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Sensitivity of *Trichodesmium erythraeum* and *Crocosphaera watsonii* abundance and N_2 fixation rates to varying NO_3^- and PO_4^{3-} concentrations in batch cultures

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ABSTRACT: Batch cultures of *Trichodesmium erythraeum*, strain IMS101, and *Crocosphaera watsonii*, strain WH8501, were grown under metal- and vitamin-replete conditions to evaluate differences in diazotroph abundance and N_2 fixation rates as well as biomass C:N:P ratios resulting from changes in the concentrations of nitrate (NO_3^-) and phosphate (PO_4^{3-}) in culture media. Holding light levels and temperature constant, variations in culture NO_3^- and PO_4^{3-} concentrations included (N:P ratios in µM) 0:0.5, 5:1, 8:0.5, and 16:1. The abundance of both diazotrophs was greatest in the 16:1 and 5:1 N:P ratio treatments (i.e. those grown with 1 µM PO_4^{3-}) while the highest N_2 fixation rates for both diazotrophs were observed in the 0:0.5 treatment (i.e. those grown in NO_3^--free media). Measurable but reduced (~25 to 50% of the rates in cultures grown with no NO_3^-) N_2 fixation rates were evident in both *T. erythraeum* and *C. watsonii* cultures grown with up to 16 µM NO_3^-. These results indicate that while diazotrophs grown in the presence of NO_3^- have significantly lower N_2 fixation rates than those not chronically exposed to NO_3^-, these lower per cell N_2 fixation rates are compensated for by a greater abundance of diazotrophs in treatments with 1 µM PO_4^{3-} and result in comparable volume-integrated rates of N_2 fixation. Additionally, N_2 fixation rates for *T. erythraeum* and *C. watsonii* were comparable when normalized to carbon (biomass). Finally, the exponential-phase C:N:P biomass ratios of both diazotrophs were similar to each other as well as to previous studies and varied little among the treatments but increased, often significantly, between exponential and stationary growth phases.

KEY WORDS: N_2 fixation · *Trichodesmium* · *Crocosphaera* · Inhibition · C:N:P ratio

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INTRODUCTION

The study of the marine nitrogen (N) cycle is in part motivated by the goal of understanding how phytoplankton meet their physiological N requirement given the scarcity of N in the surface waters of much of the world's oceans. The limited availability of N for photosynthetic organisms in the surface ocean can affect atmospheric carbon dioxide concentrations and global climate (Falkowski 1997), further motivating investigations into marine N cycling. In spite of the significance of N, there is considerable uncertainty in the rates of the biologically mediated processes that dominate the fluxes of N to and from the ocean. While some estimates suggest that fluxes of N to the ocean may only balance one-third of the fluxes of N out of the ocean (Codispoti et al. 2001, Brandes & Devol 2002, Codispoti 2007), other estimates find the marine N budget to be essentially in balance (Gruber & Sarmiento 1997, Deutsch et al. 2004, Gru-

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ber 2004). This uncertainty underscores the need to better constrain the rates, location, and sensitivities of the processes that add and remove N to and from the ocean.

The dominant process that adds N to the ocean is dinitrogen (N_2) fixation, carried out by diazotrophic prokaryotes, including cyanobacteria, bacteria, and archaea (Zehr et al. 1998, 2001, Braun et al. 1999). Based on numerous *in situ* N_2 fixation rate measurements made over the past 3 decades, a paradigm has emerged in which N_2 fixation rates are expected to be high in the warm, stratified waters of the tropical North Atlantic, especially in and adjacent to the Caribbean (e.g. Carpenter & Price 1977, Capone et al. 1997, 2005, Carpenter et al. 1999). These biological observations were followed and supported by geochemical analyses of deviations of upper thermocline nitrate (NO_3^-) to phosphate (PO_4^{3-}) concentration ratios from the empirically derived 'Redfield' ratio of 16:1 (e.g. Fanning 1992, Michaels et al. 1996, Gruber & Sarmiento 1997). Since marine diazotrophic biomass has N:P ratios typically between 25:1 to 50:1 (e.g. Letelier & Karl 1996,1998, Sañudo-Wilhelmy et al. 2001, Villareal & Carpenter 2003, Krauk et al. 2006, White et al. 2006), and thus is elevated compared to the Redfield biomass stoichiometry of ~16:1 for average phytoplankton, thermocline NO_3^-:PO_4^{3-} concentration ratios >16:1 have often been attributed to diazotrophic inputs. In contrast, ratios <16:1 have been attributed to losses of N by denitrification and/or anammox. Oceanographers have quantified these deviations in subsurface NO_3^-:PO_4^{3-} concentration ratios from Redfield stoichiometry to infer the location and magnitude of these processes that both add and remove N to and from the ocean but leave PO_4^{3-} concentrations unaltered (Fanning 1992, Gruber & Sarmiento 1997, Deutsch et al. 2001, Hansell et al. 2004, 2007). These geochemical analyses have largely supported the previous biological observations of high N_2 fixation rates in the tropical North Atlantic as well as studies of N removal processes occurring at significant rates in the Eastern Tropical Pacific and Arabian Sea (e.g. Cline & Kaplan 1975, Brandes et al. 1998, Hamersley et al. 2007, Bulow et al. 2010, Chang et al. 2010).

Recently, however, remote-sensing observations (Westberry et al. 2005, Westberry & Siegel 2006) and a new analysis of macro-nutrient (i.e. NO_3^- and PO_4^{3-}) distributions in the world's oceans (Deutsch et al. 2007) have challenged the conventional view that the largest N_2 fixation fluxes occur in the North Atlantic and instead suggest that the highest oceanic rates of N_2 fixation may occur in the Eastern Tropical North and South Pacific (ETNP and ETSP, respectively) and the Arabian Sea. These studies imply that marine N_2 fixation may have very different sensitivities than if, as traditionally thought, N_2 fixation rates are highest in the North Atlantic. For example, the N_2 fixation rate estimates of Westberry et al. (2005) and Westberry & Siegel (2006) are *Trichodesmium* spp.-specific, and the blooms of *Trichodesmium* identified in these studies are predicted to occur in regions receiving the lowest eolian dust flux to the global ocean (Wagener et al. 2008, Mahowald et al. 2009). The prediction for the highest *Trichodesmium* spp.-specific N_2 fixation rates in the global ocean to occur in regions with the lowest atmospheric dust flux challenges the well-documented high iron requirements of *Trichodesmium* spp. (e.g. Berman-Frank et al. 2001, Kustka et al. 2003). In contrast, it is thought that the high atmospheric dust flux from African deserts support the high rates of N_2 fixation documented in the tropical North Atlantic (Mills et al. 2004). The lack of an analogous eolian flux to the eastern Pacific leads to questions about how diazotrophs in general, but *Trichodesmium* spp. in particular, would have higher abundances and N_2 fixation rates in the Eastern Pacific than in the tropical North Atlantic as predicted by the recent remote sensing studies.

