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D. A. Hutchins

F.-X. Fu

Y. Zhang

M. E. Warner

Y. Feng

See next page for additional authors

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CO_2 control of *Trichodesmium* N_2 fixation, photosynthesis, growth rates, and elemental ratios: Implications for past, present, and future ocean biogeochemistry

D. A. Hutchins[1] *and F.-X. Fu*
Department of Biological Sciences, University of Southern California, 3616 Trousdale Parkway, Los Angeles, California, 90089

Y. Zhang and M. E. Warner
College of Marine and Earth Studies, University of Delaware, 700 Pilottown Road, Lewes, Delaware, 19958

Y. Feng
Department of Biological Sciences, University of Southern California, 3616 Trousdale Parkway, Los Angeles, California, 90089

K. Portune
College of Marine and Earth Studies, University of Delaware, 700 Pilottown Road, Lewes, Delaware, 19958

P. W. Bernhardt and M. R. Mulholland
Department of Ocean, Earth and Atmospheric Science, Old Dominion University, 4600 Elkhorn Avenue, Norfolk, Virginia, 23529

Abstract

Diazotrophic marine cyanobacteria in the genus *Trichodesmium* contribute a large fraction of the new nitrogen entering the oligotrophic oceans, but little is known about how they respond to shifts in global change variables such as carbon dioxide (CO_2) and temperature. We compared *Trichodesmium* dinitrogen (N_2) and CO_2 fixation rates during steady-state growth under past, current, and future CO_2 scenarios, and at two relevant temperatures. At projected CO_2 levels of year 2100 (76 Pa, 750 ppm), N_2 fixation rates of Pacific and Atlantic isolates increased 35–100%, and CO_2 fixation rates increased 15–128% relative to present day CO_2 conditions (39 Pa, 380 ppm). CO_2-mediated rate increases were of similar relative magnitude in both phosphorus (P)-replete and P-limited cultures, suggesting that this effect may be independent of resource limitation. Neither isolate could grow at 15 Pa (150 ppm) CO_2, but N_2 and CO_2 fixation rates, growth rates, and nitrogen : phosophorus (N : P) ratios all increased significantly between 39 Pa and 152 Pa (1500 ppm). In contrast, these parameters were affected only minimally or not at all by a 4°C temperature change. Photosynthesis versus irradiance parameters, however, responded to both CO_2 and temperature but in different ways for each isolate. These results suggest that by the end of this century, elevated CO_2 could substantially increase global *Trichodesmium* N_2 and CO_2 fixation, fundamentally altering the current marine N and C cycles and potentially driving some oceanic regimes towards P limitation. CO_2 limitation of *Trichodesmium* diazotrophy during past glacial periods could also have contributed to setting minimum atmospheric CO_2 levels through downregulation of the biological pump. The relationship between marine N_2 fixation and atmospheric CO_2 concentration appears to be more complex than previously realized and needs to be considered in the context of the rapidly changing oligotrophic oceans.

[1] Corresponding author: (dahutch@usc.edu).

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Trichodesmium spp. and other diazotrophic cyanobacteria support a large fraction of total biological productivity in the tropical and subtropical seas (Capone et al. 2005; Mahaffey et al. 2005). They also exert a significant influence on the global cycles of nutrients and carbon by supplying new nitrogen that enables marine phytoplankton to draw down atmospheric CO_2 (Karl et al. 2002; Arrigo

2005). Consequently, the potential for limitation of *Trichodesmium* dinitrogen (N_2) and carbon dioxide (CO_2) fixation by nutrients such as iron (Fe) and phosphorus (P) has been investigated (Sañudo-Wilhelmy et al. 2001; Mills et al. 2004). A further complication in our understanding of the interactions between oceanic N_2 fixation and the carbon (C) cycle is the unknown effect of global-scale anthropogenic perturbation of carbon biogeochemistry, following a century of unrestrained fossil fuel burning.

The atmospheric partial pressure of CO_2 (pCO_2) has already increased by roughly one-third, to the highest levels in at least half a million years (Houghton et al. 2001). Continuing emissions will nearly double current seawater pCO_2 to ~76 Pa by 2100, lowering ocean pH by about 0.35 units (Wolf-Gladrow et al. 1999). Marine phytoplankton respond to anthropogenic CO_2 increases and sea-surface warming by altering their physiology (Riebesell et al. 2000), relative abundance (Tortell et al. 2002), and biogeography (Boyd and Doney 2002). In particular, the detrimental effects of ocean acidification on marine calcifying organisms have received a great deal of recent attention (Riebesell et al. 2000; Orr et al. 2005; Feng et al. unpubl. data). Unicellular marine cyanobacteria such as *Synechococcus* and *Prochlorococcus* can also show taxon-specific responses to increasing pCO_2 and temperature (Fu et al. in press), but little information is currently available about global change effects on N_2-fixing cyanobacteria such as *Trichodesmium*.

Marine N_2 fixation may be coupled to long-term natural climate cycles through indirect feedbacks to atmospheric CO_2. For example, it has been hypothesized that increases in N_2 fixation from dust-driven iron fertilization of the ocean during glacial periods may shift the balance between global nitrogen fixation (N inputs) and denitrification (N removal). The resulting accumulation of fixed nitrogen in the ocean alleviates phytoplankton nitrogen limitation, although limitation by other nutrients such as P could potentially modify this scenario. Subsequently, increased carbon export then sequesters atmospheric CO_2, reducing greenhouse forcing and cooling the Earth. Thus, oceanic N_2 fixation may be an important driver of pCO_2 and climate regulation, even over geological timescales (Falkowski 1997; Michaels et al. 2001).

Here, we present the results of experiments examining the relationship between major global change variables (pCO_2 and temperature) and diazotrophic growth of two *Trichodesmium* ecotypes. We grew cultures of both Pacific and Atlantic Ocean isolates across a range of CO_2 concentrations, at two different temperatures, and at sufficient and limiting P concentrations. Sea-surface temperature is increasing globally along with pCO_2 (Boyd and Doney 2002), and *Trichodesmium* distributions are thought to be limited geographically by temperature. Furthermore, diazotrophic growth across most of the ocean is believed to be limited by P or Fe (Sañudo-Wilhelmy et al. 2001; Mills et al. 2004; Fu et al. 2005*a*), so determining how variables like temperature and P availability interact with changing pCO_2 is critical. Cultured populations were used for these studies out of necessity, as acclimated growth is critical to evaluating steady-state physiological responses to elevated CO_2 and natural *Trichodesmium* samples cannot be maintained for extended periods in enclosed experiments at sea (Mulholland et al. 2001). Our results suggest that N_2 and carbon fixation, growth rates, and elemental stoichiometry in *Trichodesmium* are strongly affected by CO_2 levels during steady-state growth, a finding that could have large implications for nutrient cycling and food web dynamics in the past, present, and future ocean.

Methods

Culturing techniques—Stock and experimental cultures of *Trichodesmium erythraeum* strains GBRTRLI101 from the Pacific Ocean (GBR) and IMS101 from the Atlantic Ocean (IMS) were grown without combined nitrogen in modified YBCII seawater medium (Chen et al. 1996; Fu et al. 2005*a*,*b*) supplemented with growth-replete levels of Fe and other trace metals (Fu et al. 2005*a*). Unialgal stock cultures were grown at 25°C with a light–dark cycle of 12 : 12 light : dark (LD) and an incident photon flux density of 100 μmol photons m^{-2} s^{-1}.

