in aquaria and fed live fish (Burkholder *et al.,* 2001). Vessels with relatively dense cyst accumulations were rinsed briefly with, and then immersed in, DI water to rinse away and/or lyse flagellated cells and reduce the dinoflagellate populations to cysts. Cysts were held in DI water for 24–120 h, and then were covered in sterile 15-psu artificial seawater. Percent excystment was determined by monitoring fields of view containing at least 50 cysts with light microscopy (LM; Olympus IX70, 200×), and marking excystment over time on a video monitor. Within 3 h the excysted flagellates were decanted away from remaining cysts. For flow cytometry, flagellated cell samples were fixed with 0.5% paraformaldehyde, treated with RNase A (1 μg mL^{-1}, Sigma), and stained with 5 μM SYTOX Green (Molecular Probes) (Parrow *et al.,* 2002). Multicycle Software (Phoenix Flow Systems) was used to compute peak numbers and CVs (relative standard deviation). Vacuolar DNA was assessed based on 1C peak CV values.

Effects of sodium hypochlorite treatment were tested on cysts from 2 *P. shumwayae* clones. *P. shumwayae* cysts were collected for purification in petri dishes placed in aquaria and incubated in DI water. Cysts were gently scraped free, resuspended in 40 mL of DI water, shaken vigorously, pelleted by centrifugation (2000 rpm for 10 min), then re-suspended in DI water with 0%, 0.005%, 0.05% or 0.5% sodium hypochlorite (dilution of a 6% sodium hypochlorite commercial bleach solution) and shaken. After 5 min, cysts were re-pelleted and cleaned twice by shaking then pelleting in sterile DI water, then resuspended in sterile 15-psu artificial seawater and observed for excystment and contaminants under LM. Subsamples (1 mL) were inoculated into culture tubes with 20 mL 15-psu f/2 media enriched with 1 g L^{-1} bacto-peptone and 1 g L^{-1} glucose, and checked daily

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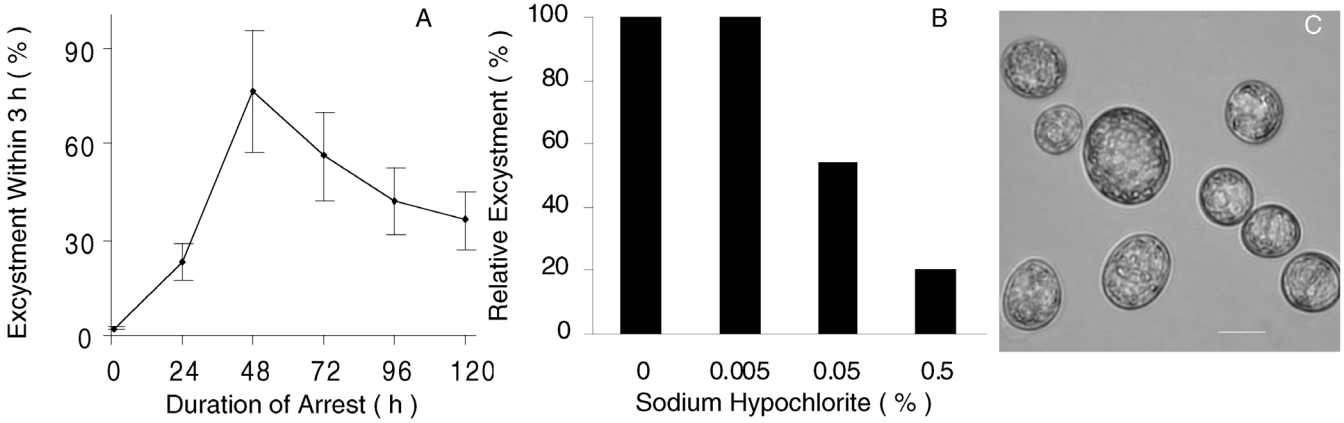

Figure 1 **A** Percent excystment within 3 h of resumed culture salinity from representative *P. piscicida* cyst beds (≥50 cysts) after increased duration of arrest in DI water (means ± 2 SE; n = 3). **B** Percent excystment of *P. shumwayae* after hypochlorite treatment relative to that of untreated cysts (n = 2). **C** Visually contaminant-free *P. shumwayae* cysts from an aquarium culture after treatment with 0.05% sodium hypochlorite (bar = 10 μm).

over 12 days for turbidity as evidence of bacterial or fungal growth; uninoculated tubes were clarity controls (Hoshaw and Rosowski, 1973).

