LIMNOLOGY
and
OCEANOGRAPHY

ASLO

Limnol. Oceanogr. 62, 2017, 846–858

doi: 10.1002/lno.10484

The effect of iron limitation on cyanobacteria major nutrient and trace element stoichiometry

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Abstract

Phytoplankton elemental stoichiometry provides a window into the interactions between marine nutrient distributions and phytoplankton growth. Here, we report the extended elemental stoichiometry of two non-diazotrophic cyanobacteria strains, *Synechococcus* WH7803 and *Prochlorococcus* MED4, in both Fe-replete and Fe-deplete media. Fe concentrations were reduced by two orders of magnitude in the Fe-deplete media, causing growth rates to decline by 38% and 24%, respectively. The average elemental composition of Fe-replete cells was $(C_{76.5}N_{19}P_1)_{1000}Fe_{52.5}Mn_{1.90}Zn_{0.86}Cu_{0.40}Ni_{0.40}Co_{0.05}Cd_{0.0020}$, while Fe-limited cells averaged $(C_{121}N_{30}P_1)_{1000}Fe_{12.2}Mn_{3.22}Zn_{1.70}Cu_{0.41}Ni_{0.36}Co_{0.11}Cd_{0.0038}$ The trace-metal stoichiometries measured here are similar to the ranges previously measured for a strain of *Synechococcus* and for many species of large eukaryotic phytoplankton. In contrast to large eukaryotic phytoplankton, which have previously exhibited either unchanged or decreased N : P under Fe-limited conditions, *Synechococcus* and *Prochlorococcus* increased N : P in Fe-deplete media by 58% and 67%, respectively. Previous studies have examined the direct role of Fe in limiting nitrogen fixation by diazotrophs, but this study suggests a second mechanism by which Fe may impact nutrient cycling, by influencing the N : P uptake ratio of non-diazotrophic cyanobacteria. A model of cyanobacteria distribution and Fe limitation is combined with the N : P stoichiometries measured here to suggest that up to 40% of the particulate organic nitrogen in the surface ocean might be associated with Fe-limited waters, with the strongest effect observed in the *Prochlorococcus* dominated subtropical Pacific. Thus, if the effect on N : P we observe in culture is widespread in the oceans it would mean that Fe-limitation could play a major role in global nitrogen and carbon cycling.

Alfred Redfield was one of the first oceanographers to study the elemental composition of plankton in seawater. He discovered a nearly constant elemental ratio of 106 carbon (C) atoms to 16 nitrogen (N) to 1 phosphorus (P), both in plankton biomass and as dissolved nutrients in seawater (Redfield 1934, 1958). This 106 : 16 : 1 ratio is commonly referred to as the Redfield Ratio and has been vital to understanding phytoplankton growth and biogeochemical cycling in the ocean. In the subsequent decades there has been an explosion of data on seawater elemental concentrations which have largely confirmed the original findings of Redfield, although later studies put the Redfield ratio closer to 117 : 16 : 1 (Anderson and Sarmiento 1994). A 16 : 1 ratio of N : P is typically considered a critical threshold between N and P limitation of phytoplankton photosynthesis, and therefore carbon sequestration (Broecker 1982; Codispoti 1989; Klausmeier et al. 2004), although more recent work has highlighted the large variability in marine N : P (Arrigo 2005; Mills and Arrigo 2010; Weber and Deutsch 2010, 2012; Martiny et al. 2013, 2014).