Experimental design—Cultures were grown semicontinuously by diluting with fresh microwave-sterilized, pCO_2-equilibrated medium every 2 days. Cell numbers were monitored microscopically to calculate growth rates (Fu et al. 2006, in press). Cultures were kept optically thin ($< \sim 150$ filaments mL^{-1}) to avoid self-shading and dissolved inorganic carbon (DIC) or nutrient depletion. Bottles were equilibrated at the target pCO_2 by gentle bubbling at uniform rates in all treatments with commercially prepared air : CO_2 mixtures (Scott Gas) for all treatments except the ambient pCO_2 bottles, which were bubbled at the same rate with fresh filtered ambient air using an aquarium pump. CO_2 equilibration was verified in all experiments by daily pH measurements and by total DIC analyses (*see* Analytical methods). Steady-state growth was considered attained when measured cell-specific growth rates were constant for 7–10 consecutive generations, at which time the cultures were sacrificed for measurements of N_2 fixation rates, CO_2 fixation rates, photosynthesis versus irradiance curves, and elemental ratios (*see* Analytical methods). Significant differences ($p < 0.05$) between means of triplicate bottles for each treatment were determined using a Student's *t*-test.

The first set of experiments used GBR grown under P- and Fe-replete conditions at 25°C, with triplicate bottles equilibrated at 15, 39, 76, 127, and 152 Pa CO_2 (150, 380, 750, 1250, and 1500 ppm). For the second set of experiments, cultures of both GBR and IMS were acclimated in triplicate 1-liter polycarbonate bottles under the stock culture growth conditions at either 25°C or 29°C. For each temperature treatment, triplicate bottles were incubated at present-day pCO_2 (~39 Pa) or at projected year 2100 pCO_2 (76 Pa) levels as described above. The four treatments used in this part of the study were 25°C and 39 Pa CO_2 (control); 29°C and 39 Pa CO_2 (high temperature); 25°C and 76 Pa CO_2 (high CO_2); and 29°C and 76 Pa CO_2 (greenhouse). Both isolates were maintained in steady-state growth, in

triplicate, under all four treatment conditions in both P-replete (20 μmol L^{-1} phosphate) and P-limited (0.2 μmol L^{-1} phosphate) media (Fu et al. 2005*a*,*b*).

Analytical methods—Rates of N_2 fixation in the experiments were estimated by the acetylene reduction method using a ratio of 3 : 1 to convert ethylene production to N_2 fixation. N_2 fixation rates were also estimated by $^{15}N_2$ incorporation in some cases (Mulholland and Bernhardt 2005). Trends in $^{15}N_2$ uptake rates were very similar to those obtained from acetylene reduction (data not shown), but are not presented as they were not performed for every experiment. Particulate organic C and N and isotope ratios were determined using a Europa 20 : 20 isotope ratio mass spectrometer equipped with an automated nitrogen and carbon analysis for gas, solids, and liquids (ANCA-GSL) preparation unit (Mulholland and Bernhardt 2005); intracellular particulate organic P and chlorophyll *a* (Chl *a*) were measured as in Fu et al. (2005*b*).

CO_2 fixation rates under experimental conditions were measured for the first GBR experiment using $H^{13}CO_3^-$ (Mulholland and Bernhardt 2005), and for the IMS experiment and the second GBR experiment using $H^{14}CO_3^-$ (Fu et al. in press). All CO_2 fixation rates were calculated using experimental DIC concentrations for each pCO_2 treatment. Total DIC was determined by injecting 1.25-mL samples into an acid sparging column, and the CO_2 resulting from acid conversion of the DIC pool was quantified with a Licor infrared analyzer with high precision flow control (Fu et al. in press). This instrument has replicate precision of $\pm 0.06\%$ for seawater samples. Triplicate subsamples for DIC analysis were preserved with $HgCl_2$ (0.44 μmol L^{-1}) and stored at 4°C in the dark until analysis.

Photosynthesis versus irradiance (P–E) curves measured ^{14}C fixation rates across a range of light intensities using a CHPT photosynthetron with a fiber optics–based light source (Fu et al. in press). Triplicate 5-mL culture samples from each treatment were placed in 7-mL glass vials, and 3,700 Bq mL^{-1} ^{14}C sodium bicarbonate was added. Samples were then incubated at appropriate experimental growth temperatures under light intensities ranging from 10 μmol photons m^{-2} s^{-1} to 900 μmol photons m^{-2} s^{-1}. To correct for dark uptake, parallel incubations were carried out in duplicate foil-covered vials. Duplicate killed control blanks were also performed for each treatment and subtracted from reported live cell activities. After 30-min incubations, ^{14}C uptake was stopped by fixation with gluteraldehyde, and the samples were filtered (0.4 μm polycarbonate), thoroughly rinsed with 0.2-μm-filtered seawater, and counted using a Wallac System 1400 liquid scintillation counter. CO_2 fixation rate calculations used measured DIC values in the seawater for each experimental treatment, as described above. P–E parameters were calculated as in Platt et al. (1980), including corrections for potential photoinhibition at elevated irradiance (β). These included the initial slope of the P–E curve α [mg C (mg Chl *a*)$^{-1}$ h^{-1} (μmol quanta m^{-2} s^{-1})$^{-1}$], the maximum Chl *a*-specific carbon fixation rate P_{Bmax} [mg C (mg Chl *a*)$^{-1}$ h^{-1}], and the light saturation point E_k ($P_{Bmax} : \alpha$).

Table 1. Measured steady-state pH and total dissolved inorganic carbon (DIC) values in semicontinuous cultures of *Trichodesmium* GBR grown across a pCO_2 range. Shown are the known pCO_2 of the air : CO_2 mixture with which the cultures were bubbled and the calculated pCO_2 in the seawater medium based on measured pH and total DIC concentrations.

Known air pCO_2 (Pa)	pH	DIC (μmol kg^{-1})	Calculated seawater pCO_2 (Pa)
15	8.52	1844	15
39	8.20	2056	40
76	7.96	2169	76
127	7.76	2283	130
152	7.69	2339	157

Results

Seawater medium pCO_2 values calculated from pH and DIC measurements were all within <3% of the known concentrations in the commercial air : CO_2 mixtures used to bubble the cultures (Table 1). Total DIC concentrations in the present-day control (2056 μmol kg^{-1}) fall within the natural annual range observed in areas where *Trichodesmium* blooms, like the Sargasso Sea (~2010–2070 μmol kg^{-1}, Bates 2001). Likewise, changes in pH and DIC values in the elevated pCO_2 treatments (Table 1) were closely comparable to those expected in future surface seawater (Houghton et al. 2001). Thus, the carbonate buffer system parameters in our artificial seawater culture medium were virtually identical to those found in natural seawater across the relevant range of pCO_2 values.