Additionally, it has been assumed that marine N_2 fixation is inhibited by NO_3^- because acquiring N via N_2 fixation requires more energy than assimilating NO_3^- (Falkowski 1983). However, the surface waters in the Eastern Tropical Pacific and Arabian Sea highlighted by Westberry et al. (2005), Westberry & Siegel (2006), and Deutsch et al. (2007) have relatively high concentrations of NO_3^-, i.e. 1 to 10 µM, throughout the year (Garcia et al. 2010) compared to the tropical North Atlantic surface ocean, where the NO_3^- concentration is typically at or below detection limits of common analytical methods (i.e. <0.1 µM). Consequently, the prediction by both the remote sensing and geochemical modeling studies that the largest N_2 fixation fluxes to the global ocean occur in waters with NO_3^- concentrations capable of inhibiting N_2 fixation (e.g. Holl & Montoya 2005) challenges our understanding of the sensitivity of N_2 fixation to exposure to NO_3^-. Given the significant differences both in the surface ocean nutrient and metal distributions between the North Atlantic and the eastern Pacific, the findings of the remote sensing and geochemical modeling studies challenge expectations of where N_2 fixation 'should' occur based on our traditional understanding of the sensitivities of N_2 fixation.

While the remote sensing and nutrient distribution studies of Westberry & Siegel (2006) and Deutsch et

al. (2007), respectively, are intriguing, they are indirect estimates of N_2 fixation rates, and very few *in situ* measurements of N_2 fixation rates have been reported in the South Pacific (Bonnet et al. 2008, Fernandez et al. 2011, Halm et al. 2012). Given the paucity of field data from the regions highlighted in the remote sensing and modeling studies, culture studies can be used to explore the viability and sensitivity of diazotrophs to environmental conditions analogous to the surface waters in these regions. However, few culturing studies have been conducted with nutrient conditions similar to those found in the ETSP. Some culture-based studies of the sensitivities of N_2 fixation have used environmentally relevant PO_4^{3-} concentrations (e.g. Berman-Frank et al. 2001, Kustka et al. 2003, White et al. 2006), but these studies did not examine the effects of NO_3^- inhibition of N_2 fixation. Other studies of the inhibition of N_2 fixation by NO_3^-, ammonium, and/or urea have used concentrations of PO_4^{3-} and/or dissolved N species that exceed those typically found in the upper ocean (e.g. Mulholland & Capone 1999, Mulholland et al. 2001, Holl & Montoya 2005, Milligan et al. 2007, Dekaezemacker & Bonnet 2011, Sandh et al. 2011). To the best of our knowledge, previous studies examining NO_3^- inhibition of N_2 fixation by marine diazotrophs have not been conducted using both environmentally relevant NO_3^- and PO_4^{3-} concentrations.

The present study investigated the effect of varying NO_3^- and PO_4^{3-} concentrations in artificial seawater media on 2 cultured strains of diazotrophic cyanobacteria: the filamentous, non-heterocystous *Trichodesmium erythraeum* and the unicellular *Crocosphaera watsonii*. The 4 variations in NO_3^- and PO_4^{3-} concentrations in the media (i.e. 'treatments') that both diazotrophs were grown on included (N:P ratios in µM) 0:0.5, 5:1, 8:0.5, and 16:1. The concentrations of macro-nutrients in the artificial seawater media were chosen to span the previously observed 10 µM NO_3^- threshold for ~50% inhibition of N_2 fixation in cultures of *Trichodesmium* (Mulholland et al. 2001, Fu & Bell 2003, Holl & Montoya 2005) as well as to mimic the NO_3^- and PO_4^{3-} concentrations found in the surface waters of the ETNP and ETSP in the regions highlighted in the geochemical and remote-sensing studies described above (Garcia et al. 2010). Below, we compare the differences that resulted among the treatments in diazotroph abundance, N_2 fixation rates, per trichome (for *T. erythraeum*) and per cell (for *C. watsonii*) chlorophyll (chl) *a*, carbon (C), N, and phosphorus (P) content, and biomass molar C:N:P ratios for both *T. erythraeum* and *C. watsonii*.

MATERIALS AND METHODS

Trichodesmium erythraeum and *Crocosphaera watsonii* cultures

Unialgal batch cultures of *Trichodesmium erythraeum,* strain IMS101, and *Crocosphaera watsonii*, strain WH8501, were grown on the N-free artificial seawater media YBC II (Chen et al. 1996) with varied NO_3^- and PO_4^{3-} concentrations as described below. To the best of our knowledge, axenic cultures of *T. erythraeum* and *C. watsonii* were not available at the time of the experiment. However, care was taken to keep all media and equipment sterile, including the inoculation of *T. erythraeum* and *C. watsonii* into the autoclaved YBC II media in a laminar flow hood and filter sterilization of nutrients, metals, and vitamins through a 0.2 µm Acrodisc filter (Pall). Cultures were grown in ~100 ml of media in sterile polycarbonate bottles that had been filled with Milli-Q water and microwaved prior to inoculation to sterilize the bottles. *T. erythraeum* and *C. watsonii* cultures were grown in triplicate at the following 4 nutrient amendments: 0.0 µM NO_3^-, 0.5 µM PO_4^{3-} ('0:0.5 N:P'); 5.0 µM NO_3^-, 1.0 µM PO_4^{3-} ('5:1 N:P'); 8.0 µM NO_3^-, 0.5 µM PO_4^{3-} ('8:0.5 N:P'); and 16.0 µM NO_3^-, 1.0 µM PO_4^{3-} ('16:1 N:P'). The cultures were grown out at their respective nutrient concentrations for 3 or more transfers, typically corresponding to ≥10 generations, before sampling began. The *T. erythraeum* and *C. watsonii* cultures were grown in an incubator at 27°C and 80 µE light on a 12 h light:12 h dark cycle.

Approximately every 3 d, trichome counts for *Trichodesmium erythraeum* cultures were done in triplicate 1 h after the incubator lights turned on by counting the number of trichomes in a 1 ml sample under green excitation using an epifluorescent microscope. *Crocosphaera watsonii* abundance was monitored every day on triplicate cultures at 17:00 h local time using *in vivo* chl *a* fluorescence (Trilogy model fluorometer, Turner Designs). Additionally, *C. watsonii* cells were enumerated at the same time as the particulate C, N, P, and N_2 fixation measurements for exponential and stationary growth phase measurements using a Zeiss Axioplan epifluorescent microscope (20×) fitted with a green (510 to 560 nm) excitation filter on a Malassez counting chamber. Duplicate counts of *C. watsonii* cells were performed for every replicate in each of the 4 treatments, and these cell counts were used to normalize the N_2 fixation rate and biomass C, N, and P content measurements. The statistical significance for differences in the average abundance of both diazotrophs among treatments was determined

using the Wilcoxon signed-ranks test for matched pairs of non-parametric data.

N_2 fixation rates

Trichodesmium erythraeum

N_2 fixation rates for the *Trichodesmium erythraeum* cultures were measured beginning 5 h after local 'sunrise' (i.e. when the lights in the incubator came on) using the acetylene reduction method described by Capone (1993), in which 10 ml of culture was pipetted into a 14 ml serum vial and capped with a rubber stopper, and 1 ml of acetylene was injected. One vial was prepared for each of 3 replicates per experimental treatment, and the increase in ethylene concentration was monitored by flame ionization gas chromatography. Acetylene reduction rates were normalized per trichome, per chl *a* content and carbon content (biomass). Statistical significance of differences in average N_2 fixation rates among treatments as well as for comparisons of average chl *a*, particulate C, N, and P content for both *T. erythraeum* and *Crocosphaera watsonii* was determined using the Kruskal-Wallis rank-sums test for non-parametric data (Triola 2001).