In recent years, phytoplankton elemental stoichiometry research has extended beyond C : N : P to also include trace metals measurements. Ho et al. (2003) measured the extended elemental stoichiometry of 15 eukaryotic phytoplankton species and found an average composition of $(C_{124}N_{16}P_1S_{1.3}K_{1.7}Mg_{0.56}Ca_{0.5})_{1000}Sr_{5.0}Fe_{7.5}Zn_{0.80}Cu_{0.38}Co_{0.19}Cd_{0.21}Mo_{0.03}$, with systematic differences between these various phytoplankton lineages attributed to the redox state of the ocean in which they arose (Ho et al. 2003; Quigg et al. 2003). A more recent similar study included the prokaryotic cyanobacterium *Synechococcus* (Quigg et al. 2011). The extended elemental stoichiometry of individual phytoplankton cells from culture has also been measured by SXRF, although without data on N (Twining et al. 2003). As the concentration of trace-metals within the culture media are varied by orders of magnitude, the resulting phytoplankton trace metal stoichiometry typically changes by similar amounts (Twining and Baines 2013). Other growth factors such as irradiance can also have a large impact on in trace metal stoichiometries (Finkel et al. 2006). Extended

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elemental stoichiometries have been measured in natural ocean samples including studies of individual phytoplankton cells (Twining et al. 2004, 2010, 2014) and whole communities (Sherrell and Boyle 1992; Twining and Baines 2013; Lam et al. 2015).

Major element stoichiometries in cells (C, N, P, and Si) vary considerably between species, and when the same species is grown under different conditions. Although the Redfield ratio holds true throughout the deep ocean, this average C : N : P masks considerable variability in nutrient ratios within surface phytoplankton communities. Measured particulate C : N : P from different world ocean regions ranges from 195 : 28 : 1 in warm oligotrophic gyres to 78 : 13 : 1 in cold high-latitude HNLC regions (Martiny et al. 2013). Modeling studies have similarly shown that the average Redfield ratio observed in the deep ocean is the result of mixing between ocean regions, with lower N : P (∼10 : 1) observed in high latitude waters and higher N : P (∼20 : 1) at lower latitudes (Weber and Deutsch 2010, 2012). Interspecies differences and variations in nutrient limitation within culture media leads to considerable variation in C : N : P composition (Geider and La Roche 2002). Limitation of P tends to cause the most drastic changes in phytoplankton elemental stoichiometry, with decreases in cellular P leading to increases in C : P and N : P in eukaryotes (Lynn et al. 2000) and prokaryotes (Sañudo-Wilhelmy et al. 2001; Bertilsson et al. 2003; Mouginot et al. 2015). Interestingly, a recent study on N- and P-limitation of *Synechococcus* found growth-dependent variability in cell size controls the relationship between nutrient-limited phytoplankton and cellular elemental stoichiometry (Garcia et al. 2016).

In addition to studies which examine changes in micronutrient and major nutrient stoichiometries separately, the availability of micronutrients, especially Fe, can change the ratio at which phytoplankton take up the major nutrients. A large number of studies have examined the changes in phytoplankton Si, N, and C ratios under Fe-replete and Fe-deplete conditions, both in culture and in the natural systems (Takeda 1998; Boyd et al. 2007; Marchetti and Cassar 2009). In many of these cases particulate P was not measured, perhaps because it is more challenging to analyze than particulate N and C, which can be easily measured on an elemental analyzer, and perhaps because N and P are expected to be taken up in amounts close to the Redfield ratio.

Culture studies examining the effect of Fe additions on phytoplankton N : P have focused mostly on diatoms, and have shown that Fe-limitation leads to either a decrease in N : P or no change in N : P. *Thalassiosira weissflogii* were found to decrease their N : P ratios from 14 : 1 under Fe-replete conditions to 10 : 1 under Fe-deplete conditions in culture (Price 2005). Cultured *Phaeodactylum tricornutum* and *Dunaliella tertiolecta* do not significantly change their N : P when Fe-limited (Greene et al. 1991; La Roche et al. 1993), although the addition of Fe to previously Fe-limited cultures caused N : P to increase from 9.0 to 17.4 and from 7.6 to 11.2 for *P. tricornutum* and *D. tertiolecta*, respectively (La Roche et al. 1993). Cultured diatoms *Pseudo-nitzschia pseudodelicatissima*, *Fragilariopsis kerguelensis*, and *Chaetoceros dichaeta* had similar N : P when cultured in Fe-replete and deplete conditions (Hoffmann et al. 2007; Sugie and Yoshimura 2013). When elemental stoichiometry of *F. kerguelensis* was determined by dissolved nutrient depletion, N : P was found to decrease under Fe-limited conditions, similar to the other studies on diatoms, although these results may be compromised by analytical problems associated with tracking dissolved rather than particulate concentrations (Timmermans and van der Wagt 2010). To our knowledge the only cyanobacteria studied are the nitrogen-fixing *Crocosphaera* (Garcia et al. 2016) and *Trichodesmium*, which decreases N : P from 13 to 4.8 when it becomes Fe-limited (Berman-Frank et al. 2001), although these results are likely associated with the particularly large Fe requirements for N-fixation and with access to newly fixed N and thus would not be expected to apply to non-diazotrophs.

