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Carbon dioxide regulation of nitrogen and phosphorus in four species of marine phytoplankton

John R. Reinfelder*

Department of Environmental Sciences, Rutgers University, 14 College Farm Road, New Brunswick, New Jersey 08901-8551, USA

ABSTRACT: The concentration of carbon dioxide in seawater may affect phytoplankton physiology and ecology and their role in marine biogeochemical cycles. In order to assess the effects of CO_2 on the elemental composition of marine phytoplankon, carbon, nitrogen, and phosphorus quotas were measured in 4 species of marine phytoplankton acclimated to 150 to 1500 ppm CO_2 (5 to 50 µM) in semi-continuous cultures. Nitrogen quotas declined steeply with increasing CO_2 in the centric diatoms *Thalassiosira pseudonana* and *T. weissflogii* acclimated to 150 to 380 ppm (5 to 13 µM), but more slowly as the CO_2 increased from 380 to 1500 ppm (13 to 50 µM). Nitrogen demand varied little with CO_2 in the pennate diatom *Phaeodactylum tricornutum*, but was positively correlated with CO_2 over the range of 150 to 770 ppm in the prymnesiophyte *Isochrysis galbana*. Based on the nitrogen–CO_2 trends in centric diatoms, relief from carbon–nitrogen colimitation could lead to 2-fold larger cells as CO_2 increases from 150 to 380 ppm, but only 15% larger cells from 380 to 770 ppm CO_2. Phosphorus quotas in the 3 diatoms decreased as CO_2 increased from 150 to 380 ppm. As previously observed in these and other species, C:N, C:P, and N:P ratios increased with increasing CO_2, but the present results show that much of this variation was due to differences in nitrogen and phosphorus rather than carbon quotas. Marine phytoplankton could provide a negative feedback against increasing CO_2 over the pCO_2 range of 150 to 380 ppm by supporting larger cells or higher biomass, but would support a smaller carbon sink as atmospheric CO_2 rises above 380 ppm.

KEY WORDS: Nitrogen · Phosphorus · Carbon dioxide · Phytoplankton

INTRODUCTION

The elemental composition of marine phytoplankton varies among species (Arrigo et al. 2002, Geider & La Roche 2002, Ho et al. 2003, Quigg et al. 2003) and within species depending on the extent of nutrient (Goldman et al. 1979, Price 2005, Moore et al. 2007, Varela et al. 2011) or light (Leonardos & Geider 2004, Finkel et al. 2006) limitation of growth. Since marine phytoplankton use various strategies to concentrate inorganic carbon for photosynthesis (Giordano et al. 2005, Raven et al. 2011, Reinfelder 2011), which carry metabolic costs (Raven & Johnston 1991, Raven et al. 2012), element ratios in phytoplankton may also depend on inorganic carbon supply, but this is only beginning to be examined (Hutchins et al. 2009, Montechiaro & Giordano 2010).

Inorganic carbon concentrating mechanisms (CCMs) in marine phytoplankton are characterized by increased activities of the enzyme carbonic anhydrase (Morel et al. 1994, Rost et al. 2003, Dason et al. 2004), an ability to use HCO_3^- for photosynthesis (Tortell et al. 1997, 2008a, Cassar et al. 2004, Hopkinson et al. 2011), and, in at least some C_3–C_4 intermediate species of marine diatoms, up-regulation of a single-cell, C_4 carbon pump (Reinfelder et al. 2000, 2004, Roberts et al. 2007, McGinn & Morel 2008). Physiological changes associated with CCMs impose resource and energy costs on cells at ambient or lower concentrations of CO_2 (Raven & Johnston 1991, Beardall & Giordano 2002, Raven et al. 2012), and as a result, the concentration of CO_2 in seawater has the potential to regulate phytoplankton physiology, ecology, and biogeochemistry (Rost et al. 2008, Raven et

*Email: reinfelder@envsci.rutgers.edu

al. 2011). Indeed, laboratory, mesocosm, and shipboard incubation experiments demonstrated that the concentration of CO_2 can affect phytoplankton growth (Morel et al. 1994, Hein & Sand-Jensen 1997, Riebesell et al. 2007, Sun et al. 2011), inorganic carbon uptake (Rost et al. 2003, Tortell et al. 2008a, Trimborn et al. 2009), nitrogen fixation (Barcelos e Ramos et al. 2007, Hutchins et al. 2007, Levitan et al. 2007), and species composition (Tortell et al. 2002, 2008b). In addition, CO_2 was shown to affect element ratios in phytoplankton (Burkhardt et al. 1999, Tortell et al. 2000, Engel et al. 2005, Montechiaro & Giordano 2010). These results allude to an important effect of CO_2 on the elemental composition of marine phytoplankton, but vary in method of CO_2 control and in only a few cases permit the effects of CO_2 on carbon, nitrogen, and phosphorus cell quota to be assessed independently.

To further examine the effects of CO_2 on the elemental composition of marine phytoplankton, cell quotas of carbon, nitrogen, and phosphorus were measured in 2 temperate centric diatoms, a temperate pennate diatom, and a temperate prymnesiophyte acclimated to CO_2 concentrations from 150 to 1500 ppm (5 to 50 µM). This range includes the approximate current atmospheric level of CO_2 (380 ppm), concentrations (150 and 280 ppm) that may be encountered during an intense phytoplankton bloom (Codispoti et al. 1982, Karl et al. 1991, Murata et al. 2002), and those in upwelling regions (770 ppm; Feely et al. 2002, Hales et al. 2005). The experimental concentrations of CO_2 are also representative of Pleistocene glacial (150 ppm) and interglacial (280 ppm) intervals (Lüthi et al. 2008) and past and future periods of elevated CO_2 (770 and 1500 ppm).

