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A comparison of future increased CO_2 and temperature effects on sympatric *Heterosigma akashiwo* and *Prorocentrum minimum*

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Abstract

Very little is known about how global anthropogenic changes will affect major harmful algal bloom groups. Shifts in the growth and physiology of HAB species like the raphidophyte *Heterosigma akashiwo* and the dinoflagellate *Prorocentrum minimum* due to rising CO_2 and temperature could alter their relative abundance and environmental impacts in estuaries where both form blooms, such as the Delaware Inland Bays (DIB). We grew semi-continuous cultures of sympatric DIB isolates of these two species under four conditions: (1) 20 °C and 375 ppm CO_2 (ambient control), (2) 20 °C and 750 ppm CO_2 (high CO_2), (3) 24 °C and 375 ppm CO_2 (high temperature), and (4) 24 °C and 750 ppm CO_2 (combined). Elevated CO_2 alone or in concert with temperature stimulated *Heterosigma* growth, but had no significant effect on *Prorocentrum* growth. P_{Bmax} (the maximum biomass-normalized light-saturated carbon fixation rate) in *Heterosigma* was increased only by simultaneous CO_2 and temperature increases, whereas P_{Bmax} in *Prorocentrum* responded significantly to CO_2 enrichment, with or without increased temperature. CO_2 and temperature affected photosynthetic parameters α, Φ_{max}, E_k, and $\Delta F/F'_m$ in both species. Increased temperature decreased and increased the Chl *a* content of *Heterosigma* and *Prorocentrum*, respectively. CO_2 availability and temperature had pronounced effects on cellular quotas of C and N in *Heterosigma*, but not in *Prorocentrum*. Ratios of C:P and N:P increased with elevated carbon dioxide in *Heterosigma* but not in *Prorocentrum*. These changes in cellular nutrient quotas and ratios imply that *Heterosigma* could be more vulnerable to N limitation but less vulnerable to P-limitation than *Prorocentrum* under future environmental conditions. In general, *Heterosigma* growth and physiology showed a much greater positive response to elevated CO_2 and temperature compared to *Prorocentrum*, consistent with what is known about their respective carbon acquisition mechanisms. Hence, rising temperature and CO_2 either alone or in combination with other limiting factors could significantly alter the relative dominance of these two co-existing HAB species over the next century.

Keywords: *Heterosigma akashiwo*; CO_2; HAB; Global change; Dinoflagellates; Raphidophytes

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1. Introduction

The frequency and intensity of harmful algal blooms (HABs) have increased, coinciding with a global surge in population density in the coastal zone (Mudie et al., 2002; Hallegraeff, 1993) due to cultural eutrophication

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in recent decades (Anderson et al., 2002). In response to these observations, a great deal of the HAB research effort has focused on the influence of eutrophication and nutrient availability on bloom establishment and growth of species such as *Aureococcus*, toxic dinoflagellates and raphidophytes (Anderson et al., 2002; Popels et al., 2003; Pustizzi et al., 2004; Gobler et al., 2005; Zhang et al., 2006).

Eutrophication is however only one of multiple global anthropogenic biogeochemical impacts. In addition to human disturbance of natural nutrient cycles, we are also causing a massive perturbation of the global carbon cycle. Atmospheric CO_2 will more than double by 2100 due to ever-accelerating rates of fossil fuel burning (IPCC, 2001). Consequently, ocean pH will decrease by as much as 0.77 units within the next several centuries (Caldeira and Wickett, 2003). At the same time, over the next 50–100 years greenhouse warming will increase average sea surface temperatures by as much as 4–5 °C (Bopp et al., 2001; Sarmiento et al., 2002).

These changes in the coming decades will drive massive changes in the biology and chemistry of the oceans. For instance, changes in CO_2 availability can exert a strong control on algal physiology, nutrient cycling and ecological interactions. Experiments with natural phytoplankton communities suggest that one response to elevated CO_2 could be increased phytoplankton primary production in the open ocean (Hein and Sand-Jensen, 1997), or that CO_2 concentrations could affect competition among major marine phytoplankton groups (Tortell et al., 2002). A recent model suggests that the growth of marine phytoplankton will increase by 40% between current CO_2 levels and 700 ppm CO_2 (Schippers et al., 2004). Laboratory studies have shown that altered CO_2 can result in species-specific changes in cellular carbon acquisition pathways (Raven, 1997; Tortell et al., 2000; Burkhardt et al., 2001) and elemental ratios (Burkhardt et al., 1999; Tortell et al., 2000; Fu et al., 2007; Hutchins et al., 2007). Important cyanobacterial functional groups such as *Synechococcus*, *Trichodesmium* and *Prochlorococcus* will all respond differently to elevated CO_2 (Fu et al., 2007; Hutchins et al., 2007). How major HAB groups such as dinoflagellates and raphidophytes will react to globally increasing CO_2 concentrations is, however, just now beginning to be examined (Rost et al., 2006).

Many algal groups, including dinoflagellates, have evolved carbon concentrating mechanisms (CCMs) to elevate the concentrations of CO_2 near the active site of Rubisco. These mechanisms include an active transport system for bicarbonate uptake (Sukenick et al., 1997), as well as a catalyzed dehydration of bicarbonate to CO_2 by the enzyme carbonic anhydrase (CA, Badger and Spalding, 2000). However, active inorganic carbon transport and CA activity is not ubiquitous throughout all algal groups. In algae, CO_2 is the preferred carbon substrate for the principle carbon-fixing enzyme Rubisco. However, Rubisco efficiencies can differ between different groups (Badger et al., 1998; Tortell, 2000) and hence carbon fixation could be stimulated by rising CO_2 for some species but not for others.

Notably, the toxic raphidophyte *Heterosigma*, which produces devastating blooms in Delaware and around the world (Whereat, 2003; Honjo, 1992), does not appear to utilize CA (Nimer et al., 1997). This suggests that *Heterosigma* and possibly other raphidophytes may be especially favored by rising CO_2 levels. Many dinoflagellates possess a form II Rubisco that has a lower affinity for carboxylation and is extremely inefficient at processing CO_2 compared to the form I Rubisco found in all other algae (Whitney and Yellowlees, 1995). However, several studies have documented effective carbonic anhydrase activity in some dinoflagellates, including *Prorocentrum micans* and *Prorocentrum minimum* (Nimer et al., 1999; Rost et al., 2006). Other species however appear to have very limited HCO_3^- uptake capabilities (Dason and Huertas, 2004), thus there is likely a potential for carbon limitation in some dinoflagellates.

Increases in sea surface temperature may also impact carbon utilization, resulting in shifts in species composition and diversity. Likewise, temperature can differentially impact growth rate, pigment content, light harvesting capacity and photosynthetic carbon fixation in microalgae and cyanobacteria (Sosik and Mitchell, 1994; Coles and Jones, 2000; Anning et al., 2001; Fu et al., 2007). It has been suggested that climate change-driven temperature increase is the main factor stimulating HAB blooms in the Pacific and Atlantic regions of Canada over the past 50 years, based on pre-industrial sedimentary cyst records of red tide histories (Mudie et al., 2002).

Global warming trends are likely to have large consequences for HAB dominance (Eppley, 1972; Raven and Geider, 1988), however, how a combination of simultaneously rising temperature and CO_2 will affect their growth is completely unknown. To our knowledge only two laboratory studies have examined potential effects of rising temperatures on HAB species (Peperzak, 2003, 2005), and none have examined interactions with CO_2.