Chl *a*–normalized N_2 fixation rates of GBR increased significantly by 63% between present day CO_2 (39 Pa) and projected year 2100 pCO_2 (76 Pa) ($p < 0.05$, Student's *t*-test), and then remained relatively steady between 76 Pa and 152 Pa ($p > 0.05$, Fig. 1a). CO_2 fixation rates also increased incrementally by 54% ($p < 0.05$) between 39 Pa CO_2 and 152 Pa CO_2 (Fig. 1b), and cellular growth rates increased by 37% ($p < 0.05$) for the same pCO_2 range (Fig. 1c). Cellular C : N ratios were relatively invariant (5.2–5.9, $p > 0.05$) between treatments (data not shown). However, cellular N : P ratios (and by extension C : P ratios, data not shown) increased significantly ($p < 0.05$) from near-Redfield values of 17 at 39 Pa CO_2 to 21–23 at 76–152 Pa (Fig. 1d). N : P ratios were not significantly different from each other in the three highest CO_2 treatments ($p > 0.05$).

Three independent trials using triplicate cultures demonstrated that neither the Pacific GBR strain nor the Atlantic *Trichodesmium* strain IMS101 were capable of surviving for extended periods at 15 Pa CO_2 (data not shown). Therefore, N_2 and CO_2 fixation and growth rates were zero at this pCO_2 (Fig. 1a–c), and elemental ratios could not be determined (Fig. 1d). After transfer to this low pCO_2 treatment, all cultures ceased growing within 2 days, individual filaments of cells fragmented, and both strains ultimately died within a week or so.

In the second experiment, the effects of 39 Pa CO_2 and 76 Pa CO_2 were examined at two temperatures during P-

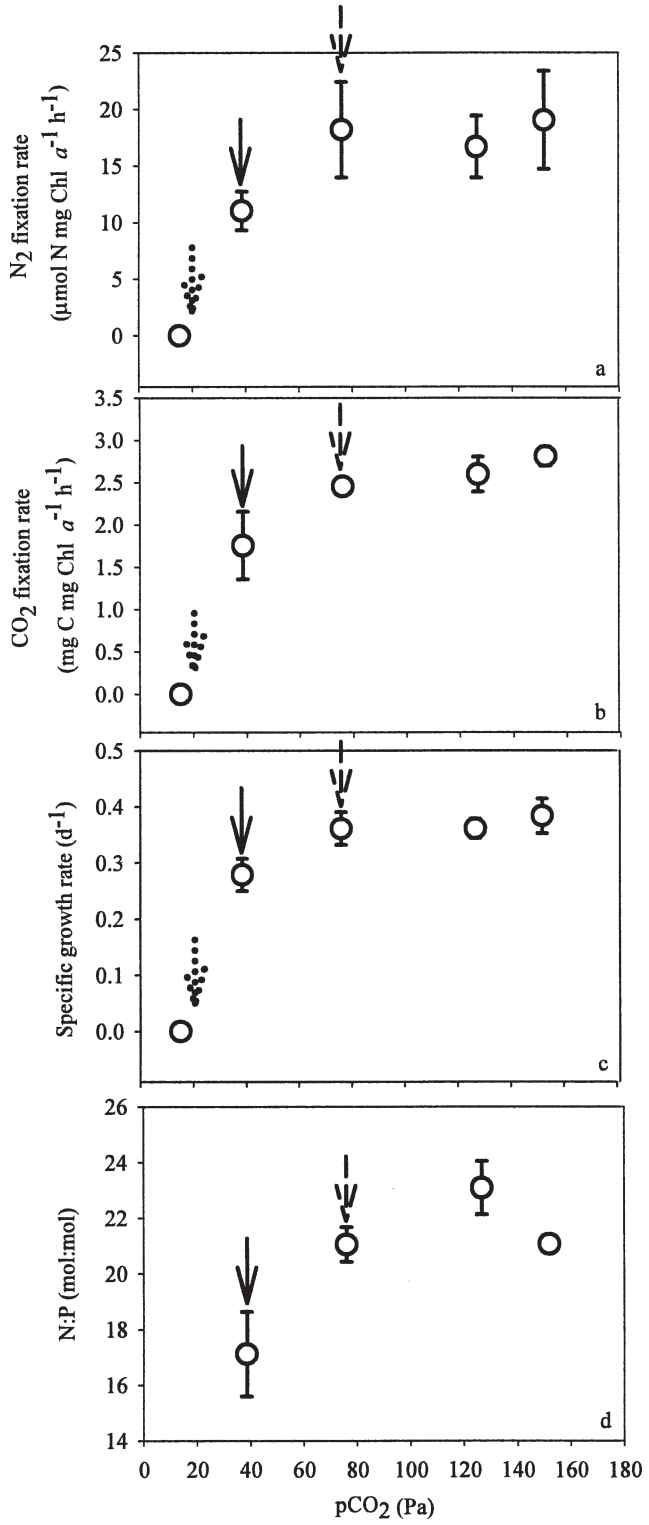

Fig. 1. N_2 fixation rates, CO_2 fixation rates, cell-specific growth rates, and elemental ratios of the Pacific *Trichodesmium* isolate GBR across a pCO_2 range from 15 Pa to 152 Pa. (a) Chl *a*–normalized N_2 fixation rates, (b) Chl *a*–normalized CO_2 fixation rates, (c) cell-specific growth rates, and (d) cellular molar N : P ratios. In panels a, b, and c arrows indicate approximate last glacial maximum pCO_2 (~19 Pa, dotted arrow), present day pCO_2 (~39 Pa, solid arrow), and projected year 2100 pCO_2 (~76 Pa, dashed arrow). N_2 fixation, CO_2 fixation, and growth rates at 15 Pa were zero, as *Trichodesmium* was unable to survive at this pCO_2. Consequently, no elemental ratio measurements were possible for the 15 Pa culture, and these data and the arrow indicating 19 Pa were omitted in panel d. Values are the means and error bars are the standard deviations of triplicate cultures for each treatment.

replete and P-limited steady-state growth for both GBR and IMS in a complete eight-treatment matrix for each isolate. In P-replete cultures of both strains, neither Chl *a*-normalized (Fig 2a,b) nor cell-normalized (Fig 2c,d) N_2 fixation rates were affected by a 4°C temperature increase for either isolate ($p > 0.05$). As in the first experiment, however, P-replete N_2 fixation rates were significantly higher in every case ($p < 0.05$) at elevated pCO_2 (Fig. 2). N_2 fixation rates in P-replete cultures grown at 76 Pa CO_2 were 42–66% (GBR) and 35% (IMS) higher than in cultures grown at the same temperatures at 39 Pa CO_2 (Table 2).

As expected, severely P-limited cultures of both the Pacific and Atlantic isolates fixed much less N_2 than P-replete cultures (Table 2). Despite this, relative increases in N_2 fixation rates under high CO_2 were similar to, or even greater than, in the P-replete cultures (Fig. 3). CO_2-mediated increases in N_2 fixation rates in the P-limited samples were 66–100% (GBR) and 35–39% (IMS) in the 76 Pa cultures relative to the 39 Pa cultures (Table 2). Again, similar increases were observed whether N_2 fixation rates were normalized to Chl *a* (Fig. 3a,b) or cell number (Fig. 3c,d). Similar results were also recorded regardless of whether N_2 fixation rates were measured using the acetylene reduction method (Fig. 3), or by $^{15}N_2$ incorporation (data not shown).