MATERIALS AND METHODS

The phytoplankton strains (Table 1) used in this study were isolated from the North Atlantic Ocean between 41° and 54° N (National Center for Marine Algae and Microbiota: https:ncma.bigelow.org). Other strains of these species have been found in tropical waters, but the strains used here are considered representative of temperate species. Phytoplankton were grown in Aquil artificial seawater media (Price et al. 1988/89) maintained at 18°C under a 12:12 h light:dark regime with 200 µmol quanta m^{-2} s^{-1} irradiance provided by cool while fluorescent lamps. Semi-continuous, non-nutrient-limited cultures were kept in exponential growth by periodic (48 or 72 h) dilution. Cultures were diluted so as to minimize the drawdown of CO_2 (see below). Nitrate was provided at an initial concentration of 30 µM, and phosphate and silicate (diatom cultures) were provided at 10 and 100 µM, respectively. Cells were acclimated to lower nitrogen concentrations than is used in standard culture media to minimize luxury consumption.

Cultures were grown in 0.5 l volumes and acclimated to various concentrations of CO_2 by bubbling (≈20 ml min^{-1}) with 0.2 µm-filtered air containing various partial pressures of CO_2 (Table 2) with an average uncertainty of 2% as analyzed by the supplier. Phytoplankton were acclimated to each concentration of CO_2 for at least 10 generations, and acclimation was assessed through the achievement of con-

Table 1. Phytoplankton species examined

Species	Strain	Class	Cell volume (μm^3)
Isochrysis galbana	CCMP1323	Prymnesiophyceae	40[a]
Phaeodactylum tricornutum	CCMP632	Baccilariophyceae	65[b]
Thalassiosira pseudonana	CCMP1335	Baccilariophyceae	41 (0.2)[c]
Thalassiosira weissflogii	CCMP1336	Baccilariophyceae	626 (6)[d]

[a]Based on the equivalent spherical diameter reported by Nakamura et al. (1995)
[b]Based on measured cell dimensions of cultures grown at 380 ppm CO_2
[c]Average (±1 SD) cell volumes measured in cultures acclimated to 150, 380, and 1500 ppm CO_2
[d]Average (±1 SD) cell volumes measured in cultures acclimated to 280 and 770 ppm CO_2

Table 2. Concentrations of aqueous carbon dioxide (CO_{2aq}) and total dissolved inorganic carbon (DIC), and pH_{NBS} (National Bureau of Standards calibration scale) for each experimental partial pressure of CO_2 (pCO_2) in parts per million by volume (ppmv). Values were calculated using the CO2SYS program (Lewis & Wallace 1998) for Aquil media with an alkalinity of 2136 µeq kg^{-1}, a salinity of 35 and temperature of 18°C (density = 1032.3 kg m^{-3}), a phosphate concentration of 5 µM, and a silicate concentration of 50 µM

pCO_2 (ppmv)	$[CO_{2aq}]$ (µM)	DIC (µM)	pH_{NBS}
150	5.2	1751	8.49
280	9.7	1887	8.28
380	13	1948	8.17
770	27	2069	7.91
1500	52	2161	7.64

stant (no longer increasing) growth rates. With this method of control, the concentrations of aqueous carbon dioxide (CO_{2aq}), dissolved inorganic carbon (DIC), and pH vary with the partial pressure of CO_2 (pCO_2) at constant alkalinity as would occur in surface ocean seawater under conditions of variable atmospheric CO_2 (Rost et al. 2008). This approach contrasts with methods in which pH, DIC, and carbonate or non-carbonate alkalinity are varied (Burkhardt et al. 1999, Clark 2001) and dampens diurnal swings in CO_2 and pH as occurs in non-bubbled cultures (Shi et al. 2009). Maintenance of nominal concentrations of CO_2 during phytoplankton growth was monitored by periodically measuring pH_{NBS} (National Bureau of Standards calibration scale) of bubbled cultures. Based on measurements of cultures at the time of harvest, CO_2 concentrations were at most 40 ppm (1.3 µM) lower than nominal values for 150, 280, and 380 ppm cultures, 100 ppm (3.4 µM) lower for 770 ppm cultures, and 200 ppm (7 µM) lower for 1500 ppm cultures. The alkalinity of the synthetic seawater media was calculated based on the balance of acids and bases in the recipe. Concentrations of CO_{2aq} were calculated with the CDIAC CO_2 system software (Lewis & Wallace 1998) using alkalinity and CO_2 partial pressures as input and the dissociation constants of Roy et al. (1993).

Biological replicates were grown for each species acclimated to 380 ppm CO_2 including triplicate cultures for 3 of 4 species and duplicate cultures for *Thalassiosira weissflogii*. At other CO_2 concentrations, duplicate or single cultures were grown. Based on the variation among biological replicates, between-culture variability was estimated to be between 2 and 6% for carbon, 4 and 13% for nitrogen, and 6 and 19% for phosphorus. Cultures were harvested after 80 to 90% of nitrate was consumed to minimize intracellular nitrogen storage. Cell samples were collected in triplicate (analytical replicates) for organic carbon and nitrogen analysis on pre-combusted glass fiber filters (GF/F) and for organic phosphorus on polycarbonate filters, and subsequently rinsed with nutrient-free synthetic seawater. Additional samples were collected for cell enumeration and sizing by Coulter Counter analysis and microscopic examination. Particulate organic carbon and nitrogen were analyzed using a Carlo-Erba element analyzer after acidification to remove inorganic carbon. Phosphorus was analyzed by the molybdate blue method after persulfate digestion (Parsons et al. 1984, Clesceri et al. 1998).