In the Delaware Inland Bays (DIB), harmful raphidophytes such as *Heterosigma akashiwo* and dinoflagellates such as *P. minimum* have come to dominate the phytoplankton community during much of the year. These various species often form mixed blooms (Zhang et al., 2006). These that include the related alga currently known as *Chattonella* cf. *verruculosa* are associated with toxins in the water (Bourdelais et al., 2002), and have caused massive mortality of fish, crabs and other marine life. Similar devastasting *Heterosigma* blooms have been observed around the world (Honjo, 1992), although to date no fish kills have been definitively attributed to this raphidophyte in the DIB.

In general, our approach was to gather information on the growth, photosynthetic physiology and elemental quotas and ratios of sympatric *H. akashiwo* and *P. minimum* isolates from the Delaware Inland Bays under conditions of elevated CO_2 and/or temperature. DIB is an estuarine ecosystem, so there are typically diel variations in aqueous pCO_2 which are dependent on tidal cycle, photosynthesis in the day, and respiration in the dark. Temperature also obviously varies over tidal, daily and seasonal timescales. As these temporal fluctuations are not possible to simulate realistically in laboratory cultures, our experiments out of necessity used mean late springtime/early summer temperature and pCO_2 values for the present day and the predicted year 2100 treatments. Cell physiological and biochemical characteristics under these conditions were then compared, with the intention of shedding light on possible effects of global change on inter-specific competition and dominance within multiple-species HAB events in the DIB.

2. Materials and methods

2.1. Isolates and culture conditions

We originally isolated *H. akashiwo* (deposited as CCMP2393 at the Center for the Culture of Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, ME, USA) and *P. minimum* (CCMP 2233) from Love Creek, Indian River Bay, Delaware, USA in August 2001. Both stock cultures were maintained in f/2 seawater medium (salinity ~23%) and grown under cool white fluorescent light at an irradiance of 100 μmol quanta m^{-2} s^{-1} with a 12:12 h light:dark cycle at 22 °C. The stock cultures were maintained in exponential growth phase by dilution using sterile techniques approximately every 10 days.

2.2. Experimental design

Experiments were performed in triplicate 1 l acid-washed and autoclaved polycarbonate bottles containing 0.6 l medium. Temperatures used in this study were 20 and 24 °C, to simulate typical present day late spring or early summertime temperatures in the DIB (Zhang et al., 2006) and expected average increases of ~4 °C over the next century (Sarmiento et al., 2002). Within each temperature, triplicate bottles were equilibrated with two different CO_2 concentrations: 375 ppm (present day average atmospheric concentrations) and 750 ppm (projected mean year 2100 concentrations, IPCC, 2001).

The four different CO_2/temperature conditions used in this study were therefore: 20 °C and present day CO_2, 375 ppm (“control”); 20 °C with high CO_2, 750 ppm (“high CO_2”); 24 °C and 375 ppm CO_2 (“high temperature”) and 24 °C with 750 ppm CO_2 (“combined”). Different CO_2 concentrations were obtained by gentle bubbling with filtered ambient air and a commercially prepared air/CO_2 mixture (Scott Gas). The pH in each bottle was monitored every day using a microprocessor pH-meter, calibrated with pH 7 and 10 buffer solutions. CO_2 equilibration was also verified using dissolved inorganic carbon (DIC) measurements (data not shown, see methods below).

2.3. Determination of growth rates and Chl a

Experiments used identical semi-continuous culturing methods with each species in order to measure temperature and CO_2 effects during acclimated, steady-state growth (Fu et al., 2007; Hutchins et al., 2007). Semi-continuous cultures were diluted daily based on growth rates calculated from *in vivo* chlorophyll fluorescence using a Turner 10AU fluorometer (Turner Designs) and volumes of dilutions were adjusted to maintain constant growth rates and allow biomass to reach pre-dilution levels. The variability in nutrient concentrations due to daily dilution was less than 20%. For all experiments, final sampling occurred once steady-state growth was obtained for each growth condition (typically after 10–14 generations). Steady-growth status was defined by no significant difference in growth rates for at least three consecutive transfers.

Daily live microscopic cell counts and in vivo fluorescence (measured with a Turner 10AU fluorometer) were used to monitor growth rates (Fu et al., 2006). Growth rates were calculated from the equation $\mu = (\ln N_2 - \ln N_1)/(t_2 - t_1)$, where N_1 and N_2 are the average values of cell numbers at times t_1 and t_2. For

chlorophyll *a* (Chl *a*) determination, duplicate culture subsamples of 10–20 ml from each of the triplicate bottles for the treatments were filtered onto a GF/F glass fiber filter, extracted in 5 ml of 90% acetone, and stored overnight in darkness at −20 °C. Chl *a* concentrations were measured using a Turner 10-AU fluorometer.

2.4. Determination of P–E curves, primary production and light absorption

Photosynthetic parameters obtained from ^{14}C photosynthesis–irradiance curves were used to evaluate mechanisms of adaptation and response of phytoplankton photosynthesis to changing temperature and CO_2. In addition, standard 24 h primary production measurements were used to assess the daily carbon production rates of the two species under the experimental conditions. Measurements of photosynthesis versus irradiance (*P–E*) curves were performed by measuring ^{14}C fixation rates at a range of light intensities using a photosynthetron (CHPT Inc.). Five milliliters culture samples were harvested into 7-ml vials. Each vial was spiked with 0.1 μCi ml^{-1} ^{14}C sodium bicarbonate (MP Biomedicals). One vial in each set was wrapped in aluminum foil and used to estimate of dark ^{14}C uptake. Light intensities in each slot varied from 10 to 900 μmol photons m^{-2} s^{-1}. All samples were incubated at the growth temperature of the experimental culture. After a 30 min incubation, the cells were killed with gluteraldehyde to terminate ^{14}C uptake and then the content of each vial was filtered onto 0.4 μm Millipore polycarbonate filters and rinsed well with 0.2 μm filtered seawater to remove adsorbed unassimilated bicarbonate from cell surfaces. The incubation time was shortened to 30 min to obtain production values as close as possible to gross production (Williams, 1993). Total added activity was determined from 100 μl aliquots in the samples and added immediately into 5-ml scintillation cocktail containing 50-μl 4% (v/v) phenylethylamine. Five milliliters of scintillation cocktail was added and the filters were incubated in the dark overnight and then counted using a Wallac System 1400 liquid scintillation counter.

All ^{14}C uptake rates were corrected for dark uptake and carbon assimilation values were subsequently normalized to Chl *a*. The initial slope of the *P* versus *E* curve, i.e. photosynthetic efficiency α [mg C (mg Chl $a)^{-1}$ h^{-1} (μmol quanta m^{-2} $s^{-1})^{-1}$] and the maximum chlorophyll specific carbon fixation rate P_{Bmax} [mg C (mg Chl $a)^{-1}$ h^{-1}] were calculated by least-squares nonlinear regression using the exponential function of Platt et al. (1980), with corrections for any down-turn in carbon fixation at elevated irradiance (β). E_k, the light saturation point and index of light adaptation, was calculated as P_{Bmax}/α.

For determination of primary production, 30 ml culture samples were harvested into 35-ml clear polycarbonate flask and 0.2 μCi ml^{-1} ^{14}C sodium bicarbonate (MP Biomedicals) was added to each flask. Triplicate sets of flasks were exposed to the growth temperature and light intensities of their growth conditions for 24 h. Each experiment used duplicate killed control blanks at each treatment; these blank values were subtracted from reported activities of live cells. The initial total DIC concentrations and algal biomass (Chl *a*) were used for calculations of primary production. Calculations were the same as those for carbon fixation in the *PE* curves.