The pCO_2-mediated trends for CO_2 fixation rates in both Pacific and Atlantic *Trichodesmium* strains were generally similar to those observed for N_2 fixation (Fig. 4). As with N_2 fixation, the relative degree of rate enhancement at high pCO_2 was similar or even greater in P-limited cultures as compared to P-replete ones. For P-replete GBR (Fig. 4a) and P-replete or P-limited IMS (Fig. 4b, d) grown at a pCO_2 of 76 Pa, CO_2 fixation rates were significantly higher than in cultures grown at the same temperatures at 39 Pa CO_2 ($p < 0.05$, Table 2). P-limited GBR cultures showed a slightly different response, with the highest CO_2 fixation rates in the greenhouse and high-temperature treatments (Fig. 4c). At the higher pCO_2, CO_2 fixation rates were elevated by 27–38% and 15–24% for P-replete and P-limited GBR, respectively, and by 39–51% and 97–128% for P-replete and P-limited IMS, respectively, relative to the cultures grown at the same temperatures at present day pCO_2 (Table 2).

Molar C : N ratios of the GBR and IMS cells in the second experiment ranged from 4.7 to 5.9, and as in the first experiment there was no systematic influence of the two pCO_2 treatments (Table 2). In contrast, paired comparisons of P-replete GBR and IMS at the same temperatures show that N : P ratios increased by 16–21% ($p < 0.05$) at 76 Pa CO_2 as compared to 39 Pa CO_2. The CO_2-

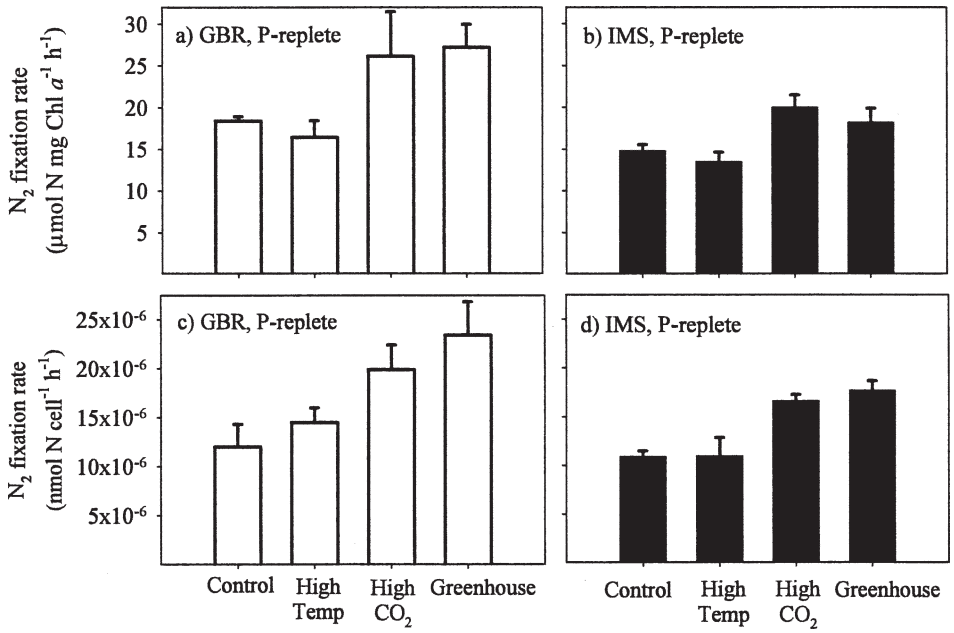

Fig. 2. N_2 fixation rates in P-replete Pacific (GBR, open bars) and Atlantic (IMS, closed bars) *Trichodesmium* cultures during steady-state growth under four experimental combinations of pCO_2 and temperature. (a) GBR, chl *a*–normalized N_2 fixation rates, (b) IMS, chl *a*–normalized N_2 fixation rates, (c) GBR, cell-normalized N_2 fixation rates, and (d) IMS, cell-normalized N_2 fixation rates. Values are the means and error bars are the standard deviations of triplicate cultures for each treatment.

mediated N : P increase in P-limited cultures was 10–27% ($p < 0.05$, Table 2).

As with the other rate measurements, short-term CO_2 fixation rates from the P–E curves were invariably much lower (~25%) in P-limited cultures than in P-replete cultures. However, CO_2- and temperature-driven changes in photosynthetic parameters were evident regardless of growth limitation by P. Maximum light–saturated Chl *a*-normalized photosynthetic rates (P_{Bmax}) in both P-replete and P-limited GBR cultures increased 27–55% in the high CO_2 and high-temperature treatments relative to the respective controls (Fig. 5a,c). In contrast, under both conditions of P availability, increases in both CO_2 and temperature in the greenhouse treatment increased P_{Bmax} significantly more ($p < 0.05$), by 88% and 64% over the control values in the P-replete and P-limited cultures, respectively (Table 2).

The response of IMS P_{Bmax} to temperature and CO_2 increases differed somewhat from that of GBR, but was again consistent between P-replete and P-limited cultures (Fig. 5b,d). Unlike the GBR isolate, the IMS P_{Bmax} was not significantly increased under high CO_2 alone as compared to the control values ($p > 0.05$). Increased temperature alone, however, did increase P_{Bmax} significantly relative to the respective control levels (100% in the P-replete and 46% in the P-limited IMS treatments, Table 2). However, as with the GBR results, the greenhouse treatments had much higher P_{Bmax} under both nutrient conditions than in any of the other treatments, 70–178% higher than in the controls (Fig. 5b,d).

Treatment-related trends in the slope of the light-limited portion of the P–E curve, α, were generally similar to those for P_{Bmax} for the GBR experiment (Table 2). In P-limited cultures of this Pacific isolate, α was marginally enhanced relative to the control by either elevated temperature or CO_2, but values in the combined greenhouse treatment were significantly greater relative to the other treatments ($p < 0.05$). P-replete GBR and IMS cultures had a slightly different trend, with significant increases in α from high temperature alone but not from high CO_2 alone, but again the greenhouse treatment had the highest α values (38% to 307% higher than in the control, $p < 0.05$). In contrast to P-replete conditions, α increased only modestly in the P-limited IMS greenhouse treatment. This slight increase was not significant, and all four treatments had similar α values ($p > 0.05$, Table 2).

E_k ($P_{Bmax} : \alpha$) represents the light saturation point, where light absorption is in balance with the capacity of the photosynthetic system to process this incoming energy. E_k was elevated in all three high-temperature and/or CO_2 treatments in P-replete GBR relative to the control, mainly because of greater increases in P_{Bmax} than in α under all of these growth conditions. These three treatments did not, however, differ significantly from each other ($p > 0.05$, Table 2). In contrast, in the P-limited GBR cultures, E_k decreased significantly in the greenhouse treatment relative to the fairly invariant values in the other three treatments, in this case because P_{Bmax} showed a smaller relative increase than α under the combined high CO_2 and high-temperature conditions. IMS E_k showed this same trend but only in the P-replete cultures; in P-limited cultures, both high-temperature treatments had higher α values than both low-temperature treatments, with no evident CO_2 effect (Table 2).