Nitrogen quotas in the 2 centric diatoms were used to estimate the maximum diatom cell volume that could support nitrate-diffusion-limited growth at each experimental CO_2 concentration. Equivalent spherical radii (R) of diatom cells capable of maintaining a specified nutrient quota under diffusion-limited nitrate supply were estimated by equating the cell volume-specific nitrate uptake rate and the diffusion-limited flux of nitrate through the cell's boundary layer:

$$\mu Q_v = 4\pi RD(N_{sw} - N_R)/4\pi R^3/3 \quad (1)$$

$$\mu Q_v = 3D(N_{sw} - N_R)/R^2 \quad (2)$$

where µ is the specific growth rate (d^{-1}), Q_v is the volume-specific nitrogen quota (mol l^{-1}), D is the diffusion coefficient of nitrate in seawater (m^2 s^{-1}), N_{sw} and N_R are the concentrations of nitrate (mol m^{-3}) in surface ocean seawater and at the surface of the cell, respectively. The diffusion coefficient for nitrate in water with the same ionic strength as seawater is 1.3×10^{-9} m^2 s^{-1} (Yeh & Wills 1970). For the criterion of growth at 2/3 of the diffusion-limited nitrate flux, an arbitrary constraint that represents the nitrate concentration gradient between the cell surface and bulk medium likely to occur under nitrogen limiting conditions, $N_R = 1/3N_{sw}$. Solving for R:

$$R = (2DN_{sw}/\mu Q_v)^{1/2} \quad (3)$$

Diffusion-limited diatom cell radii were calculated for a growth rate of 1 d^{-1} using the average volume-specific nitrogen quotas of *Thalassiosira pseudonana* and *T. weissflogii* measured at each concentration of CO_2 and a surface seawater nitrate concentration of 0.5 µM.

Relative contributions of changes in nitrogen and phosphorus quotas to changes in C:N, C:P, and N:P ratios were estimated for each CO_2 concentration interval. For example, the relative contribution of the change in nitrogen quota to the C:N ratio ($\%N_{C:N}$) was estimated as:

$$\%N_{C:N} = \Delta N/N_{ave}/(\Delta N/N_{ave} + \Delta C/C_{ave}) \quad (4)$$

where ΔN and ΔC are the differences in cell quotas in cells acclimated to 2 different concentrations of CO_2 and N_{ave} and C_{ave} are the average cell quotas for those concentrations of CO_2.

Trends in element quotas and ratios in cells acclimated to different concentrations of CO_2 were evaluated by either linear regression analysis or by comparing pooled values using 2-tailed t-tests for equal or unequal variance as determined by the F-test of variance. Correlations and differences were considered significant for $p < 0.1$. Since phosphorus quotas were measured in different physical samples than carbon and nitrogen quotas, differences in C:P and N:P ratios were compared using random pairs of carbon or nitrogen and phosphorus quotas.

Table 3. *Isochrysis galbana*, *Phaeodactylum tricornutum*, *Thalassiosira pseudonana*, *T. weissflogii*. Cell volume-specific carbon, nitrogen, and phosphorus quotas (mol l^{-1}) in 4 species of marine phytoplankton acclimated to various concentrations of aqueous CO_2 (CO_{2aq}) and corresponding partial pressures (pCO_2). Values are means (±1 SD). Within each column, groups of values marked with an asterisk or cross were correlated with the concentration of CO_2 ($p < 0.1$). nc: not cultured; nd: not determined

$[CO_{2aq}]$ (µM)	pCO_2 (ppm)	*I. galbana*			*P. tricornutum*			*T. pseudonana*			*T. weissflogii*		
		C	N	P	C	N	P	C	N	P	C	N	P
5.2	150	16.9 (0.6)	1.85* (0.06)	0.131 (0.015)	31.8 (0.6)	3.84 (0.04)	0.285* (0.027)	14.7 (0.4)	1.46* (0.08)	0.278* (0.041)	14.8 (0.3)	1.60* (0.09)	0.134* (0.013)
9.7	280	18.1 (1.6)	1.99* (0.15)	nd	nc	nc	nc	19.0* (0.1)	1.24* (0.05)	0.134* (0.009)	11.9 (0.3)	1.16* (0.12)	0.113* (0.002)
13	380	16.1* (0.3)	2.20* (0.29)	0.085 (0.016)	27.6 (1.0)	3.59 (0.2)	0.180* (0.004)	18.7* (0.3)	0.99*† (0.04)	0.106* (0.010)	10.1 (0.6)	1.00* (0.09)	0.061* (0.004)
27	770	28.1* (0.3)	2.88* (0.05)	nd	26.6 (0.1)	3.51 (0.2)	0.182 (0.018)	17.1* (0.6)	0.92† (0.03)	0.098 (0.003)	14.7 (0.7)	0.89 (0.04)	0.053 (0.004)
52	1500	32.5* (0.7)	2.46 (0.05)	0.067 (0.013)	nc	nc	nc	13.2* (0.5)	0.88† (0.14)	0.083 (0.011)	13.5 (2.0)	0.91 (0.12)	0.061 (0.011)