Crocosphaera watsonii

N_2 fixation rates for the *Crocosphaera watsonii* cultures were measured at night, 8 h after the beginning of the dark period, when N_2 fixation rates are known to be the highest (Shi et al. 2010). The rates were determined in each triplicate culture by adding 0.5 ml of $^{15}N_2$ labeled gas (99%, EURISOTOP) to 25 ml of culture and incubating for 4 h under the same darkness and temperature conditions as the original culture to measure the ^{15}N incorporation into *C. watsonii* biomass, as described by Montoya et al. (1996). After incubation, samples were filtered under low vacuum pressure (<100 mm Hg) onto pre-combusted (4 h at 450°C) glass fiber filters (GF/F; 25 mm diameter, 0.7 µm nominal porosity), placed in 2 ml glass tubes, dried at 50°C for 24 h, and then stored over desiccant until analysis. The isotopic enrichment analysis was performed by continuous-flow isotope ratio mass spectrometry using an Integra-CN mass spectrometer. The accuracy of the system was verified regularly using reference material (International Atomic Energy Agency [IAEA], Analytical Quality Control Services). The isotopic enrichment was calibrated using IAEA reference material every 10 samples. The $^{15}N_2$ fixation rates were normalized to cell counts, to chl *a*, and to carbon (biomass).

Chlorophyll *a* analysis

The chl *a* content of the *Trichodesmium erythraeum* and *Crocosphaera watsonii* cultures was measured by spectrophotometry according to Jeffrey & Humphrey (1975). Briefly, 30 ml of culture media was filtered onto a 25 mm Whatman GF/F and stored frozen for up to 2 wk until analysis. During analysis, filters were added to a 10 ml culture tube and covered with 7 ml of 90% acetone, and the culture tube was covered with Parafilm and vortexed. The test tubes were then covered with foil and stored in a freezer overnight. The next day, samples were measured on a spectrophotometer according to the wavelengths and equations specified by Jeffrey & Humphrey (1975).

Particulate C, N, and P analysis

Particulate carbon (PC) and particulate nitrogen (PN) concentrations were measured by filtering 25 ml of *Trichodesmium erythraeum* or *Crocosphaera watsonii* culture media onto a pre-combusted (4 h at 450°C) 25 mm Whatman GF/F, drying the filter in a 50°C drying oven, and pelletizing the filters for combustion analysis. For *C. watsonii* samples, PN and PC were analyzed at the same time as the ^{15}N enrichment as described above on an Integra-CN mass spectrometer. Detection and quantification limits for *C. watsonii* PN and PC samples were calculated daily as 3 times and 10 times the average + SD of 10 blanks, respectively. The detection limits were 0.42 and 0.90 µmol for PN and PC, respectively. The quantification limits were 0.61 and 1.93 µmol for PN and PC, respectively. *T. erythraeum* samples were sent to the UC Davis Stable Isotope Facility for quantification of the PC and PN content of the filters. *T. erythraeum* particulate phosphorus (PP) samples were analyzed according to standard Hawaii Ocean Time-series protocol (http://hahana.soest.hawaii.edu/hot/protocols/protocols.html). Briefly, *T. erythraeum* PP samples were collected by filtering 35 ml of culture media onto a pre-combusted 25 mm Whatman GF/F and dried at 50°C. Subsequently, filters were placed in pre-combusted 16 ml test tubes and heated for 4.5 h at 450°C. After cooling, 10 ml of 0.15 M HCl was added to the test tube to

cover the filters, and samples were heated for 30 min at 90°C and then centrifuged for 30 min at 2800 × *g*. The supernatant was then analyzed per colorimetric methods for soluble reactive P concentration measurements (Strickland & Parsons 1968). PP samples for *C. watsonii* were analyzed following the wet oxidation method described by Raimbault et al. (1999).

RESULTS

Diazotroph abundance

In both the *Trichodesmium erythraeum* and *Crocosphaera watsonii* cultures, the abundance of diazotrophs showed the following pattern within the treatments: 16:1 > 5:1 > 8:0.5 > 0:0.5 (Fig. 1). While the abundance of both diazotrophs was highest in the 16:1 treatment, it was not significantly higher than in the 5:1 treatment. However, the abundance of *T. erythraeum* in both the 16:1 and 5:1 treatments was significantly higher than the abundance of *T. erythraeum* in both the 8:0.5 and 0:0.5 treatments ($p < 0.001$); the abundance of *T. erythraeum* in the 8:0.5 and 0:0.5 treatments was similar. Exponential phase measurements of all *T. erythraeum* treatments were made on Day 18. Stationary phase measurements for the 0:0.5 *T. erythraeum* treatment were made on Day 23 and were made on Day 28 for the 5:1, 8:0.5, and 16:1 *T. erythraeum* treatments. All *T. erythraeum* measurements reported below are from exponential phase cultures unless otherwise noted.

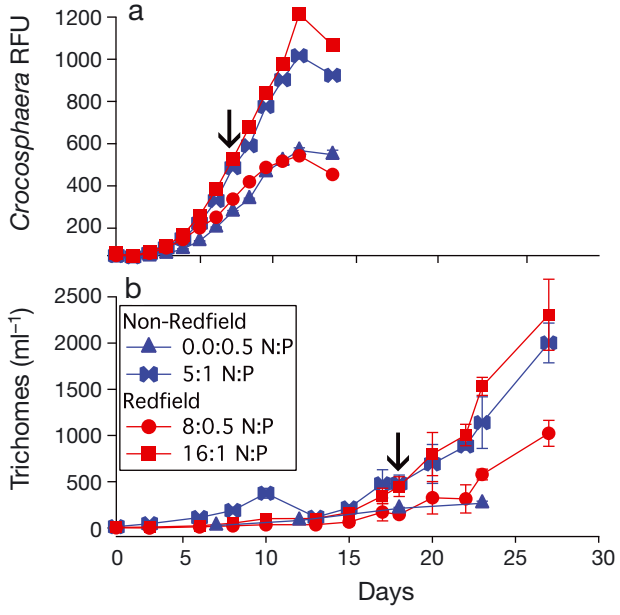

Fig. 1. Triplicate average (±1 SD) (a) relative *in vivo* chlorophyll *a* fluorescence (RFU) of culture media for *Crocosphaera watsonii* and (b) number of trichomes per 1 ml of culture media for *Trichodesmium erythraeum* cultures grown with (▲) 0.0 µM NO_3^- and 0.5 µM PO_4^{3-}, (⋈) 5.0 µM NO_3^- and 1.0 µM PO_4^{3-}, (●) 8.0 µM NO_3^- and 0.5 µM PO_4^{3-}, and (■) 16 µM NO_3^- and 1 µM PO_4^{3-}. Error bars represent 1 SD; for *C. watsonii* cultures, the error bars are typically smaller than the symbol size. Arrows represent the date of sampling for exponential phase cultures. For *C. watsonii* cultures, stationary phase samples were collected on Day 10 of the experiment, and for *T. erythraeum* cultures, the last time point represents termination of the experiment, when stationary phase samples were collected

Similar results were found for the *Crocosphaera watsonii* cultures (Fig. 1); the abundances of *C. watsonii* in the 16:1 and 5:1 treatments were not significantly different from each other, but both were significantly higher than the 8:0.5 ($p < 0.01$) and 0:0.5 ($p < 0.001$) treatments. All *C. watsonii* exponential phase measurements were made on Day 8, and stationary phase measurements were made on Day 10 of the experiment. All *C. watsonii* measurements reported below are from exponential phase cultures unless otherwise noted.