Table 2. Cell-specific growth rates μ (d^{-1}), Chl *a*-normalized N_2 fixation rates [μmol N (mg Chl *a*)$^{-1}$ h^{-1}], CO_2 fixation rates [mg C (mg Chl *a*)$^{-1}$ h^{-1}], C : N and N : P ratios (mol : mol), and the P–E parameters P_{Bmax}, [mg C (mg Chl *a*)$^{-1}$ h^{-1}], α (mg C (mg Chl *a*)$^{-1}$ h^{-1} (μmol photons^{-2} s^{-1})$^{-1}$, and E_k (μmol photons^{-2} s^{-1}) of Pacific (GBR) and Atlantic (IMS) *Trichodesmium* strains during steady-state P-replete and P-limited growth in the four CO_2 and temperature treatments. Values are the means and numbers in parentheses are the standard deviations of triplicate bottles for each treatment.

GBR									IMS							
	P-replete				P-limited				P-replete				P-limited			
	Control	High temperature	High CO_2	Greenhouse	Control	High temperature	High CO_2	Greenhouse	Control	High temperature	High CO_2	Greenhouse	Control	High temperature	High CO_2	Greenhouse
Growth rate μ	0.29	0.36	0.36	0.40	0.08	0.08	0.12	0.10	0.35	0.36	0.39	0.41	0.13	0.13	0.15	0.15
	(0.04)	(0.05)	(0.05)	(0.04)	(0.01)	(0)	(0.02)	(0)	(0.04)	(0.04)	(0.05)	(0.05)	(0.01)	(0.01)	(0.01)	(0.02)
N_2 fixation rate	18.3	16.4	26.1	27.2	5.9	5.3	11.9	8.9	14.8	13.5	20.0	18.2	6.5	6.1	9.1	8.3
	(0.5)	(2.0)	(5.4)	(2.8)	(0.2)	(0.3)	(0.9)	(0.3)	(0.8)	(1.1)	(1.5)	(1.7)	(0.3)	(0.5)	(0.9)	(0.5)
24-h CO_2 fixation rate	2.37	2.57	3.01	3.54	0.56	0.87	0.69	1.00	1.25	1.24	1.75	1.87	0.32	0.33	0.74	0.65
	(0.1)	(0.1)	(0.2)	(0.6)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.2)	(0.2)	(0.4)	(0.1)	(0)	(0)	(0.1)
C : N ratio	4.8	4.9	5.0	4.8	5.5	5.6	4.8	5.1	5.1	5.2	5.1	4.7	5.5	5.9	5.3	5.7
	(0.10)	(0.04)	(0.10)	(0.11)	(0.1)	(0.05)	(0.1)	(0.1)	(0.1)	(0)	(0)	(0.3)	(0.4)	(0.1)	(0.2)	(0.10)
N : P ratio	17.8	19.4	21.4	23.5	36.2	39.0	45.3	49.4	17.7	19.0	20.5	22.1	45.4	44.2	49.3	50.8
	(1.0)	(1.6)	(1.7)	(0.8)	(7.8)	(6.4)	(3.5)	(7.7)	(1.0)	(0.7)	(0.8)	(1.0)	(0.3)	(2.1)	(0.5)	(3.6)
P_{Bmax}	4.2	6.5	6.1	7.9	1.1	1.4	1.5	1.8	2.8	5.6	3.7	7.7	1.3	1.9	1.3	2.2
	(0.3)	(0.2)	(0.3)	(0.1)	(0.2)	(0.1)	(0)	(0.1)	(0.2)	(0.4)	(0.1)	(0.1)	(0.2)	(0)	(0.1)	(0.2)
α	0.052	0.062	0.053	0.072	0.018	0.023	0.029	0.037	0.015	0.032	0.017	0.061	0.019	0.020	0.018	0.022
	(0)	(0.01)	(0.01)	(0)	(0)	(0)	(0)	(0)	(0)	(0.01)	(0)	(0.01)	(0)	(0)	(0)	(0)
E_k	81	107	115	110	59	63	52	49	198	175	226	128	68	96	70	102
	(4)	(12)	(15)	(7)	(9)	(8)	(6)	(3)	(38)	(33)	(46)	(19)	(14)	(2)	(11)	(18)

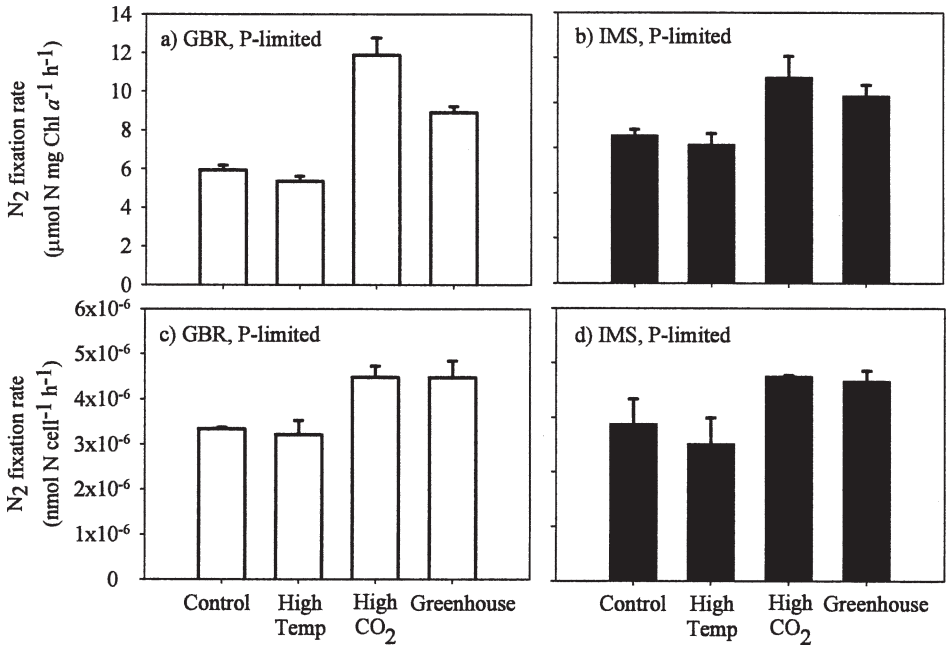

Fig. 3. N_2 fixation rates in P-limited Pacific (GBR, open bars) and Atlantic (IMS, closed bars) *Trichodesmium* cultures during steady-state growth under four experimental combinations of pCO_2 and temperature. (a) GBR, chl *a*–normalized N_2 fixation rates, (b) IMS, chl *a*–normalized N_2 fixation rates, (c) GBR, cell-normalized N_2 fixation rates, and (d) IMS, cell-normalized N_2 fixation rates. Values are the means and error bars are the standard deviations of triplicate cultures for each treatment.