Similarly, field studies on the effects of Fe on N : P have focused on regions dominated by diatoms and other large eukaryotic phytoplankton, and have found that Fe deficiency leads to either a decrease in N : P or no change in N : P. When Fe is added to incubations of Fe-limited waters from the Gulf of Alaska (Martin et al. 1989) and tropical Pacific (Hutchins et al. 2002), N was observed to be drawn down more quickly than P. Little change in particulate N : P ratios was observed after a large scale Fe addition to the Southern Ocean during the EIFEX experiment (Hoffmann et al. 2007). The natural Fe fertilization which results from sedimentary interactions near the Kerguelen Islands also did not have a large impact on phytoplankton N : P (Lasbleiz et al. 2014). Incubations in the Amundsen Sea resulted in lower N : P uptake when Fe was added with DFB, although other additions of Fe had little effect on N : P (Mills et al. 2012).

Interestingly, these earlier results on large eukaryotes and eukaryote-dominated ecosystems are at odds with traditional ecological theory, which holds that rapidly growing cells under conditions of abundant resources require higher amounts of P to build the RNA and DNA required for cell metabolism and growth, while slower growing cells under conditions of nutrient limitation require more N to build the proteins associated with nutrient acquisition (Sterner and Elser 2002). This resource allocation theory has been used to explain the generally higher N : P in marine cyanobacteria compared to large eukaryotic phytoplankton (Klausmeier et al. 2004; Arrigo 2005; Weber and Deutsch 2012).

The elemental stoichiometry of non-diazotrophic cyanobacteria and picoeukaryotes has been much less studied than large eukaryotes. The focus on large eukaryotes reflects their dominance in high-latitude high-nutrient low-chlorophyll (HNLC) regions where nutrients are abundant, so small

changes in elemental stoichiometry can have a large impact on the global distribution of micronutrients. However, cyanobacteria also play a crucial role in the global cycling of major and trace nutrients. Cyanobacteria such as *Synechococcus* and *Prochlorococcus* are the most abundant microorganisms in the global ocean, and they dominate in many oligotrophic ecosystems where they account for a large portion of total marine productivity (Flombaum et al. 2013).

Here, we present the major element and trace element stoichiometry (C, N, P, Fe, Cu, Ni, Mn, Co, Zn, and Cd) from cultured oceanic strains of two non-diazotrophic cyanobacteria under Fe-replete and -deplete conditions. *Synechococcus* WH7803 and *Prochlorococcus* MED4 were grown in media with abundant major nutrients under either Fe-replete or Fe-deplete conditions as determined by a decrease in growth rate. This represents the first combined major and trace elemental stoichiometry for *Prochlorococcus*. We also report the first study of extended elemental stoichiometry in Fe-limited cyanobacteria cultures, and find significant differences between average element : P ratios of C, N, Co, Fe, Mn, and Cd between Fe-replete and Fe-deplete conditions.

Methods

Growth of laboratory cultures

Synechococcus WH7803 and *Prochlorococcus* MED4 were axenically grown in either Fe-replete or Fe-deplete media and maintained using trace-metal clean procedures. Each culture was grown in 1 L acid cleaned polycarbonate bottles with constant bubbling from air pushed through a 0.2 μm filter to prevent Fe-contamination from airborne particles. Cultures were transferred to new media under HEPA-filtered laminar flow air to prevent contamination. All cultures were grown under a 14 : 10 light : dark cycle at 40 μmol quanta m^{-2} s^{-1} at 23°C. Cell concentrations were tracked daily using a Guava easyCyte HPL flow cytometer.