Absorption spectra were recorded by concentrating cells onto GF/F filters by gentle filtration, followed by scanning each filter from 400 to 750 nm on a Shimadzu UV-2401 recording spectrophotometer equipped with an integrating sphere. Residual scattering was corrected for differential scattering at each wavelength by subtracting the optical density measured at 750 nm. The corrected optical densities were then converted to Chl *a* normalized absorption coefficients a^*, using previously published β correction factors (Cleveland and Weidemann, 1993). The spectral mean Chl-specific absorption coefficient ($\bar{a}*$, 400–700 nm) was then calculated. This measure has been documented to be more suitable than absorption at a given wavelength to characterize the overall phytoplankton absorption (Markager and Vincent, 2001). Maximum quantum yield of photosynthesis (Φ_{max}) was calculated from the ratio of α^{chla} to $\bar{a}*$, scaled by a constant of 0.02315 to convert grams of carbon to moles of carbon and hours to seconds (Park et al., 2002).

2.5. Fluorescence induction measurements

Chlorophyll fluorescence was monitored by pulse amplitude modulation fluorometry (PAM 101, Walz, Germany) in a high sensitivity liquid suspension cuvette with constant stirring (ED-101, Walz, Germany). Fluorescence excitation was provided by a blue LED measuring light (470 nm, intensity <1 μmol photon m^{-2} s^{-1}), and emission ≥645 nm was detected by a photodiode. Actinic irradiance was provided by a blue LED array (470 nm peak, HPL-470, Walz, Germany) set to the growth irradiance (100 μmol photons m^{-2} s^{-1}), and saturation pulses (400 ms, 3000 μmol photon m^{-2} s^{-1}) were provided by the same LED array to

record dark and light acclimated maximal fluorescence. Prior to fluorescence measurements, the fluorometer was zeroed to a sample of freshly filtered (0.2 μM) media from each species. The minimum fluorescence level (F_0) was measured after dark acclimation for 10 min and then followed by a short saturating flash to determine the maximal PSII quantum yield (F_v/F_m). Fluorescence induction was then recorded by switching on the actinic light, and the effective quantum yield of PSII in the light acclimated state ($\Delta F/F'_m$ or Φ_{PSII}) was recorded after 8 min of illumination.

2.6. Analysis of POC, PON and POP

Samples for the analysis of particulate organic carbon (POC), particulate organic nitrogen (PON) and particulate organic phosphorus (POP) were collected on precombusted (450 °C for 5 h) GF/F glass fiber filters under low vacuum. POC and PON were analyzed on a 440 Elemental Analyzer follow the protocol of Fu et al. (2006). POP was measured as in Fu et al. (2005).

2.7. Analysis of dissolved inorganic carbon

Sub-samples from each bottle for dissolved inorganic carbon (DIC) analysis were preserved with 0.12 mg Hg Cl_2 l^{-1}, stored at 4 °C, and analyzed in triplicate. All DIC samples were analyzed within 1 week after final sampling. Total dissolved inorganic carbon (DIC) was determined using an acid sparging instrument built in the laboratory of J.H. Sharp at the University of Delaware that is a modified from an instrument developed at the Monterey Bay Aquarium Research Institute by G. Friederich (Walz and Friederich, 1996; analytical details are described Fu et al., 2007).

2.8. Statistical analysis

Growth rates, photosynthetic parameters, and cell quotas and elemental ratios in different experiments were assessed using a one-way ANOVA or Student's *t*-test (Zar, 1999). Differences between treatment groups were tested by Tukey HSD multiple comparisons. Differences are termed significant when $p < 0.05$.

3. Results

3.1. Growth

Cell-specific growth rates of *Heterosigma* and *Prorocentrum* under the four CO_2 and temperature conditions are shown in Fig. 1. *Heterosigma* specific growth rates were significantly lower in the controls relative to the other three treatments ($p < 0.05$), but were not significantly different among the other three treatments at values of ~0.7 day^{-1} ($p > 0.05$). Increases in either temperature or CO_2 alone, or both together, stimulated growth rates about 33% compared to controls (Fig. 1A). In contrast with *Heterosigma*, neither elevated CO_2 nor increased temperature significantly affected growth rates of *Prorocentrum* ($p > 0.05$, Fig. 1B).

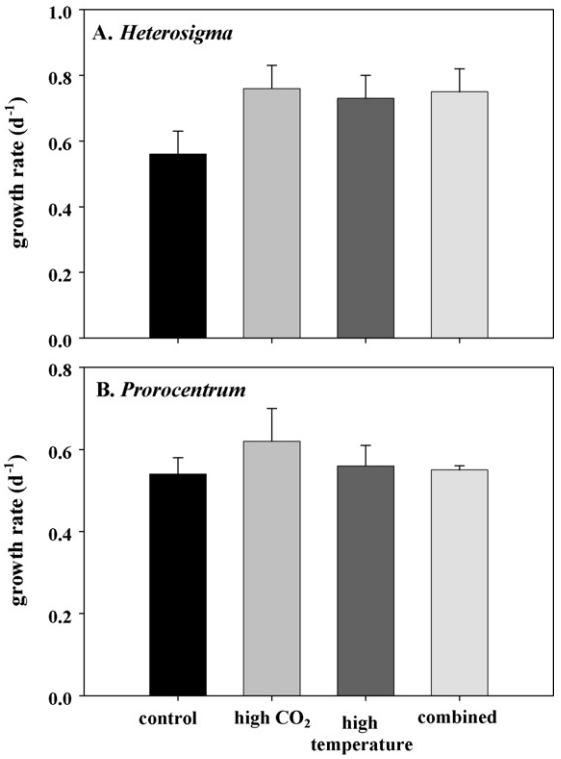

Fig. 1. Growth rates of *Heterosigma akashiwo* (A) and *Prorocentrum minimum* (B) in the four temperature and CO_2 treatments. Error bars denote standard deviations of averaged results from three replicate bottles.

3.2. Photosynthesis and primary production

The response curve of photosynthetic carbon fixation as a function of irradiance (*P–E* curves) in *Heterosigma* was affected by both CO_2 and temperature (Fig. 2A). The photosynthetic parameters obtained from curve fitting are shown in Table 1. Photosynthetic rates in both cultures reached a plateau with no sign of photoinhibition at higher irradiance. For *Heterosigma*, P_{Bmax} increased, albeit not significantly when grown at 24 °C ($p < 0.05$, Table 1). However, the effect of CO_2