Results and Discussion

Cell division(s) occurred in cysts submerged in DI water, but flagellated offspring did not emerge in the osmotically unfavorable medium. The duration of arrest was longer than the time typically required for completion of divisions and excystment (Parrow *et al.,* 2002; Parrow and Burkholder, 2003a,b), forcing many cysts into developmental synchrony. A robust excystment response typically occurred within 3 h of resumed salinity (Fig. 1A). Although the arrest conditions did not appear harmful over shorter durations, periods of arrest longer than optimal typically resulted in less vigorous excystment (Fig. 1A), perhaps due to cell impairment, death, or induced dormancy. In this study, around 48 h of arrest optimized excystment for *P. piscicida* (Fig. 1A) and the cryptoperidiniopsoid (ca. 60–90% excystment), and around 72 h was optimal for *P. shumwayae* (ca. 60–80% excystment). *P. shumwayae* cysts were somewhat resistant to sodium hypochlorite under these conditions (Fig. 1B). Initial cyst populations from aquarium cultures contained visible bacterial, fungal, and protozoan contaminants, some of which survived treatment with DI water (Parrow and Burkholder, 2003b) and 0.005% hypochlorite, and enriched broth tests from those treatments clouded visibly in 1–3 days. Treatments with 0.05% and 0.5% hypochlorite visually reduced contaminants (Fig. 1C), and broth tests indicated decreased contaminant abundance/diversity based on longer durations (ca. 4–7 days) of broth clarity. The inoculated broth remained clear over the observation period in 40% of treatments with 0.5% hypochlorite, indicating that demonstrable contaminants were eliminated.

The synchronously excysted flagellated cells were similar in size, colorless, and rapidly swimming. Cell pairs in apparent fusion were observed with variable frequency (ca. 0–5% of flagellated cells) in excysted populations, but were highly motile and thus escaped continuous observation. Some isolated flagellated cells produced small cysts that were morphologically consistent with non-reproductive temporary cysts (Parrow *et al.,* 2002). Flow cytometric DNA analysis showed that the released flagellated cell populations of each taxon were composed primarily (≥90%) of 1C DNA cells (Fig. 2). The 2C DNA cells present in each population most likely consisted of diploid planozygotes

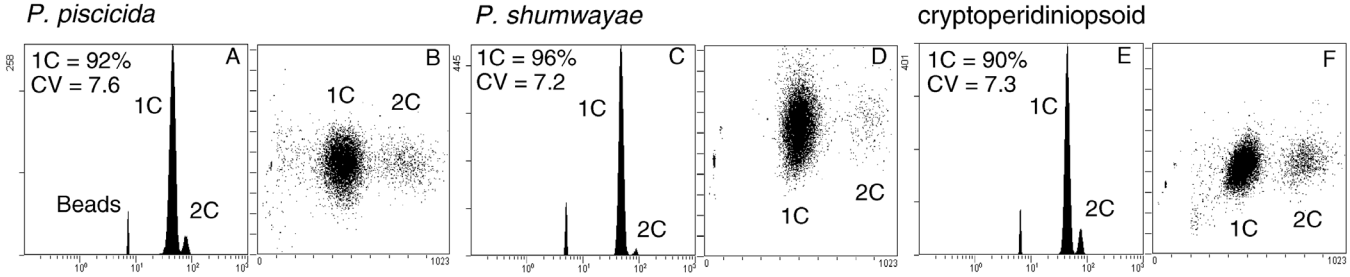

Figure 2 Representative cellular DNA-fluorescence plots for synchronously excysted flagellated cell populations. Bottom axes are arbitrary units of relative DNA in all plots (**A**, **C**, **E**, logarithmic scale; **B**, **D**, **F**, linear scale); vertical axes in two-parameter scatter plots (**B**, **D**, **F**) are linear forward scatter (a sizing parameter). **A**, **B**, *P. piscicida*; **C**, **D**, *P. shumwayae;* **E**, **F**, cryptoperidiniopsoid (beads = fluorescence standard).

and/or G2 cell cycle phase haploid cells. Cells with 2C DNA were not detectably larger than 1C cells, based on flow cytometric forward scatter signals (Fig. 2). The 1C peak CVs were <8.3, comparable to whole-cell values obtained for photosynthetic dinoflagellates using the same DNA stain (Veldhuis *et al.,* 1997), and lower than values reported for *Pfiesteria* spp. populations analyzed without use of this synchronization technique (Parrow and Burkholder, 2002). Thus vacuolar DNA from previous phagotrophy apparently was minimized by digestion during the forced quiescence in DI water. Observation and population DNA measurements indicate that flagellated cells of *Pfiesteria* spp. and cryptoperidiniopsoids, whether haploid or diploid, are primarily in interphase of the cell cycle (Parrow *et al.,* 2002, Parrow and Burkholder, 2003a,b). Determination of the full cell cycle progression of these taxa will require inclusion of encysted cells or their nuclei in population DNA analyses since all or most nuclear divisions occur in cysts. The presence of ingested and exogenous DNA complicates genomic DNA analyses of these phagotrophic microorganisms. The synchronization and purification methods described here resulted in cells with minimized vacuolar DNA and external contaminants, which would be useful in cell cycle, biochemical, and molecular investigations. The synchronization technique was relatively simple but effective, and could be beneficial in studies of the cell biology, physiology, and ecology of these heterotrophic dinoflagellates.

Acknowledgments

We thank Dr. H. Marshall for providing a cryptoperidiniopsoid culture, and Dr. P. Rublee for assistance in molecular taxonomy. Funding was provided by the NSF (#9912089) and the NC General Assembly.

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