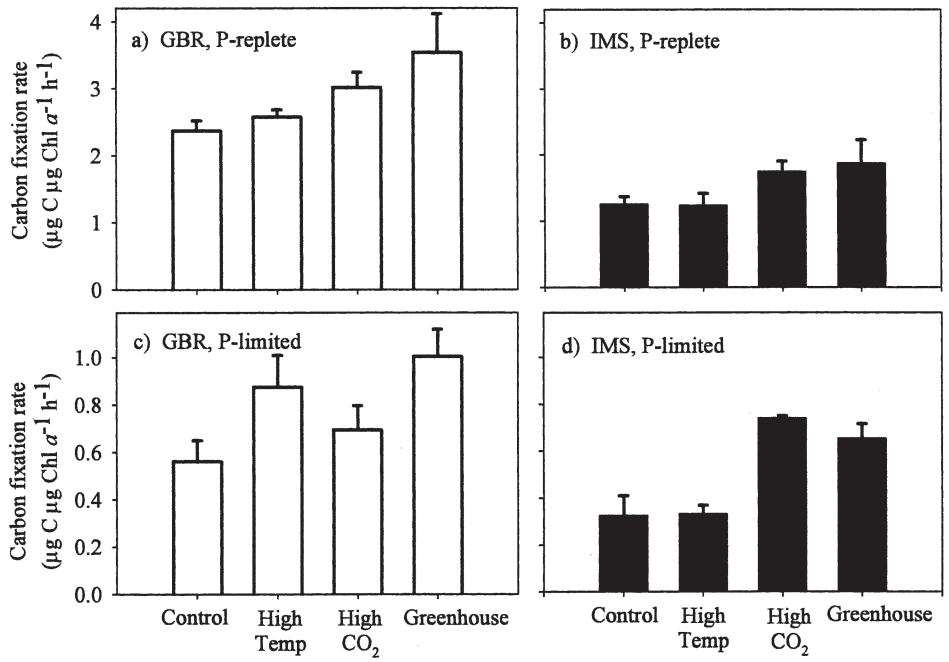

Fig. 4. Chl *a*-normalized CO_2 fixation rates of P-replete and P-limited Pacific (GBR) and Atlantic (IMS) *Trichodesmium* cultures during steady-state growth under four experimental combinations of pCO_2 and temperature. (a) P-replete GBR, (b) P-replete IMS, (c) P-limited GBR, and (d) P-limited IMS. Note differences in y-axis scales for P-replete cultures (a and b) as compared to P-limited cultures (c and d). Values are the means,and error bars are the standard deviations of triplicate cultures for each treatment.

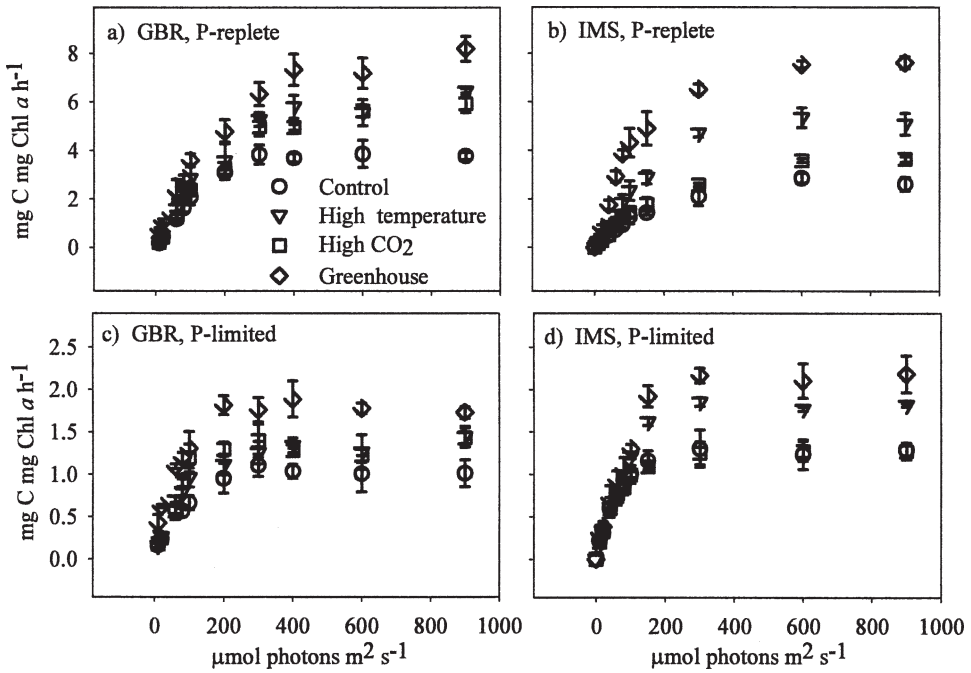

Fig. 5. P–E curves for P-replete and P-limited Pacific (GBR) and Atlantic (IMS) *Trichodesmium* cultures during steady-state growth in control (circle), high temperature (triangle), high CO_2 (square), and greenhouse (diamond) treatments. (a) P-replete GBR, (b) P-replete IMS, (c) P-limited GBR, and (d) P-limited IMS. Note differences in y-axis scales for P-replete cultures (a and b) as compared to P-limited cultures (c and d). Values are the means and error bars are the standard deviations of triplicate cultures for each treatment.

Discussion

Our results demonstrate that *Trichodesmium* N_2 and CO_2 fixation rates, growth rate, and N : P ratios are all strongly dependent on the CO_2 concentrations experienced during growth. We found remarkably consistent responses to pCO_2 changes in two physiologically different *Trichodesmium* isolates from distinct biogeochemical regimes in two major ocean basins (Fu et al. 2005*a*), under varying temperature and nutrient regimes. This suggests that these results may be broadly applicable to natural *Trichodesmium* populations in the oligotrophic oceans. Enhanced N_2 fixation in response to increasing CO_2 has been reported in some terrestrial ecosystems such as agricultural soils (Cheng et al. 2001), but these results provide evidence that marine nitrogen fixation may also be sensitive to ongoing rapid changes in atmospheric CO_2 levels.

In contrast, nitrogen fixation in our cultures was not responsive to a 4°C temperature increase, consistent with results previously reported for the IMS strain (Mulholland and Bernhardt 2005; Breitbarth et al. 2006). It has, however, been suggested that the spatial extent and thus the magnitude of *Trichodesmium* N_2 fixation could increase in the future because of sea-surface warming and consequent expansion of the subtropical biome into higher latitudes (Boyd and Doney 2002). It is also suggested that *Trichodesmium* N_2 fixation could decrease, if warming exceeds optimal temperatures for growth (Breitbarth et al. 2006). Increased stratification and mixed layer shoaling are other global change processes not investigated here that may either enhance or inhibit future *Trichodesmium* diazotrophy by leading to higher mean light exposures or reduced vertical supplies of nutrients like P or Fe.

Current global estimates of N_2 fixation by *Trichodesmium* are about 60×10^9 kg N yr^{-1} (Mahaffey et al. 2005), and diazotrophic growth represents upward of 50% of the new production in some oligotrophic tropical and subtropical oceans (Capone et al. 2005; Mahaffey et al. 2005). If our experimental results can be directly extrapolated to the ocean (which remains to be tested), by 2100 this amount could increase to between 81×10^9 kg N yr^{-1} (35% increase) and 120×10^9 kg N yr^{-1} (100% increase) because of anticipated CO_2 increases alone. This calculation assumes that the relative influences of other limiting factors such as P, Fe, and light availability do not change.