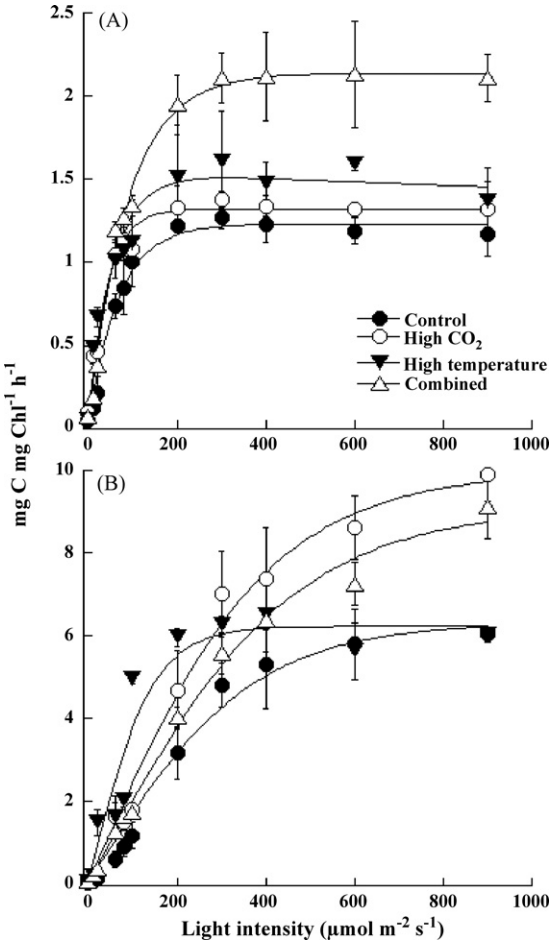

Fig. 2. *PE* curves (photosynthesis vs. irradiance curves) for *H. akashiwo* (A) and *P. minimum* (B) in the four temperature and CO_2 treatments. Error bars denote standard deviations of averaged results from three replicate bottles.

enrichment on P_{Bmax} was dependent on temperature. At 20 °C, there was no significant difference in P_{Bmax} between the control and high CO_2 treatment, while increasing both CO_2 and temperature together in the combined treatment yielded the greatest enhancement of P_{Bmax} ($p < 0.001$, 60% higher than the control).

Similar to the trend of P_{Bmax} obtained from short-term *P–E* curves, increases in temperature or CO_2 either alone or together stimulated the 24 h primary productivity of *Heterosigma* under the experimental culture light conditions (Fig. 3A). Increased CO_2 alone increased carbon fixation rates slightly compared to the control, while raising temperature increased rates still further. Increases in primary production rates in the combined treatment were roughly equivalent to the sum of the individual CO_2 and temperature-induced increases, yielding primary productivity rates that were 69% higher than in the control.

There was a significant rise in the Chl *a*-specific photosynthetic efficiency (α) ($p < 0.002$) when *Heterosigma* was grown under elevated CO_2 or elevated temperature alone, or when both variables were combined as compared to α in algae grown at 20 °C (Table 1). The differential response in α and P_{Bmax} between culture conditions resulted in unique shifts in the saturation intensity for photosynthesis (E_k). In the elevated CO_2 and elevated temperature treatments, E_k fell significantly compared to the control treatment due to a significant rise in α and only a slight increase in P_{Bmax}. Meanwhile, combined conditions resulted in no significant change in E_k, as P_{Bmax} increased to a greater extent than α (Table 1). Generally, the ratios of E_k to the culture irradiance were <1, suggesting that no light limitation occurred in *Heterosigma*. Maximal photosynthetic quantum yield (Φ_{max}) increased in all treatments, but this was significant ($p = 0.02$) only

Table 1
Functional photosynthetic performance parameters and pigment content for *Heterosigma* and *Prorocentrum* cells grown different conditions

	P_{Bmax}	α	E_k	F_v/F_m	$\tilde{a}*$	Φ_{max}	Chl *a*
Heterosigma							
Control	1.29 a (0.08)	0.018 a (0.002)	75 a (10)	0.77 a (0.01)	0.0108 a (0.0009)	0.059 a (0.003)	3.59 (0.25)
High CO_2	1.33 a (0.03)	0.032 b (0.005)	42 b (7)	0.76 a (0.01)	0.0122 a (0.0021)	0.086 bc (0.003)	3.34 (0.49)
High temp.	1.49 a (0.25)	0.033 b (0.005)	47 b (13)	0.79 a (0.05)	0.0124 a (0.0009)	0.086 ac (0.0190)	2.76 (0.43)
Combined	2.14 b (0.12)	0.024 b (0.002)	88 a (1)	0.76 a (0.03)	0.0132 a (0.0019)	0.061 ac (0.014)	2.77 (0.26)
Prorocentrum							
Control	6.40 a (0.30)	0.017 a (0.004)	384 a (85)	0.52 ac (0.01)	0.0159 ac (0.0009)	0.039 a (0.006)	1.16 (0.16)
High CO_2	10.11 b (0.37)	0.026 bcd (0.002)	396 a (51)	0.46 a (0.05)	0.0175 bc (0.0023)	0.052 ac (0.007)	0.98 (0.09)
High temp.	5.81 a (0.58)	0.030 d (0.004)	197 b (21)	0.60 b (0.01)	0.0132 a (0.0014)	0.074 bc (0.015)	1.49 (0.53)
Combined	9.23 b (0.78)	0.019 ac (0.001)	451 a (18)	0.55 bc (0.01)	0.0138 ac (0.0014)	0.049 a (0.003)	1.78 (0.31)

Units: P_{Bmax} (mg C mg Chl a^{-1} h^{-1}), α (mg C h^{-1} (mg Chl a)$^{-1}$ (μmol m^{-2} s^{-1})$^{-1}$, E_k (μmol m^{-2} s^{-1}), $\tilde{a}*$ (m^2 (mg Chl a)$^{-1}$), Φ_{max} (mol C (mol quanta)$^{-1}$), Chl *a* (pg cell^{-1}). Values sharing the same letters are not significantly different from each other. Values are the mean of triplicate measurements (±S.D.). Different letters designate significant difference between variables ($p < 0.05$) for multiple comparison tests.

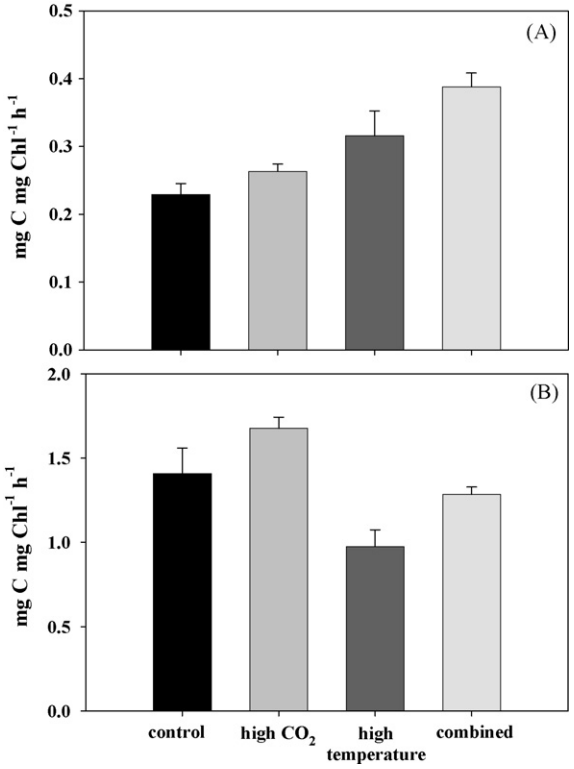

Fig. 3. Primary production for *H. akashiwo* (A) and *P. minimum* (B) in the four temperature and CO_2 treatments. Error bars denote standard deviations of averaged results from three replicate bottles.