RESULTS

Growth rates of the 4 phytoplankton species did not vary with the concentration of CO_2 to which each was acclimated in these light- and nutrient-replete cultures (Morel et al. 1994, Tortell et al. 1997, Burkhardt et al. 1999) and averaged 0.72 ± 0.1 d^{-1} for *Isochrysis galbana*, 1.3 ± 0.04 d^{-1} for *Phaeodactylum tricornutum*, 1.6 ± 0.2 d^{-1} for *Thalassiosira pseudonana*, and 0.90 ± 0.06 d^{-1} for *T. weissflogii*. However, element quotas in the 4 species varied with respect to the concentration of CO_2, and patterns of variation differed among species (Table 3). For example, carbon quotas decreased as the acclimation concentration of CO_2 increased from 10 to 50 µM in *T. pseudonana* ($R^2 = 0.989$, $p < 0.01$), but increased in *T. weissflogii* as CO_2 increased from low (10 and 13 µM) to high (27 and 50 µM) values ($p < 0.01$, *t*-test) and in *I. galbana* as CO_2 increased from 13 to 50 µM ($R^2 = 0.875$, $p < 0.05$). Carbon quotas did not vary significantly with increasing CO_2 ($R^2 = 0.573$, $p = 0.14$) in *P. tricornutum*.

Nitrogen and phosphorus quotas also varied with acclimation CO_2 (Table 3). For the centric diatoms *Thalassiosira pseudonana* and *T. weissflogii*, nitrogen quotas declined steeply with increasing CO_2 from 5 to 13 µM, but more slowly or not at all in cells acclimated to 13 to 50 µM CO_2. Thus as the concentration of CO_2 increased from 5 to 13 µM, nitrogen quotas in *T. pseudonana* and *T. weissflogii* decreased by 61 ($R^2 = 0.973$, $p < 0.01$) and 76 ($R^2 = 0.938$, $p < 0.05$) mmol l^{-1} cell volume per µM CO_2, respectively. However, from 13 to 50 µM CO_2, nitrogen quotas declined by only 3 mmol l^{-1} cell volume per µM CO_2 in *T. pseudonana* ($R^2 = 0.752$, $p = 0.06$) and showed no significant decline in *T. weissflogii* ($R^2 = 0.298$, $p > 0.1$). Nitrogen quotas in the pennate diatom *Phaeodactylum tricornutum* did not vary significantly with CO_2 ($R^2 = 0.425$, $p = 0.23$). Unlike nitrogen quotas in the centric diatoms, cellular nitrogen in *Isochrysis galbana* increased as CO_2 increased from 5 to 27 µM. *I. galbana* cells acclimated to 27 µM CO_2 had a 45% higher nitrogen quota than cells acclimated to 10 µM, and nitrogen quotas were positively correlated with CO_2 over the range of 5 to 27 µM CO_2 ($R^2 = 0.785$, $p < 0.05$).

Higher nitrogen quotas at low CO_2 increased the nitrogen costs of photosynthesis (mol N required to support the fixation of 1 mol C d^{-1}) in *Thalassiosira weissflogii* and *T. pseudonana* as CO_2 decreased from 27 to 5 µM (Table 4). The nitrogen costs of pho-

Table 4. *Isochrysis galbana*, *Phaeodactylum tricornutum*, *Thalassiosira pseudonana*, *T. weissflogii*. Nitrogen cost of photosynthesis in 4 species of marine phytoplankton acclimated to various concentrations of aqueous CO_2 (CO_{2aq}) and corresponding partial pressures (pCO_2) in parts per million (ppm). Values are means (±1 SD) in units of mmol N mol^{-1} C fixed d^{-1} (i.e. mmol mol^{-1} d^{-1}). nc: not cultured

$[CO_{2aq}]$ (µM)	pCO_2 (ppm)	*I. galbana*	*P. tricornutum*	*T. pseudonana*	*T. weissflogii*
5.2	150	152 (22)	93 (4)	62 (9)	120 (11)
9.7	280	153 (27)	nc	41 (5)	108 (13)
13	380	187 (36)	100 (7)	33 (4)	108 (14)
27	770	142 (20)	102 (6)	34 (5)	67 (6)
52	1500	105 (15)	nc	41 (8)	70 (13)