If these estimates are coupled with modeling predictions of a 27% warming-induced expansion of suitable habitat (Boyd and Doney 2002), calculations suggest that global N_2 fixation by *Trichodesmium* alone could range from 103×10^9 kg N yr^{-1} to 152×10^9 kg N yr^{-1} by the end of this century. Because *Trichodesmium* carries out 40–59% of the geochemically inferred N_2 fixation in the North Atlantic and Pacific (Mahaffey et al. 2005), these values would substantially increase recent estimates for total pelagic N_2 fixation of $\sim$100–200 $\times 10^9$ kg N yr^{-1} (Galloway et al. 2004). Our results also suggest, however, that N_2 fixation rates may saturate at about 76 Pa CO_2 (Fig. 1a) and that further enhancement due to subsequent CO_2 increases may be unlikely.

Free-living unicellular cyanobacteria are now believed to fix at least as much nitrogen as *Trichodesmium* in the ocean (Montoya et al. 2004). In addition, endosymbiotic cyanobacteria contribute substantially to N_2 fixation, at least regionally (Carpenter et al. 1999). If N_2 fixation rates in these groups show commensurate increases with rising CO_2, the cumulative effect on the global nitrogen cycle could be considerably larger (e.g., a doubling). Thus, determining the influence of pCO_2 on all important ocean N_2-fixing groups, including free-living and symbiotic cyanobacteria, heterotrophic bacteria, and other common *Trichodesmium* species such as *Trichodesmium thiebautii,* should be a priority for future research. Of course, the net effect of increased N_2 fixation on the future fixed nitrogen inventory of the ocean will also depend on how global change variables such as temperature and stratification affect the availability of nutrients such as P or Fe or major N removal processes such as denitrification.

Our results predict that, like N_2 fixation, CO_2 fixation by *Trichodesmium* should also increase dramatically in the future because of CO_2 enrichment. Unlike N_2 fixation, CO_2 fixation did respond somewhat to increases in temperature. For example, CO_2 fixation rates in P-limited GBR (Fig. 4c) were dependent on temperature as well as pCO_2. Likewise, maximum photosynthetic rates from the P–E curves (P_{Bmax}) increased with either temperature alone or pCO_2 alone (GBR) or with temperature alone but not pCO_2 alone (IMS). Although this observation suggests interesting differences in the short-term photosynthetic physiological responses of the two isolates, the highest P_{Bmax} values in both IMS and GBR were always found in the combined high-temperature and high CO_2 greenhouse treatment. This trend was also observed in the longer-term productivity estimates.

The temperature effect on maximum photosynthetic rates is not surprising, because carbon fixation is enzymatically controlled under saturating light conditions (Falkowski and Raven 1997). Temperature effects on light-related photosynthetic parameters, however, did not translate into temperature-mediated effects on growth or N_2 fixation rates (Table 2). Carbon fixation and growth rates have often been observed to be decoupled in other phytoplankton (Raven et al. 1993; Beardall et al. 1998), which has been attributed to processes such as dissolved organic carbon exudation (Fu et al. in press; Mulholland 2007). Differences in time scales between short-term CO_2 fixation measurements and longer-term growth rate measurements could also account in part for this difference.

The slope of the light-limited portion of the P–E curve (α) increased under greenhouse conditions for both strains, suggesting that *Trichodesmium* light-harvesting efficiency for photosynthesis may be more efficient under future sea-surface warming and high CO_2 regimes. Likewise, the light saturation constant E_k also changed under our experimental treatments in both *Trichodesmium* strains. In nutrient replete cultures, shifts in E_k under greenhouse conditions in the GBR isolate were largely driven by greater changes in P_{Bmax} relative to changes in α, whereas the opposite effect was noted in the IMS isolate. Interestingly, these corresponding changes were the direct opposite for each strain when maintained under P-limited conditions (e.g., P_{Bmax} changed less in the GBR isolate, whereas the IMS isolate had a greater change in P_{Bmax} relative to α). Although beyond the scope of this present study, analyses of pigment quotas and absorption efficiencies should provide further insight into how these variables may change in light of altered nitrogen assimilation (discussed below) and help determine their effect on photosynthetic capacity in different isolates of *Trichodesmium*.

Whereas the effect of N_2 fixation on C budgets has not been widely assessed, the available paired estimates of N_2 and CO_2 fixation suggest that C : N fixation ratios often exceed the cellular C : N ratio (Mulholland et al. 2006). Assuming conservatively that C : N fixation proceeds at rates similar to the cellular C : N ratio of about 5.0 (Table 2), the projected increases in global *Trichodesmium* N_2 fixation rates calculated above would support CO_2 fixation rates of 515–760 $\times$ 10^9 kg C yr^{-1} by this genus under end-of-the-century conditions. Although the ultimate biogeochemical fate of *Trichodesmium*-fixed elements is not fully understood (Mahaffey et al. 2005; Mulholland et al. 2006; Mulholland 2007), any fraction of this enhanced primary productivity that is exported to underlying waters will contribute to sequestration of atmospheric carbon.

Increases in algal carbon fixation rates at elevated CO_2 concentration are well documented (Riebesell 2004), but our work demonstrates that increased pCO_2 can also stimulate N_2 fixation. Similar enhancement of N_2 fixation at elevated pCO_2 was also reported for the IMS *Trichodesmium* strain by Levitan et al. (2007). Genomic evidence suggests that this strain possesses homologues for genes encoding a low-affinity HCO_3^- transport system (BicA) and a low affinity form of the NDH-1 complex (a dehydrogenase possibly involved in active CO_2 uptake), although it apparently lacks the high-affinity DIC transport mechanisms present in some other marine cyanobacteria (Badger et al. 2006). Regardless of genetic evidence for putative *Trichodesmium* inorganic carbon transport systems, our physiological data emphatically demonstrate that this species is vulnerable to DIC limitation at present day (or lower) CO_2 levels.

We suggest that the enhancement of N_2 fixation at higher pCO_2 may be an indirect effect resulting from alleviation of C-limitation of CO_2 fixation, rather than being a consequence of direct CO_2 regulation of N_2 fixation pathways such as *nifH* gene transcription. Carbon and nitrogen fixation are both energy-intensive processes that compete directly for the products of the light reactions of photosynthesis (Berman-Frank et al. 2001). Growth at elevated CO_2 may thus provide a photosynthetic subsidy that enables diversion of increased amounts of energy to support diazotrophy. Our results with both Atlantic and Pacific *Trichodesmium* strains in multiple experiments robustly confirm the phenomenon, but further elucidation of the cellular genetic and metabolic pathways involved is still needed. In ecological and biogeochemical terms, though, it is immaterial whether CO_2 control of diazotrophy is mediated through direct genetic regulation or indirectly through other physiological processes. The salient fact is that it has the potential to profoundly affect global ocean nutrient and carbon cycling.

Carbon limitation of growth and N_2 fixation is also strongly suggested by the inability of either *Trichodesmium* strain to grow at 15 Pa CO_2. This indicates that under the last glacial maximum atmospheric CO_2 levels of ~19 Pa (Petit et al. 1999), growth and N_2 fixation by *Trichodesmium* were likely to have been marginal (Fig. 1). It is conceivable that the interaction between CO_2 limitation and N_2 fixation could contribute to determining a set point for minimum CO_2 levels during glacial periods. When atmospheric CO_2 falls to some critical level (above 15 Pa but much lower than 39 Pa, our experiments have insufficient resolution to determine the exact value), *Trichodesmium* becomes severely carbon-limited. Whether this is also the case for other marine diazotroph groups still remains to be tested. At this point, new N inputs by oceanic N_2 fixation might be significantly reduced. This in turn would tend to downregulate oceanic CO_2 sequestration by the biological pump, thus stabilizing atmospheric CO_2 against further decreases. Reduced glacial atmospheric CO_2 levels could thus act as a governor on oceanic N_2 fixation, regardless of other external drivers like Fe or P supply. Thus, CO_2 control of marine diazotrophy offers a possible corollary to the ''glacial N_2 fixation hypothesis'' (Falkowski 1997; Michaels et al. 2001) that provides a crucial missing element: e.g., an ''off switch'' mechanism for increased glacial productivity.