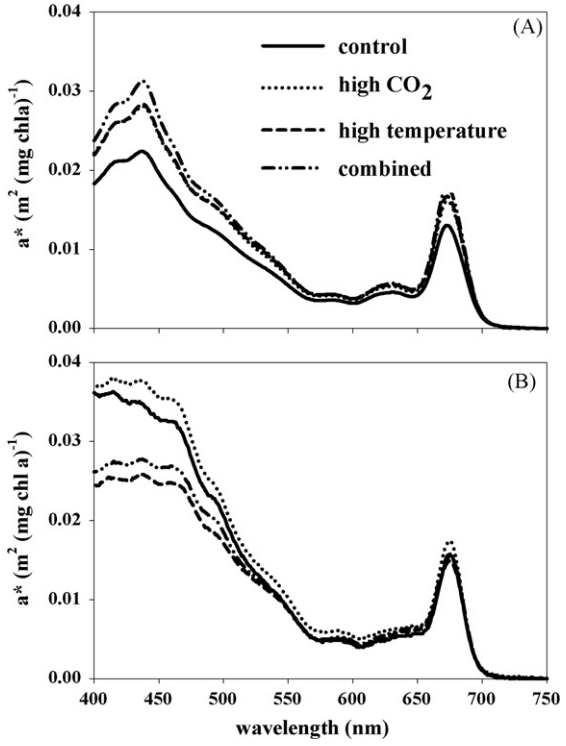

Fig. 4. Spectral absorption for *H. akashiwo* (A) and *P. minimum* (B) in the four temperature and CO_2 treatments.

when comparing the high CO_2 treatment against the controls (Table 1). There were no significant changes in the maximal quantum yield of PSII (F_v/F_m) (Table 1) or in the light acclimated PSII effective quantum yield (not shown).

Cellular Chl *a* content of *Heterosigma* was significantly higher in control cultures than in elevated temperature treatments, irrespective of CO_2 conditions (Table 1). Chl *a*-specific absorption spectra (a^*) are presented in Fig. 4. The increase in the average absorption coefficient ($\bar{a}*$) at elevated temperature or elevated CO_2 exposure was not statistically significant in *Heterosigma* (Table 1). However, comparisons of a^* at 676 nm, which results solely from Chlorophyll *a* absorption, did reveal significantly higher values ($p = 0.015$) for CO_2, elevated temperature, and combined treatments when compared to control values (Fig. 4A).

In contrast to the photosynthetic response of *Heterosigma*, *Prorocentrum* photosynthetic rates reached a plateau at an irradiance of about 380 μmol photons m^{-2} s^{-1} only in the control and high temperature treatments. Light saturation was not observed under high CO_2 conditions at either temperature, even at the maximum light intensity tested (900 μmol photons m^{-2} s^{-1}) (Fig. 2B). Hence, calculated P_{Bmax} discussed here does not represent the true maximum photosynthetic rate for these latter two treatments. Despite this lack of light saturation, it was evident that P_{Bmax} was increased by elevated CO_2 either alone (by 58%, $p < 0.001$) or in concert with rising temperature (by 59%, $p = 0.001$) for *Prorocentrum* (Fig. 2B, Table 1). Maximum photosynthetic rates in the combined treatment were not significantly different from those in the high CO_2 treatments.

In contrast to maximal photosynthetic rates derived by the PE curves, the effects of temperature were more apparent on 24 h primary productivity rates of *Prorocentrum* compared to ambient culture conditions (Fig. 3B). Primary productivity was reduced by 30% and 23%, respectively, in *Prorocentrum* grown at high temperature and combined conditions, compared with the controls and high CO_2 treatments. Within each temperature treatment, elevated CO_2 also induced a significant increase in 24 h carbon fixation rates, from 1.40 to 1.68 mg C mg Chl^{-1} h^{-1} at 20 °C and from 0.98 to 1.28 mg C mg Chl^{-1} h^{-1} at 24 °C.

The value of α was similar for *Prorocentrum* grown under control and combined conditions, but photosynthetic efficiency was significantly higher in the high CO_2 treatment at 20 °C as well as at elevated temperature ($p < 0.02$) (Table 1). The quantum yield for photosynthesis at elevated temperature was significantly higher than in the control and combined treatments, while there was no difference in Φ_{max} between the elevated temperature and elevated CO_2 treatments. There was no significant change in F_v/F_m between the control and CO_2 treatments, or between the elevated temperature and combined treatments (Table 1). However, both the high temperature and combined treatments had significantly higher F_v/F_m values relative to the CO_2 treatment (Table 1). Likewise, there was a significant rise ($p = 0.001$) in the effective quantum yield of PSII ($\Delta F/F'_m$) when comparing the elevated temperature and combined treatments to the CO_2 and control treatments (0.32 ± 0.01, 0.30 ± 0.03 versus 0.21 ± 0.04, 0.22 ± 0.01, respectively). There was a significant decline in E_k in the cultures grown at elevated temperature compared with the rest of treatments, while there was no difference in E_k between the other treatments (Table 1). In comparison with *Heterosigma*, E_k for the dinoflagellate was much higher than the growth irradiance, indicating that *Prorocentrum* was grown under light limited conditions. Chlorophyll *a* content increased significantly ($p > 0.05$) in the combined conditions, while no other differences were noted between treatments (Table 1). While the only significant difference detected in $\bar{a}*$ was between the CO_2 and high temperature treatments (Table 1), the overall pattern of spectral absorption indicated a slight reduction in a^* that was largely driven by temperature (Fig. 4B).

3.3. Cell quotas and elemental ratios

Generally, either elevated CO_2 or increased temperature increased the cellular carbon quota of *Heterosigma*, and Q_C reached the highest value in the combined treatment (Fig. 5A). Increasing CO_2 resulted

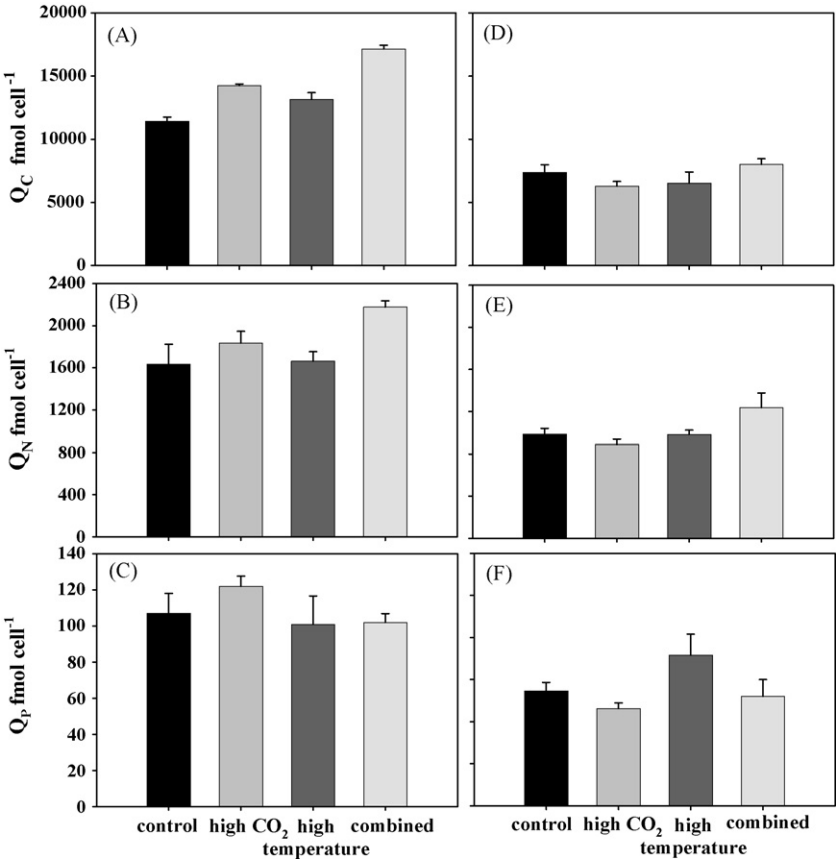

Fig. 5. Cell quota of C (Q_C), N (Q_N), and P (Q_P) of *H. akashiwo* (A–C) and *P. minimum* (D–F) in the four temperature and CO_2 treatments. Error bars denote standard deviations of averaged results from three replicate bottles.

in a significant increase of the Q_C of *Heterosigma* cells under both temperature conditions, with an increase of 25% and 30%, respectively, over cells in the control or high temperature treatments (Fig. 5A). The effects of rising temperature were also evident. Q_C for *Heterosigma* cells grown at 24 °C was 15–20% higher than at 20 °C in the same CO_2 concentration.