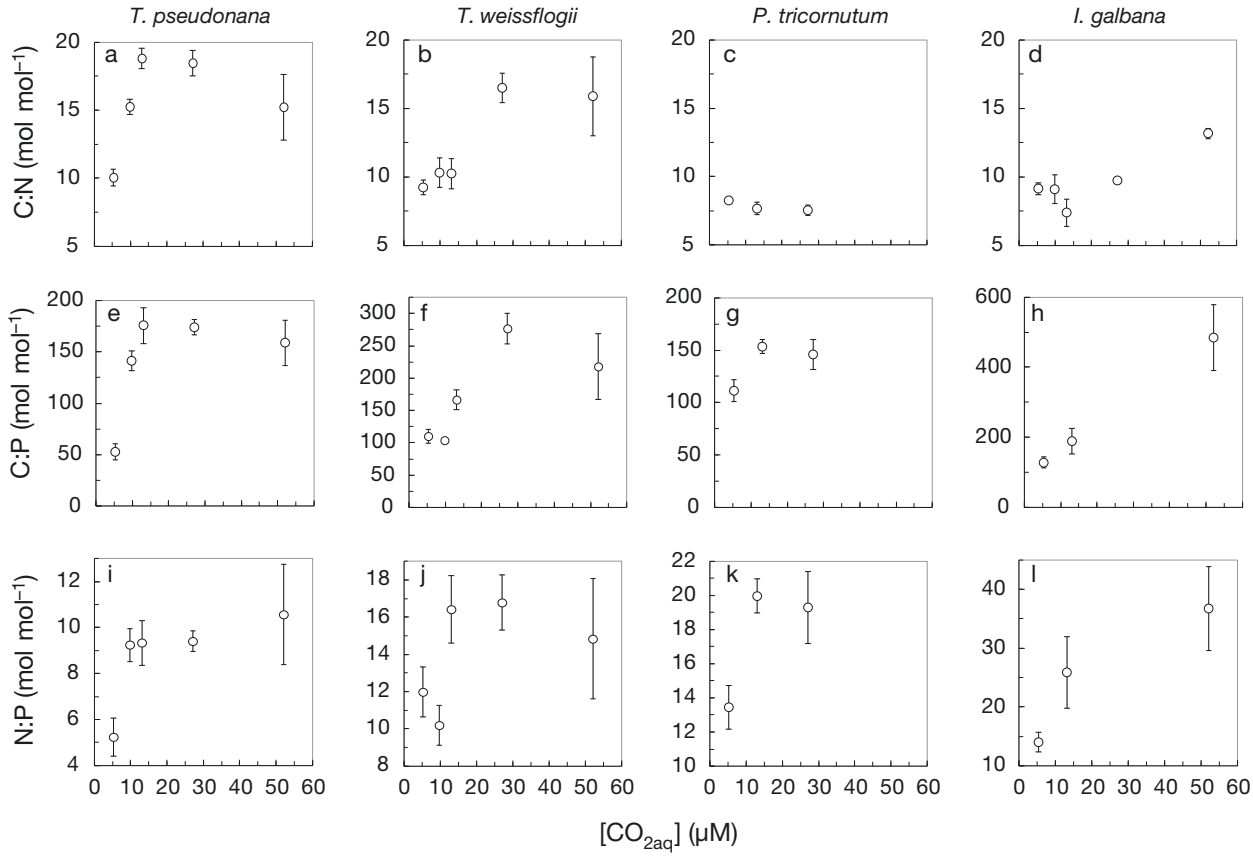

Fig. 1. Element ratios in the marine diatoms (a,e,i) *Thalassiosira pseudonana*, (b,f,j) *T. weissflogii*, and (c,g,k) *Phaeodactylum tricornutum*, and (d,h,l) the prymnesiophyte *Isochrysis galbana* acclimated to various concentrations of CO_2. Values are ratios of mean element quotas ± propagated standard deviations

tosynthesis did not vary in *Phaeodactylum tricornutum* and increased over a higher range of CO_2 (50 to 13 µM) in *Isochrysis galbana*.

All 4 species of phytoplankton examined showed declines in phosphorus quotas as CO_2 increased from 5 to 13 µM (Table 3). Over this range of CO_2 concentrations, phosphorus quotas decreased by 62 and 54% in *Thalassiosira pseudonana* and *T. weissflogii*, respectively, and were significantly inversely correlated with CO_2 ($R^2 = 0.917$, $p < 0.05$; $R^2 = 0.918$, $p < 0.05$). The phosphorus quota in *Phaeodactylum tricornutum* decreased by more than 37% in cells acclimated to 13 µM CO_2 compared with 5 µM CO_2 and were also inversely correlated with CO_2 ($R^2 = 0.997$, $p < 0.01$). In *Isochrysis galbana*, the phosphorus quota decreased by 35% as the acclimation concentration of CO_2 increased from 5 to 13 µM, but this decline was not significant ($R^2 = 0.749$, $p = 0.13$).

Declines in nitrogen and phosphorus quotas in cultures acclimated to increasing concentrations of CO_2 generally resulted in higher C:N, C:P, and N:P ratios (Fig. 1). In *Thalassiosira pseudonana*, the C:N ratio was positively correlated with CO_2 from 5 to 13 µM ($R^2 = 0.993$, $p < 0.01$). However, as acclimation CO_2 increased from 13 to 50 µM, the C:N ratio in *T. pseudonana* declined slightly ($R^2 = 0.902$, $p < 0.05$). Over the CO_2 interval of 5 to 10 µM, 40% of the increase in the C:N ratio in *T. pseudonana* was due to a decrease in nitrogen quota, but 100% of the increase in C:N from 10 to 13 µM CO_2 was due to a decrease in nitrogen quota since the carbon quota was constant over this interval.

C:P ratios in *Thalassiosira pseudonana* were also correlated with CO_2 over the range of 5 to 13 µM ($R^2 = 0.945$, $p < 0.01$), but were constant at higher concentrations of CO_2 ($R^2 = 0.325$, $p = 0.32$; Fig. 1e). Over the CO_2 interval of 5 to 10 µM CO_2, 70% of the increase in C:P was due to a decrease in phosphorus quota, while from 10 to 13 µM, 100% of the increase in the C:P ratio was due to a decrease in phosphorus quota.

C:N and C:P ratios increased at higher concentrations of CO_2 in *Thalassiosira weissflogii* than in *T. pseudonana* (Fig. 1b,f,j). Both C:N ($R^2 = 0.847$, $p <$

0.05) and C:P (R^2 = 0.938, p < 0.01) ratios in *T. weissflogii* were positively correlated with CO_2 over the range of 5 to 27 µM, with the largest increases occurring between 13 and 27 µM. Decreases in nitrogen and phosphorus quotas accounted for nearly 100% of the increase in C:N and C:P ratios in *T. weissflogii* acclimated to 27 µM compared with 5 µM CO_2 and declines in cellular phosphorus accounted for 65% of the increase in N:P ratios in *T. weissflogii* acclimated to high (13 to 50 µM) compared with low (5 to 10 µM) CO_2.