N_2 fixation rates

The highest N_2 fixation rates in both the *Trichodesmium erythraeum* and *Crocosphaera watsonii* cultures were observed in the 0:0.5 treatments (Fig. 2, Table S1 in the supplement at www.int-res.com/articles/suppl/a066p223_supp.pdf). The average (±1 SD) of triplicate N_2 fixation rate measurements for both *T. erythraeum* and *C. watsonii* cultures were normalized per trichome and per cell, respectively (Fig. 2a,b), to chl *a* (Fig. 2c,d), and to carbon (biomass) (Fig. 2e,f). For *T. erythraeum* cultures, the trichome-normalized N_2 fixation rates exhibited the following pattern, with significant ($p < 0.05$) differences among each treatment: 0:0.5 > 5:1 > 16:1 > 8:0.5 (Fig. 2a, Table S1). When normalized to chl *a*, the *T. erythraeum* N_2 fixation rates show a similar pattern; again, the N_2 fixation rate in the 0:0.5 treatment was significantly ($p < 0.05$) higher than the N_2 fixation rates in the 5:1, 8:0.5, and 16:1 treatments, and the 5:1 treatment N_2 fixation rate was significantly ($p < 0.05$) higher than in the 16:1 treatment (Fig. 2c, Table S1). However, there was too much variability in the 8:0.5 *T. erythraeum* treatment to distinguish the chl *a* normalized N_2 fixation rates in that treatment from those in either the 5:1 or the 16:1 treatment; we suspect that this is due to the combination of low trichome abundance and low chl *a* content in the 8:0.5 treatment relative to the 5:1 and 16:1

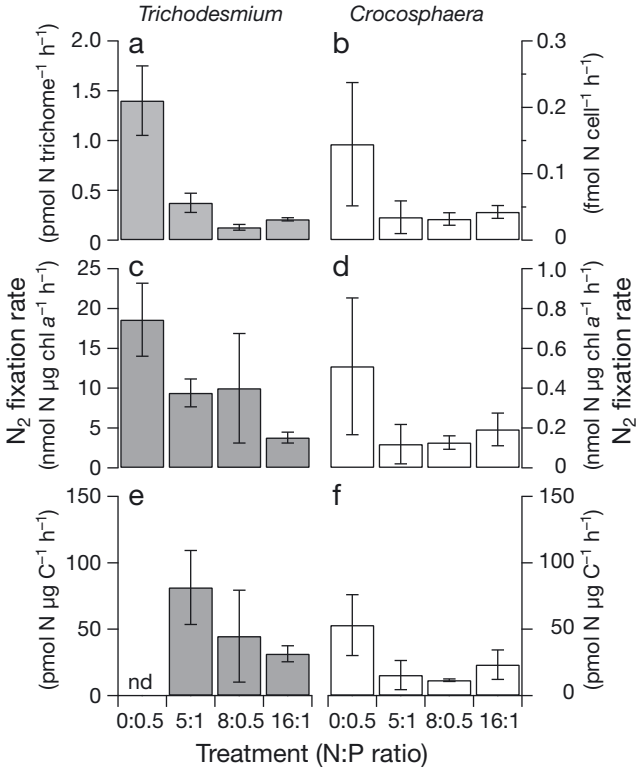

Fig. 2. Triplicate average (±1 SD) of exponential-phase N_2 fixation rates of (a,c,e) *Trichodesmium erythraeum* and (b,d,f) *Crocosphaera watsonii*, normalized (a) per trichome (pmol N trichome^{-1} h^{-1}), (b) per cell (fmol N cell^{-1} h^{-1}), (c,d) to chl *a* (nmol N ug chl *a*$^{-1}$ h^{-1}), and (e,f) to carbon (pmol N µg C^{-1} h^{-1}). nd: no data available

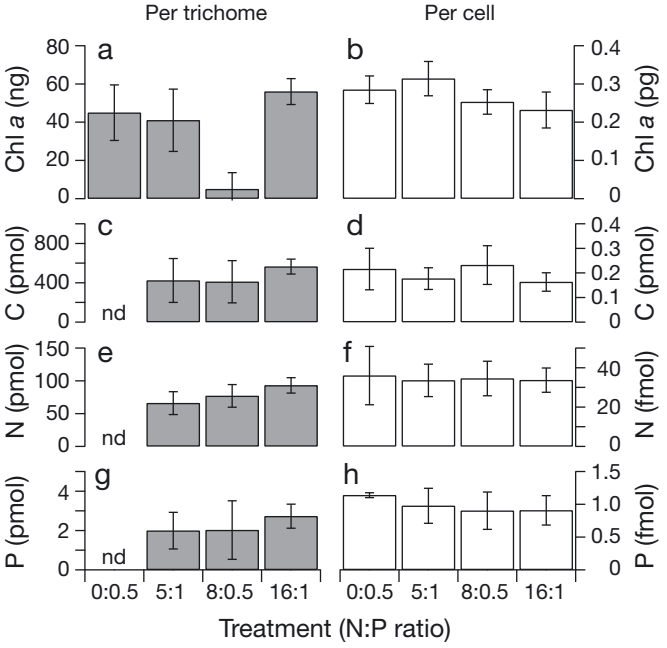

Fig. 3. Biomass content of exponential phase (a,c,e,g) *Trichodesmium erythraeum* and (b,d,f,h) *Crocosphaera watsonii* cultures. Triplicate average (±1 SD) (a,b) chl *a* content of cultures and particulate (c,d) C, (e,f) N, and (g,h) P content normalized (a,c,e,g) per trichome and (b,d,f,h) per *C. watsonii* cell. nd: no data available

T. erythraeum treatments (Figs. 1 & 3). Finally, *T. erythraeum* N_2 fixation rates normalized to C (biomass) showed a similar trend: the N_2 fixation rates in the 5:1 treatment were significantly ($p < 0.05$) higher than rates in the 16:1 treatment, although the rates in the 8:0.5 treatment were too variable to distinguish from those of the 5:1 or 16:1 treatment (Fig. 2e, Table S1). PC blanks were lost for the 0:0.5 *T. erythraeum* treatment (see below), so N_2 fixation rates normalized per C are not available for that treatment.

While the average cell-normalized N_2 fixation rate for the 0:0.5 *Crocosphaera watsonii* treatment was higher than the cell-normalized N_2 fixation rate for the 5:1 treatment (Fig. 2b, Table S1 in the supplement), the difference was not significant. However, the cell-normalized N_2 fixation rate for the 0:0.5 *C. watsonii* treatment was significantly ($p < 0.05$) higher than the cell-normalized N_2 fixation rates for the 8:0.5 and 16:1 *C. watsonii* treatments (Fig. 2b, Table S1). Similarly, the chl *a*-normalized N_2 fixation rate for the 0:0.5 *C. watsonii* treatment was significantly ($p < 0.05$) higher than that of the 8:0.5 treatment but not the 5:1 or 16:1 treatments (Fig. 2d, Table S1). Finally, the C-normalized N_2 fixation rate for the 0:0.5 *C. watsonii* treatment was significantly ($p < 0.05$) higher than the 5:1, 8:0.5, and 16:1 treatment N_2 fixation rates (Fig. 2f, Table S1). Additionally, the C-normalized 16:1 *C. watsonii* N_2 fixation rate was significantly higher than the 8:0.5 N_2 fixation rate (Fig. 2f, Table S1).