CO_2 enrichment affected Q_N in much the same manner as Q_C (Fig. 5B). Q_N in *Heterosigma* increased with increasing CO_2 by 12% and 31% compared to cells grown in control and high temperature treatments, respectively. Increased temperature without the combined effects of elevated CO_2 did not affect the Q_N, but cells grown under combined conditions had significantly higher cellular N quotas compared to all three other treatments ($p < 0.05$, Fig. 5B). Neither increased temperature nor CO_2, alone or together, affected the cellular phosphorus quota (Q_P) of *Heterosigma* (Fig. 5C).

Contrary to *Heterosigma*, growth at either elevated CO_2 or temperature did not result in significant changes in Q_C in *Prorocentrum* (Fig. 5D). Q_N was significantly higher in the combined treatment than in the other three treatments, but Q_N was relatively invariant within these three treatments (Fig. 5E), suggesting that only the combination of simultaneously increased CO_2 and temperature affected Q_N in *Prorocentrum*. Rising temperature did not affect the Q_P of *Prorocentrum*, but within each temperature treatment, Q_P was decreased at the higher CO_2 concentration (Fig. 5F). The highest values of Q_P were observed for the cells grown under high temperature conditions.

The C:N ratio in *Heterosigma* controls was slightly but significantly lower than in the other three treatments, which were not significantly different from each other (Fig. 6A). As a result of higher cellular C quotas but unchanged cellular P quotas, either increasing temperature alone or in concert with CO_2 increased the C:P ratio. This was especially evident in the combined treatment, in which this ratio was increased 1.6-fold relative to the control (Fig. 6B). The ratio of N:P in the combined treatment was significantly higher

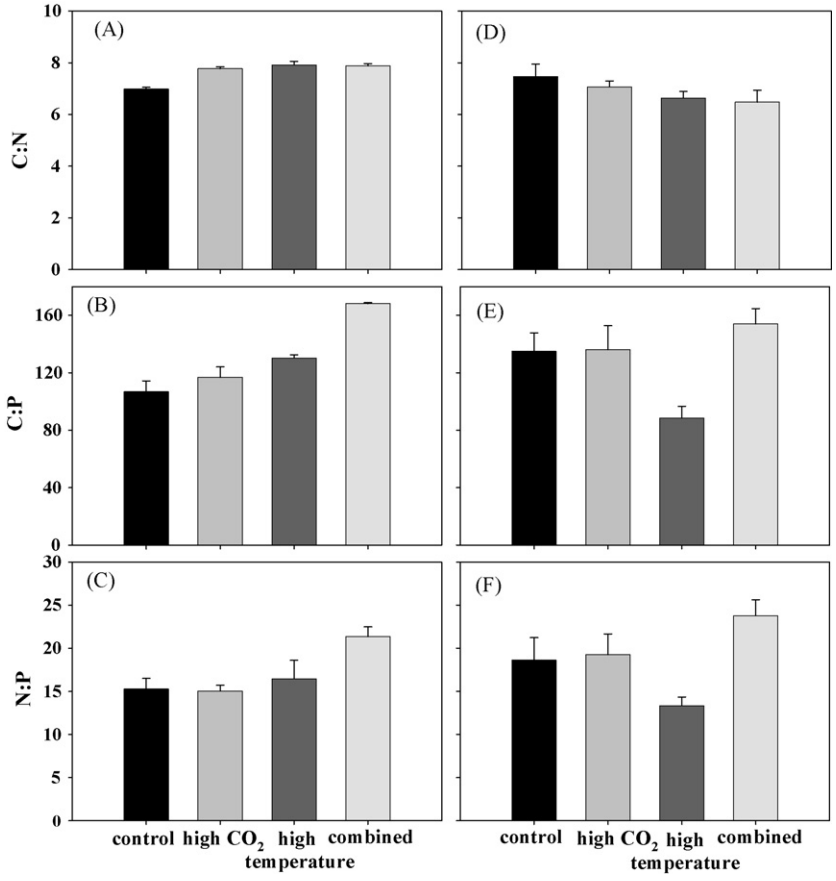

Fig. 6. Elemental ratios of *H. akashiwo* (A–C) and *P. minimum* (D–F) in the four temperature and CO_2 treatments. Error bars denote standard deviations of averaged results from three replicate bottles.

than in the other three treatments, and N:P ratios were unchanged among these (Fig. 6C). *Prorocentrum* C:N ratios did not change significantly from values of 6.5–7.5 in any of the four treatments (Fig. 6D). Both C:P and N:P in *Prorocentrum* showed a similar trend. These ratios in the high temperature treatment were significantly reduced relative to the other three conditions, with relatively constant ratios for the rest of treatments (Fig. 6E and F).

4. Discussion

Our experiments revealed fundamentally different responses of *Heterosigma* and *Prorocentrum* to increased CO_2 and temperature. Neither increased CO_2 nor elevated temperature affected the growth rates of *Prorocentrum*. In contrast, increases in either parameter increased the growth rates of *Heterosigma* to a similar degree, but no further enhancement was seen in the combined treatment.

The stimulation of *Heterosigma* growth by elevated CO_2 may be explained by the fact that this alga lacks CCMs that would allow it to take up HCO_3^- directly or indirectly, and hence probably depends only on CO_2 uptake (Nimer et al., 1997). Clark and Flynn (2000) reported a growth rate increase of 15% for *Heterosigma* grown under CO_2 concentrations two to three times above present levels. A recent model predicts that *Heterosigma* growth rates will increase by 40% between current CO_2 levels and 700 ppm CO_2 (Schippers et al., 2004). Our experimental results agreed fairly well with this prediction, as we found that elevating CO_2 to 750 ppm stimulated the growth rates of *Heterosigma* by 33% compared with current CO_2 levels.

Growth limitation by CO_2 in marine diatoms, green algae and cyanobacteria has been reported from both field and lab work (Riebesell et al., 1993; Chen and Durbin, 1994; Hutchins et al., 2007). Stimulation of algal growth rates by elevated CO_2 has also been observed in other phytoplankton, such as the diatoms *P. tricornutum* (a few percent increase, Clark and Flynn, 2000) and *Chaetoceros* (24% increase), and the chlorophyte *Dunaliella tertiolecta* (35% increase, Beardall and Raven, 2004). Increased growth of some phytoplankton under CO_2 enrichment may be explained by enhanced photosynthesis resulting in increases in carbohydrate production (Loehle, 1995).