In the pennate diatom *Phaeodactylum tricornutum*, C:N ratios were not correlated with CO_2 (R^2 = 0.45, p = 0.21; Fig. 1c). C:P ratios in *P. tricornutum* increased from 112 to 154 in cells acclimated to 13 µM CO_2 compared with 5 µM CO_2, and a lower phosphorus quota accounted for 100% of this increase (Fig. 1g).

The C:N ratio in *Isochrysis galbana* did not vary with CO_2 in cells acclimated to 5 to 13 µM CO_2 (R^2 = 0.551, p = 0.15), but was positively correlated with CO_2 over the concentration range of 13 to 50 µM (R^2 = 0.937, p < 0.01; Fig. 1d). Higher carbon quotas accounted for 50 to 100% of the increases in C:N. C:P ratios in *I. galbana* were positively correlated with CO_2 over the entire range of 5 to 50 µM (R^2 = 0.956, p < 0.01; Fig. 1h), and about half of this increase was due to lower phosphorus quotas at high CO_2.

N:P ratios in all 4 species increased by factors of 1.4 to 1.9 as the concentration of CO_2 increased over the range of 5 to 13 µM (Fig. 1i–l). In *Thalassiosira weissflogii*, N:P ratios were significantly higher in cells acclimated to CO_2 concentrations at or above 13 µM than at 5 to 10 µM (p < 0.05, *t*-test; Fig. 1j). In *T. pseudonana*, *T. weissflogii*, and *Phaeodactylum tricornutum*, 100% of the increases in N:P with higher CO_2 were due to greater declines in phosphorus than nitrogen quotas, while in *Isochrysis galbana*, 70% of the increase in N:P as CO_2 increased from 5 to 13 µM was due to a decrease in phosphorus quota.

DISCUSSION

The present results show that nitrogen and phosphorus quotas vary with CO_2 and are generally higher in non-nutrient-limited phytoplankton acclimated to low CO_2 compared with cells acclimated to current atmospheric or higher levels of CO_2. Nitrogen in phytoplankton is primarily associated with protein and amino acids, although nucleic acids could account for up to 18% (Lourenço et al. 1998). Phosphorus is used in assembly (ribosomal RNA), structural support (phospholipid), and storage (polyphosphate) components, but the phosphorus contents of marine phytoplankton are not well constrained by biochemical composition (Laws et al. 1983, Geider & La Roche 2002). The additional nitrogen needed to support inorganic carbon acquisition in phytoplankton cells with CCMs through the production of membrane-bound inorganic carbon transporters and carbonic anhydrase was estimated to account for less than 1% of cellular nitrogen (Raven 1991, Raven & Johnston 1991). Most of the cellular nitrogen increment in the centric diatoms *Thalassiosira pseudonana* and *T. weissflogii* grown at low CO_2 was therefore likely due to increased production of proteins responsible for cellular functions (nutrient and light acquisition, photorespiration) other than inorganic carbon accumulation (Raven & Johnston 1991, Raven et al. 2012). In contrast, while the affinity for inorganic carbon in the pennate diatom *Phaeodactylum tricornutum* is regulated by CO_2 (Rees 1984, Burkhardt et al. 2001), its demand for nitrogen apparently is not.

Increasing carbon and nitrogen quotas in *Isochrysis galbana* acclimated to 13 to 50 µM CO_2 (380 to 1500 ppm) is consistent with results for another marine prymnesiophyte, *Phaeocystis globosa* grown at 380 and 750 ppm (13 and 25 µM) CO_2 (Wang et al. 2010). Although cellular nitrogen decreased at low CO_2 in *I. galbana*, the associated decline in cellular carbon resulted in nitrogen costs of photosynthesis that increased over a higher range of CO_2 concentrations (50 to 13 µM) than in the centric diatoms (13 to 5 µM). This indicates that the carbon-specific nitrogen demand in *I. galbana* is more sensitive to declining CO_2 than in the centric diatoms. The CCM of *I. galbana* may therefore help this species conserve nitrogen as CO_2 declines through a lower investment in RubisCO and other proteins associated with carbon fixation (Beardall et al. 1998), perhaps at the expense of a lower maximum growth rate compared with diatoms.

Greater phosphorus demand in phytoplankton acclimated to low CO_2 is somewhat surprising and not easily explained physiologically (Raven et al. 2011). Higher phosphorus demand at low CO_2 may involve increased production of ribosomal RNA to support higher rates of protein turnover, increased phospholipid production to stabilize membranes, or greater phosphate demand for energy storage and use. For the centric diatoms, phosphorus quota of had its largest decline at a higher CO_2 concentration in *Thalassiosira weissflogii* (46% decrease between 10 and 13 µM) than in *T. pseudonana* (52% decrease

between 5 and 10 µM). Since relief from CO_2-diffusion limitation will occur at a lower concentration of CO_2 for the smaller *T. pseudonana* than for the larger *T. weissflogii* (Wolf-Gladrow & Riebesell 1997), *T. pseudonana* is able to lower its phosphorus requirement at a lower concentration of CO_2 than *T. weissflogii*.