While the different normalizations of *Trichodesmium erythraeum* and *Crocosphaera watsonii* N_2 fixation rates yield somewhat different patterns among the treatments, some general trends emerge. First, the average N_2 fixation rates in the 0:0.5 treatments for both *T. erythraeum* and *C. watsonii* were always higher, and often significantly so, than the average N_2 fixation rates in the treatments with NO_3^- in the media. Taking the 0:0.5 treatment as the 'maximum' N_2 fixation rate in the present study, the *T. erythraeum* chl *a*-normalized N_2 fixation rates in the other treatments were ~20 to 50% of the N_2 fixation rate measured in the 0:0.5 treatment, while trichome-normalized N_2 fixation rates in the 5:1, 8:0.5, and 16:1 treatments were ~10% to ~30% of the 0:0.5 trichome-normalized N_2 fixation rate (Fig. 2, Table S1 in the supplement). The trends in the *C. watsonii* N_2 fixation rates were less dependent upon the normalization metric, with N_2 fixation rates for the 5:1, 8:0.5, and 16:1 treatments typically between 20 and 40% of

the 0:0.5 treatment N_2 fixation rate (Fig. 2, Table S1). These data are generally consistent with previous work that has shown NO_3^- inhibition of N_2 fixation (e.g. Mulholland et al. 2001, Fu & Bell 2003, Holl & Montoya 2005, Milligan et al. 2007, Moisander et al. 2008, Sandh et al. 2011), although the results of the present study also confirm that N_2 fixation continues to occur in cultures grown on as much as 16 µM NO_3^- as well as after NO_3^- in the cultures has been drawn down (data not shown). Finally, we note that stationary phase N_2 fixation rates were significantly lower than exponential phase N_2 fixation rates for both *T. erythraeum* and *C. watsonii* (data not shown).

Chlorophyll *a* content

In the *Trichodesmium erythraeum* cultures, the triplicate-average (±1 SD) chl *a* content in the 16:1, 5:1, and 0:0.5 treatments was 56 ± 7, 41 ± 16, and 45 ± 15 ng chl *a* trichome^{-1}, respectively, which were all significantly higher ($p < 0.05$) than the chl *a* content in the 8:0.5 treatment of 5 ± 8 ng chl *a* trichome^{-1}, which as described above, may have resulted from a combination of low trichome abundance and low chl *a* content in this treatment (Fig. 3a, Table S2 in the supplement at www.int-res.com/articles/suppl/a066p223_supp.pdf). The chl *a* content per trichome in these experiments was comparable to previous culturing work (Mulholland et al. 2004). In the *Crocosphaera watsonii* cultures, the per-cell chl *a* content was similar among the treatments, ranging from 0.23 ± 0.04 to 0.31 ± 0.04 pg chl *a* cell^{-1} (Fig. 3b, Table S2), but was lower than that found in previous studies (Webb et al. 2009), potentially due to the lower PO_4^{3-} concentration or to differences in irradiance levels used in the present study relative to previous studies.

Biomass C, N, and P content

C content

The triplicate average (±1 SD) C content per trichome in the 5:1, 8:0.5, and 16:1 *Trichodesmium erythraeum* treatments was similar and ranged from 410 ± 215 to 565 ± 76 pmol C trichome^{-1} (Fig. 3c, Table S2 in the supplement). Unfortunately, the 0:0.5 *T. erythraeum* treatment blanks were lost, so data are not reported for that treatment. However, if the average blank values used for biomass C content from the 5:1, 8:0.5, and 16:1 *T. erythraeum* treatments are used to correct the 0:0.5 *T. erythraeum* treatment biomass C data, then the 0:0.5 treatment per-trichome C content values fall within the range of the other *T. erythraeum* treatments (data not shown). No significant variations were found among the per trichome C content of the *T. erythraeum* treatments. The average C content per exponential phase *Crocosphaera watsonii* cell did not vary among the treatments either and ranged from 0.16 ± 0.04 to 0.23 ± 0.08 pmol C cell^{-1} (Fig. 3d, Table S2); these values are similar to those reported by Dron et al. (2012) but are lower than those found in previous studies (Webb et al. 2009), potentially due to the lower PO_4^{3-} concentration and/or to differences in the irradiance level used in these experiments relative to previous studies.

N content

The triplicate average (±1 SD) per trichome N content in the exponential phase *Trichodesmium erythraeum* cultures was similar among the 5:1, 8:0.5, and 16:1 treatments and ranged from 66 ± 17 to 93 ± 12 pmol N trichome^{-1} (Fig. 3e, Table S2 in the supplement), comparable to previous culture work (White et al. 2006). Because the blanks for the 0:0.5 *T. erythraeum* treatment were lost, biomass N content data are not reported for that treatment. However, if the blank values used for biomass N content from the 5:1, 8:0.5, and 16:1 *T. erythraeum* treatments are used to correct the 0:0.5 treatment biomass N data, then the per trichome N content for the 0:0.5 treatment falls within the range of the other *T. erythraeum* treatments (data not shown). No significant variations in per trichome N content were found among the *T. erythraeum* treatments. For the exponential phase *Crocosphaera watsonii* cultures, the cellular N content was similar among the treatments, ranging from 34 ± 6 to 36 ± 15 fmol N cell^{-1} (Fig. 3f, Table S2), and was similar to that reported by Dron et al. (2012).

P content

The per trichome P content for the exponential phase *Trichodesmium erythraeum* cultures was similar among the 5:1, 8:0.5, and 16:1 treatments, and triplicate averages (±1 SD) ranged from 2.0 ± 0.9 to 2.7 ± 0.6 pmol P trichome^{-1} (Fig. 3g, Table S2 in the supplement), comparable to previous culture work (White et al. 2006). Because the blanks for the 0:0.5 *T. erythraeum* treatment were lost, no biomass P content data are reported for that treatment. For expo-

nential phase *Crocosphaera watsonii* cultures, the per cell P content was similar among the treatments and ranged from 0.90 ± 0.2 to 1.1 ± 0.04 fmol P cell^{-1} (Fig. 3h, Table S2).

Biomass C:N:P ratios

The triplicate average (±1 SD) exponential phase C:N (mol:mol) biomass ratios ranged from 4.8 ± 0.3 to 6.6 ± 0.6 for both *Trichodesmium erythraeum* and *Crocosphaera watsonii* (Fig. 4, Table S3 in the supplement at www.int-res.com/articles/suppl/a066p223_supp.pdf) (biomass C:N ratio data are not available for the 0:0.5 *T. erythraeum* treatment; see 'C content'). This range in biomass mol:mol C:N ratios is consistent with previously reported ranges for both *T. erythraeum* IMS101 (Berman-Frank et al. 2001, Mulholland & Capone 2001, White et al. 2006, Holl & Montoya 2008) and *C. watsonii* WH8501 (Mohr et al. 2010, Dron et al. 2012). While no significant differences in C:N ratios were observed among *T. erythraeum* treatments, *C. watsonii* cultures grown with 0.5 µM PO_4^{3-} (i.e. the 0:0.5 and 8:0.5 treatments) had significantly ($p < 0.05$) higher average exponential phase C:N ratios, i.e. 6.1 ± 0.2 and 6.6 ± 0.6, than the *C. watsonii* cultures grown with 1 µM PO_4^{3-} (i.e. the 5:1 and 16:1 treatments), which had ratios of 5.3 ± 0.2 and 4.8 ± 0.3, respectively (Fig. 4, Table S3). Additionally, the C:N ratio of exponential phase *C. watsonii* in the 5:1 treatment was significantly ($p < 0.05$) higher than the C:N ratio of the 16:1 treatment.