On the other hand, growth rates were not enhanced at 750 ppm CO_2 relative to present day levels in the cyanobacterium *Prochlorococcus* (Fu et al., 2007), as with *Prorocentrum* in the experiments presented here. The growth of marine dinoflagellates has been suggested to be CO_2-limited in the open ocean (Colman et al., 2002; Dason and Huertas, 2004), suggesting that their growth could be stimulated by increasing CO_2 concentrations. However, our data do not support this viewpoint. *Prorocentrum* predominately uses HCO_3^- as an inorganic carbon source (Rost et al., 2006), which could explain why increasing CO_2 does not significantly affect its growth. Although a future doubled atmospheric CO_2 level will approximately double surface seawater CO_2 concentrations, the concomitant increase in bicarbonate and total DIC concentrations will be less than 10% (Beardall and Raven, 2004). Consequently, the same elevated CO_2 levels that will strongly stimulate the growth of the CO_2-dependent *Heterosigma* may have little or no effect on the growth of bicarbonate-utilizing algae like *Prorocentrum*.

In addition to the effect of elevated CO_2, higher temperature also has an obvious effect on the growth of *Heterosigma*, whereas no effect of our 4 °C experimental increase was observed on *Prorocentrum* growth. This observation is consistent with the natural temporal patterns of blooms typically seen in the DIB during the growing season. *Prorocentrum* often dominates in the early spring, followed by blooms of *Heterosigma* in the summer (Whereat, 2003), and hence it is not surprising that the growth of *Heterosigma* was stimulated by warmer temperatures while that of *Prorocentrum* was not. Previous laboratory culture work has shown that a temperature increase of 4 °C can double the growth rates of some harmful dinoflagellates and raphidophytes (Peperzak, 2003, 2005). Our results suggest that both elevated CO_2 and temperature within the range expected over the next 100 years (IPCC, 2001) will stimulate the growth rates of *Heterosigma*, but will likely not affect the growth rates of *Prorocentrum* significantly.

Different responses of *Heterosigma* and *Prorocentrum* under elevated CO_2 and temperature conditions were also reflected in P_{Bmax}. For *Heterosigma*, P_{Bmax} was raised only by the combined effects of simultaneous CO_2 and temperature increases (the combined treatment value was ~1.7 times the control value) but there were no significant differences in the other three treatments. However, unlike its lack of growth response, P_{Bmax} in *Prorocentrum* responded significantly to CO_2 enrichment, with or without increased temperature. These results suggest possible changes in carbon fixation by both HAB species in the future.

We also determined 24 h primary productivity rates as a function of the four CO_2 and temperature treatments under ambient culture illumination conditions. In general, rising CO_2 or temperature alone or

together stimulated carbon fixation rates of *Heterosigma*. In contrast, higher temperature actually decreased the primary productivity rates of *Prorocentrum*, although its response to elevated CO_2 was similar to *Heterosigma*. Some phytoplankton can regulate their inorganic carbon acquisition to shift from HCO_3^- to CO_2 utilization under high-CO_2 growth conditions (Rost et al., 2003), because CO_2 uptake is less energetically expensive than HCO_3^- uptake (Raven, 1990). Such a shift could explain why photosynthesis of *Prorocentrum* was stimulated under elevated CO_2 conditions, but this observation seems inconsistent with the lack of growth rate stimulation discussed above.

In fact, it is quite apparent that growth and carbon fixation responded differently to elevated CO_2 in *Prorocentrum*. Cell division rates of the dinoflagellate remained constant, while carbon fixation was strongly dependent on increasing CO_2. However, differences in photosynthetic affinity for CO_2 sometimes are not predictive of the trends in growth rate responses to CO_2 (Raven et al., 1993; Beardall et al., 1998; Fu et al., 2007; Hutchins et al., 2007). One explanation for the lack of growth rate enhancement despite higher carbon fixation rates is increased exudation of dissolved organic carbon under high CO_2 conditions (Fu et al., 2007; Hutchins et al., 2007), as discussed below.

P_{Bmax} and primary production, and to a lesser extent, light use efficiency (α) of our local *H. akashiwo* isolate are much higher when CO_2 and temperature are raised together, than under present day CO_2/temperature conditions. However, there was no significant difference in primary production between control and combined conditions for *Prorocentrum* and this was also reflected in the values of α, which remained constant between these two treatments. Despite the enhanced maximal photosynthetic rates noted in *Prorocentrum* when grown under combined conditions, it appears that photosynthetic efficiency and quantum yield were more dependent on the growth temperature than the level of CO_2.

It is notable that in our study *Prorocentrum* did not reach real light saturation, and the values of E_k were far above the growth illumination, suggesting that *Prorocentrum* was light-limited. The highest E_k values were found in the combined treatment, suggesting that *Prorocentrum* bloom development under future environmental conditions may be tightly linked with the availability of light, and that this species could be especially vulnerable to self-shading effects in dense aggregations. Although also somewhat higher in the combined treatment, the values of *Heterosigma* E_k are much lower than those of *Prorocentrum*, suggesting that light is less likely to be a limiting factor for the growth of *Heterosigma* either now or in the future.

For *Heterosigma*, cellular chlorophyll content remained stable under CO_2-enriched conditions. Similar responses were observed in *Scenedesmus obtusius* and *Dunaliella viridis*, in which no variations in pigment content were detected under different DIC concentrations (Larsson et al., 1985; Gordillo et al., 2003). Gordillo et al. (2003) suggested that this could be because qualitative rather than quantitative changes in the light harvesting machinery are involved in reaching higher photosynthetic rates under CO_2 enrichment. A similar phenomenon may have been the proximal cause of the response of *Heterosigma* to elevated CO_2 at 20 °C, as photosynthetic efficiency and quantum yield increased substantially in this treatment while P_{Bmax} did not. Interestingly, a similar trend was noted for both *Heterosigma* and *Prorocentrum* in response to elevated temperature alone, which appeared to result in a limitation of carbon fixation by $P_{Bmax.}$ This result may have been due to the thermally enhanced activity of Rubisco and/or other enzymes involved in Rubisco activation (Anning et al., 2001; Raven and Geider, 1988) and subsequent limitation in carbon supply. Such a scenario could lead to an imbalance between light harvesting and utilization, which was not apparent when algae were grown under elevated temperature and CO_2. Nevertheless, it is also clear that the decrease in Chlorophyll *a* and increasing trend in *a** in *Heterosigma* compared to the increase in Chlorophyll *a* and declining trend in *a** in *Prorocentrum* demonstrates that these algal species adjusted light harvesting at elevated temperature and/or CO_2 in markedly different ways. This implies that light-regulated growth and bloom development in each species may respond quite differently under future conditions.

CO_2 availability and temperature had pronounced effects on cellular quotas of C and N in *Heterosigma*. Either elevated CO_2 or temperature increased the cellular carbon quota (Q_C) and nitrogen quota (Q_N) of *Heterosigma*, especially in the combined treatment, whereas there was no significant increase in these quotas for *Prorocentrum*. The increased carbon and nitrogen contents for *Heterosigma* grown under elevated CO_2 could be due to increased carbon fixation and a higher uptake of NO_3^-, or to increased nitrate reductase activity. Similar results have been reported with increasing CO_2 levels in freshwater green algae such as *Chlamydomonas*, *Chlorella* and *Scenedesmus*, in laboratory cultures of the marine cyanobacteria *Synechococcus* and *Trichodesmium*, and the eukaryotes *Thalassiosira weissflogii* and *Emiliania huxleyi*, and for natural algal populations (Larsson et al., 1985; Xia and

Gao, 2005; Engel, 2002; Engel et al., 2005; Fu et al., 2007; Hutchins et al., 2007).