Contrary to the present results and those of Clark (2001), in which the concentration of CO_2 was set by varying the concentration of DIC at constant pH, nitrogen quotas decreased and phosphorus quotas were relatively constant as CO_2 increased from 2 to 31 µM CO_2 in closed system batch cultures of the diatom *Skeletonema costatum* in which the concentration of CO_2 was controlled by varying pH (and alkalinity) at constant DIC (Burkhardt & Riebesell 1997). An important difference between these experimental set-ups is that in the closed system with variable pH and alkalinity, the concentration of bicarbonate decreases as CO_2 is increased, while in the closed system with varying DIC (Clark 2001) or in a gas-equilibrated open system (the present study), the concentrations of CO_2 and bicarbonate vary together. Although bicarbonate transport may be saturated at concentrations of 500 to 1000 µM (Rost et al. 2003), variation in external bicarbonate concentration, and the pH buffering it provides, could participate in the regulation of the cell's affinity for inorganic carbon and other aspects of its CCM. As a result, the decrease in bicarbonate concentration at high CO_2 in pH-controlled cultures may dampen the effects of inorganic carbon supply on cellular carbon and nutrient demands.

Another possible confounding factor in evaluating the effects of CO_2 on nutrient quotas is the concentration of nitrogen in culture media and its intracellular storage. Nitrogen quotas follow cell volume in marine diatoms (Marchetti & Harrison 2007, Timmermans & van der Wagt 2010). Indeed, cell volumes of *Thalassiosira pseudonana* and *T. weissflogii* were up to 50% higher in cells grown with high nitrate (300 µM) than low nitrate (30 µM), and in high nitrate cultures of *T. weissflogii*, cell volume increased from 600 to 900 μm^3, but nitrogen quota did not vary (2.1 ± 0.1 mol l^{-1}) as CO_2 increased from 5 to 27 µM (data not shown). Thus increased cell size and potentially greater nitrogen storage capacity in cells grown with high nitrogen may mask the effects of CO_2 on nitrogen demand.

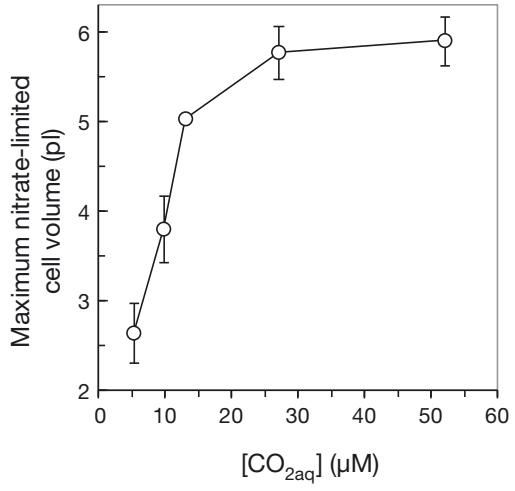

Fig. 2. *Thalassiosira pseudonana* and *T. weissflogii.* Carbon–nitrogen co-limitation of diatom cell volume. Equivalent spherical cell volumes were calculated from nitrate-diffusion-limited maximum radii that could support the average CO_2-dependent nitrogen quotas measured in the centric diatoms *T. pseudonana* and *T. weissflogii* at a growth rate of 1 d^{-1} and 2/3 of the diffusion-limited nitrate flux with a surface seawater nitrate concentration of 0.5 µM (see 'Materials and methods'). Error bars are based on the range of CO_2-dependent nitrogen quotas measured in *T. pseudonana* and *T. weissflogii*

Carbon–nitrogen co-limitation of diatom cell size

Greater demand for nitrogen at low CO_2 as observed in the 2 centric diatoms in this study could lead to carbon–nitrogen co-limitation of growth and may explain why primary production of some natural phytoplankton communities is apparently limited by CO_2 (Hein & Sand-Jensen 1997, Riebesell et al. 2007). Assuming other nutrients are not limiting, as CO_2 increases, lower nitrogen demand could support higher diatom biomass per unit of nitrogen supplied to the surface ocean. Alternatively, CO_2 regulation of nitrogen demand could limit the average size of diatoms able to survive in nitrogen-limited surface waters. To explore this possibility, nitrate-diffusion-limited cell volumes of diatoms were estimated based on the CO_2-regulated nitrogen quotas measured in *Thalassiosira pseudonana* and *T. weissflogii* (Fig. 2). This analysis showed that maximum nitrate-limited diatom cell volume increased from 2.6 to 5.9 pl (equivalent spherical radius of 8.6 to 11.2 µm) as CO_2 increased from 5 to 50 µM (150 to 1500 ppm), with the greatest increase expected between 5 and 13 µM (150 to 380 ppm). According to these trends, higher CO_2 would support nearly 2-fold larger centric diatoms as CO_2 increases from 5 to 13 µM (150 to 380 ppm), but only 15% larger cells from 13 to 27 µM (380 to 770 ppm) CO_2. Thus, relatively large in-

creases in nitrogen-limited diatom cell size could have been supported as the concentration of atmospheric CO_2 increased from glacial (180 ppm) to interglacial (280 ppm) values and from interglacial to current post-industrial (380 ppm) values, but a much smaller increase in diatom cell size could be supported by CO_2 increases from 380 to 750 ppm, as might occur over the next century (IPCC 2007). The effects of carbon–nitrogen co-limitation on diatom cell size may also have played a role in the macroevolution of diatoms during the period of declining atmospheric CO_2 from the early to late Cenozoic when there was a marked decline in the size of fossil diatoms (Finkel et al. 2005). The constraint of greater nitrogen demand at low atmospheric CO_2 beginning in the early Miocene (24 Ma) to the present (Pagani et al. 2009) may have contributed to the selective pressures favoring smaller diatom cell size (in addition to that caused by low CO_2 itself).