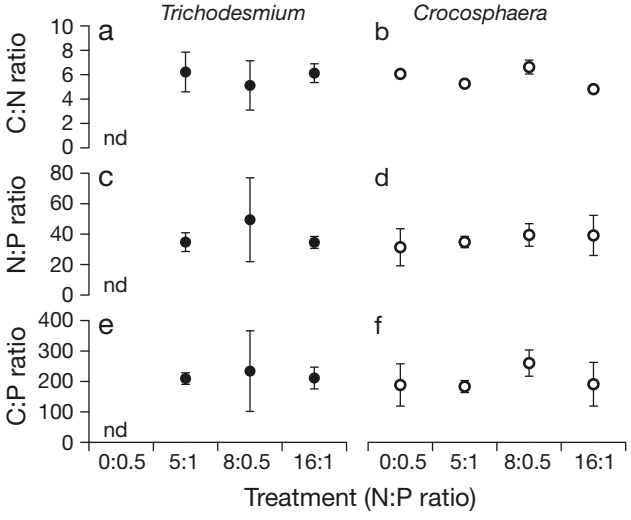

Fig. 4. (a,c,e) *Trichodesmium erythraeum* (●) and (b,d,f) *Crocosphaera watsonii* (○) cultures. Triplicate average (±1 SD) exponential phase biomass (a,b) C:N, (c,d) N:P, and (e,f) C:P (mol:mol) ratios. nd: no data available

The biomass N:P (mol:mol) ratios for exponential phase *Trichodesmium erythraeum* and *Crocosphaera watsonii* cultures did not vary significantly among treatments and ranged (triplicate average ± 1 SD) from 34.5 ± 3.9 to 49.5 ± 27.5 for *T. erythraeum* cultures and from 31.4 ± 12.2 to 39.5 ± 7.4 for *C. watsonii* cultures (Fig. 4, Table S3 in the supplement). These N:P ratios are similar to previously reported N:P ratios of ~30:1 to 60:1 found in field- and culture-based samples of *Trichodesmium* spp. (Kustka et al. 2003, Fu et al. 2005, Krauk et al. 2006, White et al. 2006, Holl & Montoya 2008).

Similarly, there were no significant differences among treatments in exponential phase biomass C:P (mol:mol) ratios for either *Trichodesmium erythraeum* or *Crocosphaera watsonii* experiments. The range in triplicate average (±1 SD) *T. erythraeum* biomass C:P ratios was 209 ± 19.0 to 234 ± 132 and was 183 ± 19.6 to 260 ± 43.2 for *C. watsonii* cultures (Fig. 4, Table S3). These C:P mol:mol biomass ratios for both diazotrophs are consistent with previously reported ranges in *Trichodesmium* C:P ratios (Berman-Frank et al. 2001, Kustka et al. 2003, Fu et al. 2005, White et al. 2006).

With the exception of *Crocosphaera watsonii* C:N ratios, there was little difference observed among treatments for exponential phase biomass C:N:P ratios in these experiments. However, biomass C:N:P ratios for both *Trichodesmium erythraeum* and *C. watsonii* increased within treatments between exponential and stationary phases, often significantly (Figs. 4 & 5, Tables S3 & S4 in the supplement). For example, the increase in C:N ratios between exponential and stationary phases for both the 5:1 and 16:1 *C. watsonii* treatments, from 5.3 ± 0.02 to 6.2 ± 0.4 and 4.8 ± 0.3 to 7.3 ± 0.7, respectively, was significant ($p < 0.05$) (Figs. 4 & 5, Tables S3 & S4). The increase in N:P ratios for both the 5:1 and 16:1 *T. erythraeum* treatments between exponential and stationary phases, from 34.7 ± 6.2 to 114 ± 13.1 and 34.5 ± 3.9 to 127 ± 10.2, respectively, was also significant ($p < 0.05$) (Figs. 4 & 5, Tables S3 & S4). Similarly, the increase in N:P ratios in both the 0:0.5 and 5:1 *C. watsonii* treatments between exponential and stationary phases, from 31.4 ± 12.2 to 62.0 ± 4.3 and 34.8 ± 3.7 to 46.7 ± 9.7, respectively, was significant ($p < 0.05$) (Figs. 4 & 5, Tables S3 & S4). Finally, the increase in C:P ratios between exponential and stationary phases for *T. erythraeum* was significant ($p < 0.05$) for the 5:1 and 16:1 treatments, from 209 ± 19.0 to 602 ± 22.1 and 211 ± 35.6 to 790 ± 118, respectively. The change in *C. watsonii* C:P ratios for the 0:0.5 and 5:1 treatments between exponential and

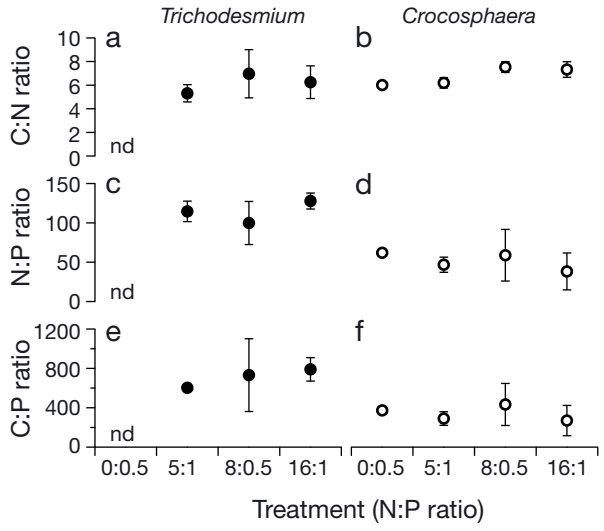

Fig. 5. (a,c,e) *Trichodesmium erythraeum* (●) and (b,d,f) *Crocosphaera watsonii* (○) cultures. Triplicate average (±1 SD) stationary phase biomass (a,b) C:N, (c,d) N:P, and (e,f) C:P (mol:mol) ratios nd: no data available

stationary phases, although not as large as for *T. erythraeum*, was also significant, increasing from 188 ± 69.4 to 373 ± 34.7 and 183 ± 19.6 to 290 ± 69.9 ($p < 0.05$), respectively (Figs. 4 & 5, Tables S3 & S4).

DISCUSSION

Diazotroph abundance and N_2 fixation rates

In these experiments, both *Trichodesmium erythraeum* and *Crocosphaera watsonii* show the same pattern: in batch cultures grown with environmentally relevant NO_3^- and PO_4^{3-} concentrations, diazotroph abundance was highest in the treatments with higher PO_4^{3-} concentration (i.e. 1.0 vs. 0.5 µM PO_4^{3-}), regardless of NO_3^- concentration, while N_2 fixation rates were highest in the treatment without NO_3^-. However, the reduction in trichome normalized N_2 fixation rates in *T. erythraeum* treatments grown with NO_3^- was nearly compensated for in the 5:1 and 16:1 treatments, where the abundance of trichomes was ~2- to 3-fold greater than in the 0:0.5 treatment, yielding comparable quantities of N_2 fixed per volume of culture media. Similar results were found in the *C. watsonii* experiments (using fluorescence as a proxy for cell abundance).