Whether increasing Q_C and Q_N under CO_2 enrichment in *Heterosigma* is caused by increasing carbohydrate or protein synthesis is unknown, since the biochemical composition of these two species was not determined in this study. Neither Q_C nor Q_N were affected by rising temperature or CO_2 in *Prorocentrum*, but this alga fixes carbon faster with increasing CO_2 levels (see above). One possibility is that fixed carbon may be more rapidly lost as dissolved organic carbon under future environmental conditions, as seen for other algae by Engel et al. (2005). The release of organic carbon to the medium at elevated CO_2 seems to be a common response in phytoplankton (Engel et al., 2005; Gordillo et al., 2003; Fu et al., 2007; Hutchins et al., 2007). The release of organic carbon may help to maintain the metabolic integrity of the cell (Fogg, 1983; Ormerod, 1983), protect the photosynthetic apparatus from accumulated organic carbon in excess of growth needs (Wood and Van Valen, 1990), and maintain the cellular balance of C:N (Gordillo et al., 2003).

It is noteworthy that *Heterosigma* but not *Prorocentrum* had an increased cellular nitrogen quota under either elevated CO_2 or temperature. This observation may suggest that the former alga may be more vulnerable to nitrogen limitation under future environmental conditions. Thus, the apparent competitive advantage that *Heterosigma* enjoys due to higher growth rates and photosynthesis under elevated temperature and CO_2 could potentially be negated in estuaries where N is the limiting nutrient for bloom development.

Contrary to the pronounced effects of CO_2 and temperature on Q_C and Q_N in *Heterosigma*, neither elevated CO_2 nor temperature affected its cellular phosphorus quota (Q_P). This suggests that most likely rising CO_2 does not affect its phosphate uptake. This hypothesis is in agreement with the observation that natural populations of *E. huxleyi* do not show effects of CO_2 on phosphate uptake either (Engel et al., 2005). Q_P is also unaffected by high CO_2 in other phytoplankton such as the cyanobacterium *Synechococcus*, *Prochlorococcus*, *Trichodesmium* and the diatom *Skeletonema costatum* (Burkhardt and Riebesell, 1997; Fu et al., 2007; Hutchins et al., 2007). Elevated CO_2 decreased the cellular P quota of *Prorocentrum*, but temperature had no effect. Decreased P quotas may suggest less likelihood of this species being limited by this nutrient under future CO_2 regimes.

It has been suggested that elemental ratios would be sensitive to changes in CO_2 if phytoplankton predominantly use CO_2 as an inorganic carbon source, but if HCO_3^- can be taken up much less sensitivity would be expected (Burkhardt et al., 1999). Extracellular carbonic anhydrase has been detected in *P. minimum* grown under carbon-replete or limited conditions, indicating that *Prorocentrum* preferred to use mainly HCO_3^- (Miao and Wu, 2002; Rost et al., 2006), while *Heterosigma* does not have the potential for either direct HCO_3^- uptake or indirect HCO_3^- utilization using extracellular carbonic anhydrase (Nimer et al., 1997; Miao and Wu, 2002). *Heterosigma* in fact appears to be an obligate "CO_2 user". It is thus perhaps not surprising that cell quotas and elemental composition were greatly affected by variations in CO_2 in *Heterosigma* but not in *Prorocentrum*, since our seawater perturbations greatly changed the partial pressure of CO_2 but would have caused only a relatively small shift in HCO_3^- availability. Future atmospheric pCO_2 increases will have this same effect.

C:P and N:P ratios increased with rising CO_2 and temperature in *Heterosigma*, suggesting this genus may have lower phosphorus requirements relative to other major elemental components under projected year 2100 conditions. Contrarily, *Prorocentrum* showed a decreased ratios of C:P and N:P due to elevated temperature, implying that it might be at a competitive disadvantage under future P-limited growth conditions. Increased ratios of C:P of phytoplankton due to elevated CO_2 such as those we observed in *Heterosigma* also have broad food web implications (Leonardos and Geider, 2005). For instance, lower zooplankton community N:P ratios have been observed due to elevated CO_2^- related changes in the ecological stoichiometry of C, N and P in the pelagic zone of lakes (Elser et al., 2000). Substantial micrograzing rates have been measured for *Heterosigma* populations in the DIB (Demir et al., 2007), suggesting that blooms of this raphidophyte do support higher trophic level production. C:P increases under future high CO_2 conditions will likely decrease the nutritional quality and mass transfer efficiency of *Heterosigma* to herbivores, thus resulting in lower zooplankton growth and production rates (Urabe et al., 2003).

During HAB blooms in coastal waters, pH may increase to 9 or even higher due to the rapid photosynthetic consumption of inorganic carbon (Hansen, 2002). Dinoflagellates such as *P. minimum* can tolerate pH values up to 9.2 during exponential growth phase (Hansen, 2002), suggesting that *P. minimum* maintains carbon fixation at elevated pH by increasing its affinity for HCO_3^- as a carbon source (Rost et al., 2006). However, the growth of *Heterosigma* ceases

completely when pH reaches 9.15 (Hansen, 2002), suggesting that during intense bloom events *Prorocentrum* could outcompete *Heterosigma* at present day CO_2 levels. However, future increasing CO_2 levels could erode this advantage that *Prorocentrum* currently enjoys, and tend to favor instead the growth of *Heterosigma*.

Our results suggest that future temperature and CO_2 changes may have major effects on present day patterns of harmful algal carbon fixation and growth, and may therefore drive large shifts in estuarine community structure. Raphidophytes may benefit disproportionately from future increases in CO_2 and temperature, as evidenced by the significantly increased growth and primary productivity rates we saw in *Heterosigma* under future environmental conditions. Current HAB species are especially dependent on their regulated CCM to maintain high growth rates under reduced CO_2 concentrations, due to the fast photosynthetic CO_2 consumption during blooms (Rost et al., 2003). Two-fold increases in atmospheric CO_2 over the coming century may well bring the "CO_2 user" *Heterosigma* a relative advantage over other phytoplankton that prefer HCO_3^- such as *Prorocentrum*.

The responses of these two sympatric harmful algal bloom species to anticipated global changes were quite different, underscoring the fact that future impacts of increasing CO_2 and temperature will be taxon- or even species-specific. Rising CO_2 and temperature over the coming decades may stimulate the growth of the harmful raphidophyte *Heterosigma* more than that of the dinoflagellate *Prorocentrum*, possibly favoring the former in interspecific competition. CO_2-driven changes in cell nutrient quotas and ratios also suggest an interaction between global change variables and ongoing eutrophication, which is already implicated in the HAB blooms occurring in the DIB (Zhang et al., 2006).

If the differences in cell physiology we observed in the raphidophyte *Heterosigma* and (to a lesser degree) the dinoflagellate *Prorocentrum* are representative of the corresponding HAB functional groups in the natural environment, ongoing CO_2- and temperature-related changes in estuarine systems are likely to accelerate and worsen blooms of harmful algal species in general. However, further work will be necessary to predict the full impact of changes in not only temperature and CO_2 but also other variables that are likely to change in the future, such as rainfall, stratification, light, nutrient inputs, and top-down controls. Only when we understand the complete interactive effects of all of these variables on the physiology of HAB species will be able to predict their ability to compete in the future phytoplankton communities within the context of a globally changing environment.

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