Unlike the centric diatoms, the pennate diatoms *Phaeodactylum tricornutum* (this study) and *Pseudonitzschia multiseries* (Sun et al. 2011) showed little and no response of nitrogen quotas to CO_2, respectively. If these species are representative of other pennates, cell size of this subclass of diatoms would not be expected to vary with CO_2, and higher CO_2 should favor centric over pennate diatoms as was observed during incubations of Ross Sea phytoplankton (Tortell et al. 2008b, Feng et al. 2010).

CO_2 regulation of element ratios in marine phytoplankton

A rise in C:N ratios with increasing CO_2, such as that found here in 2 centric diatoms and *Isochrysis glabana*, was observed previously in marine and freshwater phytoplankton (Clark et al. 1999, Tortell et al. 2000, Clark 2001, Urabe & Waki 2009, Montechiaro & Giordano 2010). The lack of an effect of CO_2 on C:N in *Phaeodactylum tricornutum* is consistent with previous studies of pennate diatoms which showed little to no effect of CO_2 on C:N ratios (Burkhardt et al. 1999, Sun et al. 2011).

Consistent with the present results, C:P and N:P ratios were found to increase with higher CO_2 in marine and freshwater diatoms (Burkhardt et al. 1999, Urabe & Waki 2009, King et al. 2011). Interestingly, there was no effect of CO_2 on C:P or N:P ratios in vitamin B_{12}-limited *Attheya* sp. acclimated to 208, 380, or 680 ppm (12, 22, 39 µM) CO_2 (King et al. 2011) or in phosphorus-limited *Skeletonema costatum* acclimated to 4.5 or 21 µM CO_2 (Gervais & Riebesell 2001). Nutrient limitation therefore appears to dampen CO_2-driven changes in C:P and N:P ratios in centric diatoms, perhaps by lowering the need for a CCM at low growth rate, although it is not clear whether the reported effects were due to a lack of variation in individual element quotas or to tightly coupled variation among these elements with varying CO_2.

The effects of CO_2 on element ratios appear to vary widely among marine prymnesiophytes. While the present results show that increasing CO_2 leads to higher C:N, C:P, and N:P ratios in *Isochrysis galbana*, relatively minor increases in C:N ratios were observed in *Phaeocystis globosa* or the coccolithophore *Emiliania huxleyi* grown with elevated CO_2 (Engel et al. 2005, Wang et al. 2010). However, increases in C:P and N:P ratios in cultures of *E. huxleyi* acclimated to 750 ppm (24 µM) compared to 375 ppm (12 µM) CO_2 (Feng et al. 2008) are similar to those observed here in *I. galbana*. Element ratios did not vary with CO_2 in *E. huxleyi* cultures in which growth was reduced by light limitation (Feng et al. 2008).

Much of the observed CO_2-driven changes in C:N and C:P ratios (20 to 100%) in the species examined here was due to variation in nitrogen and phosphorus quotas rather than changes in cell carbon. Indeed, cellular phosphorus varied with CO_2 to a greater extent than nitrogen, accounting for 50 to 100% of observed changes in C:P ratios and 70 to 100% of observed changes in N:P ratios. How the cellular use or storage of phosphorus in marine phytoplankton varies with CO_2 is currently unknown.

Element ratios in marine phytoplankton regulate the nutrient composition of the deep sea (Arrigo 2005, Lenton & Klausmeier 2007) and as such are critical to ocean biogeochemistry and its response to global change (Riebesell et al. 2007). These ratios vary with growth conditions including nutrient supply (Laws et al. 1983), temperature (Berges et al. 2002), and light (Finkel et al. 2006), and the present results add to a growing body of evidence that major element ratios in phytoplankton also vary with CO_2. In marine diatoms, present and prior results indicate that C:N ratios increase as pCO_2 increases from 150 to 380 ppm, but that these ratios are relatively constant at higher pCO_2. CO_2-modulated nitrogen demand in diatoms, and perhaps other classes of phytoplankton (Engel et al. 2005, Barcelos e Ramos et al. 2007, Hutchins et al. 2007, Levitan et al. 2007, Feng et al. 2008), could therefore provide a negative feedback against changing CO_2 over the pCO_2 range of 150 to 380 ppm by supporting larger cells or higher biomass as CO_2 increases and vice versa. However,

since C:N ratios of most marine phytoplankton do not appear to increase above a pCO_2 of 380 ppm, it is less clear that this phenomenon could provide a carbon sink as atmospheric CO_2 rises above 380 ppm over the next 50 to 100 yr (IPCC 2007). In mixed diatom-prymnesiophyte mesocosms initiated with 2 and 3 times current atmospheric concentrations of CO_2, higher DIC:nitrate drawdown ratios resulted in additional production of extracellular carbon, but no change in particulate C:N compared with mesocosms initiated at 350 ppm CO_2 (Riebesell et al. 2007). If this result is representative of natural phytoplankton communities, increased carbon fixation at elevated CO_2 could enhance the oceanic biological carbon pump, but the effectiveness of extracellular dissolved and particulate organic carbon as a sink for atmospheric CO_2 requires further evaluation.

CO_2-driven variation in the element contents and ratios of marine phytoplankton reflect the biochemical resource demands that potentially limit photosynthetic carbon fixation and growth at present and lower concentrations of CO_2 in the surface ocean. Understanding how the demands for nitrogen, phosphorus, and other nutrients in marine phytoplankton respond to CO_2, and how these responses vary with the degree of nutrient or light limitation of growth, is critical to our understanding of the ecological dynamics of marine phytoplankton communities and biogeochemical cycles of nutrient elements in the sea.

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