One of our most striking results was that increased pCO_2 enhanced N_2 and CO_2 fixation and growth rates even under severely P-limited steady-state growth conditions (Fig. 2; Table 2). This finding has major implications, because limitation of *Trichodesmium* growth and N_2 fixation by P, Fe, or light appears to be widespread (Fu et al. 2005*a*; Moutin et al. 2005; Mulholland and Bernhardt 2005). Although P-limited cultures fix much less N_2 and CO_2 and have lower growth rates than P-replete cultures, higher pCO_2 leads to rate increases of similar relative magnitude in both. Thus, P-limited cells can also be considered to be simultaneously limited by CO_2. This surprising observation is difficult to reconcile with the concept of a single Liebig-type limiting nutrient and lends support to the emerging paradigm and abundant evidence for various types of nutrient colimitation (Arrigo 2005; Hutchins and Fu in press). Our results with both *Trichodesmium* isolates conclusively demonstrate that CO_2 availability exerts an overarching control on N and C acquisition, superimposed upon the growth-regulating effects of P availability.

Future CO_2 increases may have the potential to at least partially offset any intensified P-limitation of *Trichodesmium* from expected stratification-driven decreases in P supply. For instance, our P-limited cultures grown at present-day pCO_2 fixed only 34–39% as much N_2 as P-replete cultures grown at the same temperatures (Table 2). When pCO_2 was raised to 2100 levels, however, P-limited cultures were able to fix N_2 at rates that were 61% (IMS) and 48–73% (GBR) of those measured in the P-replete present-day pCO_2 cultures. Higher pCO_2 thus compensates to a large extent for P-limited growth, offering yet another potential feedback mechanism between atmospheric CO_2 and ocean nutrient biogeochemistry. Further work will be required to determine whether pCO_2 increases also promote N_2 and CO_2 fixation regardless of limitation by other resources such as Fe or light.

Based on our results with P-limited cultures, it seems likely that elevated atmospheric CO_2 levels will boost new nitrogen inputs by *Trichodesmium* despite widespread P limitation in regimes like the Sargasso Sea or the North Pacific Central Gyre (Sañudo-Wilhelmy et al. 2001; Karl et al. 2002). This may increase biological N : P ratios in these areas and tend to drive the biota further toward P limitation. Indeed, just such a progressive shift toward P limitation has been observed in the North Pacific since the early 1990s, driven by apparent increases in N_2 fixation. The reasons for this shift may include changing climate regimes and aeolian iron fluxes (Karl et al. 2001), but the fact that atmospheric pCO_2 has increased from ~35 Pa to ~39 Pa in the intervening years (Houghton et al. 2001) could also be contributing to this trend.

C : N ratios did not change with pCO_2 in our experiments, likely because both N_2 and CO_2 fixation increase simultaneously with rising CO_2. Both isolates did exhibit markedly increased N : P ratios at higher pCO_2 under all growth conditions tested. Although we did not measure phosphate uptake rates in our experiments, this observation suggests that unlike N_2 and CO_2 fixation, P incorporation is not responsive to varying CO_2 concentrations. Changing pCO_2 does not affect phosphate uptake in incubated natural populations of the coccolithophorid *Emiliania huxleyi* either (Engel et al. 2005).

The observed increases in N : P ratios and proportional trends in C : P ratios (not shown) match closely with observed paired increases of 11–25% in cell-specific growth rates between 39 Pa CO_2 and 76 Pa CO_2 (Table 2, discounting a single outlier increase of 50% at 25°C in P-limited GBR). This is consistent with the suggestion that elemental ratio changes are driven by increases in cellular N and C incorporation, with P quotas remaining relatively constant under elevated pCO_2. The relative degree of cellular N : P ratio and growth rate enhancement by increased pCO_2 is, however, somewhat smaller than the observed increases in N_2 and CO_2 fixation rates, suggesting that an increased fraction of fixed N and C must be released from the cells at elevated pCO_2.

Trichodesmium often exudes a significant and highly variable amount of recently fixed N as dissolved organic nitrogen and NH_4^+ even under present-day pCO_2 conditions, and this flux is a major route by which new N enters the marine food web (Mulholland and Bernhardt 2005; Mulholland 2007). The ''missing'' fixed N in our experiments suggests that N exudation may be further increased at elevated pCO_2. Fixed N release can be indirectly estimated by the difference between paired N_2 fixation rates measured using acetylene reduction (gross N_2 fixation) and $^{15}N_2$ incorporation (net N_2 fixation, Mulholland 2007). This calculation suggested that in some of our experiments, N release increased from negligible to as much as 50% of total fixed N between 39 Pa CO_2 and 76 Pa CO_2 (data not shown).

The magnitude of the changes in N and C release in our first GBR experiment can also be estimated by comparing the relative increases in N_2 and CO_2 fixation rates with the increases in cellular growth rate (Fig. 1), presuming that

any C and N not appearing in cell biomass was lost to exudation. These calculations suggest that N and C exudation accounted for 41% and 31% of the increase in N_2 and CO_2 fixation rates, respectively, as pCO_2 increased from 39 Pa to 152 Pa. Similar increases in dissolved organic carbon release have been observed in eukaryotic phytoplankton communities incubated at elevated pCO_2 (Engels et al. 2005). For *Trichodesmium*, direct measurements of fixed N and C release are needed in future work to confirm our preliminary indirect estimates and to determine exactly how newly fixed N and C is partitioned under varying CO_2 availability.

Together, our observations suggest that globally increasing pCO_2 could have large consequences both for biological elemental stoichiometry and for supplies of *Trichodesmium*-derived dissolved organic N and C available to other phytoplankton and to the marine microbial food web (Mulholland et al. 2006; Mulholland 2007). Such C and N flux changes could alter the taxonomic and trophic structures of present day marine biological communities and affect the strength of the biological pump relative to the remineralizing microbial loop. Remineralization of fixed carbon by an enhanced microbial foodweb could also at least partially offset any elevated carbon sequestration from increased new production by N_2 fixation under future CO_2 regimes.

CO_2 control of N_2 fixation by *Trichodesmium* could therefore have implications for understanding the future responses of the marine biosphere and global ecosystem to ongoing anthropogenic change. Likewise, carbon limitation of diazotrophy may be linked with the mechanisms controlling climate and CO_2 levels across glacial and interglacial timescales. Many of our current concepts describing the interactions between oceanic nitrogen fixation, atmospheric CO_2, nutrient biogeochemistry, and global climate may need re-evaluation to take into account these previously unrecognized feedback mechanisms between atmospheric composition and ocean biology.

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