These results are important for 2 reasons. First, they help illustrate how the response of diazotrophs to the separate effects of variations in ambient NO_3^- and PO_4^{3-} concentrations can result in a perceived effect of variations in 'N:P ratios' on diazotrophs. Specifically, for equivalent concentrations of PO_4^{3-}, lower NO_3^- concentrations will result in higher volume-integrated rates of N_2 fixation due to decreased inhibition of N_2 fixation; conversely, for comparable concentrations of NO_3^-, higher concentrations of PO_4^{3-} will yield higher volume-integrated rates of N_2 fixation due to increased diazotroph abundance. Second, these results suggest a potential feedback mechanism for these 2 diazotrophs to respond to increases in denitrification and/or anammox, thereby helping to stabilize the marine N inventory. If rates of marine denitrification increase, as the paleoceanographic record suggests happens coming out of a glacial period and going into an interglacial period, (e.g. Haug et al. 1998, Ganeshram et al. 2000, Altabet et al. 2002), our results imply that surface ocean diazotrophs may be able to compensate for the increasing rate of NO_3^- loss in subsurface waters. For example, if surface waters overlying regions of denitrification and/or anammox have NO_3^-:PO_4^{3-} concentration ratios of ~16:1 during glacial times, but those surface ocean nutrient ratios are reduced during interglacial periods when rates of denitrification increase, equivalent concentrations of PO_4^{3-} should remain in interglacial surface waters (yielding the same biomass of diazotrophs as during glacial times) but less NO_3^- would be present to inhibit N_2 fixation, thereby resulting in higher volume-integrated rates of N_2 fixation during interglacial periods compared to glacial times.

We note, however, that the ability of diazotrophs to thrive in nutrient-rich environments still depends upon their ability to compete with other phytoplankton and heterotrophic bacteria for P, either as PO_4^{3-} and/or dissolved organic phosphorus (DOP) (e.g. Dyhrman et al. 2006, Sohm & Capone 2006, Dyhrman et al. 2009), as well as for trace metals (e.g. Berman-Frank et al. 2001, 2007, Kustka et al. 2003, Saito et al. 2011). Thus, while the present culture work suggests that NO_3^- inhibition alone is not a reason to preclude the highest rates of N_2 fixation occurring in ocean waters with significant NO_3^- concentrations, as predicted by the remote sensing (Westberry et al. 2005, Westberry & Siegel 2006) and geochemical modeling (Deutsch et al. 2007) studies described earlier, the present work does not unequivocally support these studies either. Indeed, recent *in situ* measurements made in the surface waters of the ETSP gyre suggest that N_2 fixation rates there are low and are potentially iron-limited (Bonnet et al. 2008, Moutin et al. 2008), which is perhaps to be expected given the low atmospheric dust fluxes to the South Pacific gyre (Wagener et al. 2008, Mahowald et al. 2009). Moreover, given the high iron requirements of *Tri-*

chodesmium and *Crocosphaera watsonii* (Berman-Frank et al. 2001, 2007, Kustka et al. 2003, Tuit et al. 2004, Saito et al. 2011), it is not surprising that we were unable to carry out these experiments for *T. erythraeum* and *C. watsonii* under trace-metal limiting conditions; *T. erythraeum* cultures did not grow at these PO_4^{3-} concentrations with 25 or 50 nM iron (with 2 µM EDTA), nor did *C. watsonii* cultures grow at these same PO_4^{3-} concentrations with 50 nM iron (with 2 µM EDTA) (the results presented were from cultures grown with the traditional YBC II metal concentrations, including 400 nM iron with 2 µM EDTA).

However, we expect that the recent work of Fernandez et al. (2011) and Sohm et al. (2011), who document significant rates of N_2 fixation in the near-shore waters off of Chile and Africa, respectively, where surface NO_3^- ranged from ~5 to 21 µM, and surface PO_4^{3-} concentrations ranged from ~0.5 to 2.0 µM, broadly supports our results: their measurements were made in relatively close proximity to the coast, where iron stress is less severe, permitting N_2 fixation (apparently by organisms other than *Trichodesmium* and *Crocosphaera*) to occur at rates comparable to those measured in oligotrophic regions of the North Pacific gyre. Thus, while the results presented here suggest that NO_3^- inhibition of N_2 fixation may be largely compensated for by PO_4^{3-} stimulation of diazotroph abundance, we emphasize that we were only able to demonstrate this when these batch cultures were grown under trace-metal replete conditions, consistent with previous findings for the importance of both metals and P for diazotroph success (Mills et al. 2004). We also note that the responses of *T. erythraeum* and *C. watsonii* to the nutrient conditions explored in the present study almost certainly do not reflect the full capacity of the diverse marine diazotrophic community to cope with various environmental conditions. While our study was limited to the marine diazotrophs present in culture at the time, we encourage similar culturing studies of other marine diazotrophs as they become available.

Finally, the NO_3^- inhibition of N_2 fixation in *Crocosphaera watsonii* observed in the present study is in apparent contrast to recent work (Dekaezemacker & Bonnet 2011) that shows little to no reduction in N_2 fixation by *C. watsonii* when cultures are exposed to 0.2 to 10.0 µM NO_3^-. However, we note that there are 2 significant differences between the culturing conditions in that study and those used here. First, Dekaezemacker & Bonnet (2011) grew *C. watsonii* cultures with the standard YBC II PO_4^{3-} concentration of 50 µM, while in the present study, *C. watsonii* were grown with 0.5 or 1.0 µM PO_4^{3-}. Second, the cultures in the present study were grown out for multiple generations at the nutrient conditions specified in each treatment and therefore had ample time to acclimate to those macro-nutrient concentrations. However, in the Dekaezemacker & Bonnet (2011) study, *C. watsonii* were grown and acclimated in media with no NO_3^-, and N_2 fixation rate measurements were made within hours of the *C. watsonii* cultures receiving a 'dose' of 0.2 to 10.0 µM NO_3^-.

Several recent studies have explored the mechanisms of NO_3^- inhibition of N_2 fixation and provide some insight into the apparent difference in the culturing results of Dekaezemacker & Bonnet (2011) and those presented here. First, Milligan et al. (2007) show not only significantly lower N_2 fixation rates but also lower rates of Mehler activity in *Trichodesmium erythraeum* IMS 101 and *Anabaena flos-aquae* cultures grown with NO_3^- compared with those using only N_2 as a N source. For diazotrophs, the Mehler reaction has been proposed to protect nitrogenase, the enzyme carrying out N_2 fixation, which is irreparably damaged by oxygen generated during photosynthesis. Milligan et al. (2007) suggest that when these 2 diazotrophs are grown on NO_3^-, and nitrogenase activity is reduced, the need for oxygen consumption by the Mehler reaction is also reduced. In a novel proteomic study, Sandh et al. (2011) show a down-regulation of diazocyte development in *T. erythraeum* IMS 101 cultures grown on NO_3^-, as well as other proteomic differences between *T. erythraeum* IMS 101 grown under obligate diazotrophy compared with those grown in NO_3^--bearing media. The results of Milligan et al. (2007) and Sandh et al. (2011) suggest that the acclimation time of the cultures to NO_3^- in the experiments presented in the present study allowed sufficient time to induce mechanisms that would de-emphasize the importance of N_2 as a source of assimilatory N, which may have not been the case in the Dekaezemacker & Bonnet (2011) study. This interpretation is consistent with previous work by Mulholland et al. (2001) and Ohki et al. (1991), who have shown that *T. erythraeum* NIBB 1067 cultures grown for 10 generations on 2 mM NO_3^- or 20 µM NH_4^+ did not fix N_2, but *T. erythraeum* NIBB 1067 cultures grown on N-free media and then exposed to these concentrations of inorganic N for 7 h were still capable of fixing N after this relatively brief exposure time. Consequently, the culturing work of Dekaezemacker & Bonnet (2011) and that presented here provide support for a similar sensitivity of N_2 fixation by *Crocosphaera watsonii* to chronic vs. short-term exposure to dissolved inorganic N to that previously observed in *Trichodesmium*.

Biomass content and C:N:P ratios

In our experiments, relatively little variation in the biomass content of C, N, or P was observed among exponential phase treatments of either *Trichodesmium erythraeum* or *Crocosphaera watsonii* (Fig. 3, Table S2 in the supplement). Similarly, exponential phase biomass C:N:P ratios did not vary among the treatments or even between *T. erythraeum* and *C. watsonii* (Fig. 4, Table S3). These results demonstrate that when grown and acclimated under metal-replete conditions, these 2 diazotrophs make exponential phase biomass with the same elemental stoichiometry and apparently did not induce any mechanisms to preferentially store N or P as a result of differences in the 4 macro-nutrient conditions tested in these experiments. Consequently, the range in biomass content and C:N:P ratios observed here for *T. erythraeum* and especially for *C. watsonii*, for which there are comparatively few data, provide reasonably robust constraints on their biomass C:N:P content and ratios, holding other variables (e.g. light, metals, and temperature) constant. Additionally, the finding that *T. erythraeum* and *C. watsonii* respond similarly in terms of abundance and N_2 fixation rates to variations in NO_3^- and PO_4^{3-} concentrations and/ or ratios is not necessarily to be expected based upon the emerging picture of the biogeography of these diazotrophs; Moisander et al. (2010) found different distributions of *Trichodesmium* and *Crocosphaera* in the global ocean. Presumably, the difference in geographic distribution of the 2 diazotrophs is related to sensitivities to parameters not tested in these experiments, potentially including the ability to meet metal requirements (e.g. Saito et al. 2011). Nonetheless, these experiments demonstrate that under metal-replete conditions, *T. erythraeum* IMS 101 and *C. watsonii* WH 8501 fix comparable quantities of N when normalized to C (biomass). Thus, given their similar sensitivities to NO_3^- and PO_4^{3-} based on the culturing work presented here, this suggests that from a geochemical and/or modeling perspective, these 2 diazotrophs may be 'interchangeable' in terms of quantities of N fixed per unit C, at least when trace metal requirements are met.

While biomass C:N:P ratios were similar among exponential phase treatments of both *Trichodesmium erythraeum* and *Crocosphaera watsonii*, when the cultures were no longer in exponential phase, the biomass C:N, N:P, and C:P ratios increased, often significantly. Consistent with previous findings (e.g. Krauk et al. 2006, White et al. 2006), exponential phase biomass N:P ratios in both diazotrophs ranged from ~30:1 to ~50:1; consequently, biomass N:P ratios >60:1 observed in both *T. erythraeum* IMS 101 and *C. watsonii* WH 8501 are not likely to be representative of biomass N:P ratios when N_2 fixation rates are highest and are thus inappropriate choices to represent diazotrophic biomass in modeling studies. Similar to the elevation in N:P biomass ratios relative to Redfield stoichiometry, both diazotrophs showed an elevated biomass C:P ratio (i.e. ~200:1 vs. 106:1) in exponential phase cultures (Fig. 4, Table S3). While we again note that these cultures were carried out under metal-replete conditions, the elevation in biomass C:P ratios provides a mechanism for these diazotrophs to fix more C per unit P relative to phytoplankton with biomass C:P ratios closer to the Redfield stoichiometry biomass C:P ratios of ~106:1, especially in regions of the ocean with very low surface-ocean PO_4^{3-} concentrations, such as the North Atlantic.

CONCLUSIONS

Presented here are the results from batch culture experiments of the diazotrophs *Trichodesmium erythraeum* and *Crocosphaera watsonii* grown under trace metal- and vitamin-replete conditions. In these experiments, the concentration and ratios of the macro-nutrients NO_3^- and PO_4^{3-} were varied to examine how these changes would affect the abundance, N_2 fixation rates, and biomass C:N:P ratios and content of both diazotrophs. Four N:P ratios were tested, 2 at and 2 below the Redfield ratio stoichiometry of 16:1 NO_3^-:PO_4^{3-} concentration (NO_3^-:PO_4^{3-} concentrations, in µM) 0:0.5, 5:1, 8:0.5, and 16:1, all at 'environmentally relevant' concentrations that can be found in the surface waters of different oceanic and/or coastal regions (Garcia et al. 2010).

While there was little variation in biomass C:N:P ratios among treatments, exponential phase cultures of both diazotrophs had elevated biomass N:P and C:P ratios relative to Redfield stoichiometry (i.e. N:P ratios of ~30:1 to 60:1 vs. 16:1 and C:P ratios of ~200:1 vs. 106:1), consistent with previous studies. Moreover, the C:N, N:P, and C:P ratios all increased, often significantly, after both *Trichodesmium erythraeum* and *Crocosphaera watsonii* left exponential phase. These findings provide physiological context for interpreting field reports of *Trichodesmium* N:P biomass ratios >100 (i.e. Karl et al. 1992) and suggest that N:P and C:P ratios that are significantly greater than the exponential phase biomass C:N:P ratios most commonly observed in culture studies are not likely to reflect diazotrophs in the exponential growth phase, when N_2 fixation rates are highest.

Additionally, while the abundance of both diazotrophs was significantly higher in the 16:1 and 5:1 treatments, N_2 fixation rates normalized per trichome (for *Trichodesmium erythraeum*) or per cell (for *Crocosphaera watsonii*) were significantly higher in the 0:0.5 treatment (although the diazotrophs continued to both fix N_2 and assimilate NO_3^- in all treatments). These results are consistent with previous work showing inhibition of N_2 fixation after prolonged exposure to NO_3^-. However, our results also show that the ~3-fold reduction in per cell or per trichome N_2 fixation rates by NO_3^- inhibition in the 5:1 and 16:1 treatments relative to the 0:0.5 treatment was offset by the ~3-fold increase in diazotrophic abundance resulting from higher PO_4^{3-} concentrations in the 5:1 and 16:1 treatments relative to the 0:0.5 treatment, yielding comparable quantities of N_2 fixed per volume of culture media in all 3 treatments.

Thus, these experiments start to disentangle the competing effects of NO_3^- and PO_4^{3-} on N_2 fixation rates and suggest that waters with progressively lower N:P ratios can increasingly favor N_2 fixation: if 2 waters with equivalent PO_4^{3-} concentrations result in equivalent diazotrophic biomass (Fig. 1), the water with a lower NO_3^- concentration (and thus lower N:P ratio) can support higher per cell (or per trichome) rates of N_2 fixation due to decreased NO_3^- inhibition of N_2 fixation. Importantly, these results also indicate that NO_3^- inhibition alone is not a reason to discount significant N_2 fixation fluxes occurring in surface ocean waters with significant concentrations of NO_3^-. While these results suggest a potential feedback mechanism for diazotrophs to respond to increases in marine denitrification rates and thereby help stabilize the marine N inventory, these data do not unequivocally support the remote sensing (Westberry et al. 2005, Westberry & Siegel 2006) and geochemical modeling (Deutsch et al. 2007) studies described earlier; our cultures were unable to be maintained under metal-limited conditions, consistent with previous work demonstrating the requirements of iron and other trace metals for diazotrophic success. However, if diazotrophs can meet their P and metal requirements, our data show that N_2 fixation is perhaps less sensitive to NO_3^- than traditionally thought. Thus, in addition to previous work suggesting that N_2 fixation is more common in colder waters than previously thought (e.g. Holl et al. 2007, Needoba et al. 2007, Rees et al. 2009), these findings imply that N_2 fixation could also be occurring at significant rates in environments with significant concentrations of NO_3^-, previously considered incompatible with N_2 fixation, and consequently have important implications for how this process is parameterized in models.

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