Both strains were grown in a slightly different media, although each media had similar excess macronutrient concentrations (N and P), and contained similar amounts of EDTA and trace-metals so that the concentration of bioavailable trace-metals was similar. EDTA-containing media ensures that the concentration of available Fe in seawater is buffered to a constant value. The phytoplankton can only take up the small portion of free iron (not bound to EDTA), and thus their growth may be limited in "low-Fe" media even although there is still plenty of Fe-EDTA in the media, which will soon re-equilibrate to release more free Fe. Thus, even in batch cultures such as the ones we use here, phytoplankton experience a constant concentration of free Fe, and thus they experience a constant amount of Fe-stress (or Fe-sufficiency).

Fe-replete media were created according to published recipes (see below) and Fe-deplete media were created using the same recipes except decreasing the concentration of Fe by a factor of 100, creating a free Fe concentration of ~1 nM in both media. Before preparing media, artificial seawater and nutrient stocks were cleaned of trace-metal contamination by passing them through Chelex-100 resin according to established procedures (Sunda et al. 2005).

Synechococcus WH7803 (CCMP1334) was grown in SN media (Waterbury et al. 1986). The media was prepared with trace-metal clean artificial sweater (ASW) containing final concentrations of 409 mM NaCl, 53 mM $MgCl_2$ • $6H_2O$, 28 mM Na_2SO_4, 10 mM $CaCl_2$ • $2H_2O$, 9 mM KCl, 2.7 mM $NaHCO_3$, 824 μM KBr, 420 μM H_3BO_3, 90 μM $SrCl_2$ • $6H_2O$, and 71 μM NaF. To create SN media in a 1 L polycarbonate bottle, 750 mL ASW was combined with 236 mL ultrapure water (UPW), nutrients and trace metals to achieve final concentrations of 9 mM $NaNO_3$, 99 μM K_2HPO_4, 15 μM Na_2EDTA • $2H_2O$, 100 μM Na_2CO_3, 738 pM cyanocobalamin, 32.5 μM citric acid • H_2O, 23 μM ferric ammonium citrate, 7.08 μM $MnCl_2$ • $2H_2O$, 772 nM $ZnSO_4$ • $7H_2O$, 85.9 nM $Co(NO_3)_2$ • $6H_2O$. *Prochlorococcus* MED4 (CCMP1986) was grown in Pro99 Media (Moore et al. 2007) made with low-nutrient 0.2 μm filtered and microwave sterilized seawater from the Baruch field station in South Carolina. The seawater was combined with macronutrients to achieve final concentrations of 50 μM NaH_2PO_4 •H_2O, 800 μM NH_4Cl, 1.17 μM Na_2EDTA • $2H_2O$, 1.17 μM $FeCl_3$ • $6H_2O$, 8 nM $ZnSO_4$ • $7H_2O$, 5 nM $CoCl_2$ • $6H_2O$, 90 nM $MnCl_2$ • $4H_2O$, 3 nM $NaMoO_4$ • $2H_2O$, 10 nM Na_2SeO_3, 10 nM $NiSO_4$ • $6H_2O$.

Prior to experiments, cultures were maintained in Fe-replete media under the growth conditions described above, then transferred to Fe-deplete media to produce the experimental inoculum. The two species were grown and analyzed at different times, however in both cases the Fe-replete and -deplete cultures were inoculated simultaneously with 0.1% of the same of Fe-deplete stock culture, processed simultaneously, and analyzed for major and trace-elements during the same analytical session. The experiments were ended by harvesting cells during mid-log phase growth based on cell abundance, at different times but similar cell abundances. The cell populations performed at least five doublings while in culture (~10–15 d) before harvest. Each of the four treatments (two species, two Fe concentrations) was grown in duplicate.

Particulate carbon and nitrogen measurements

Measurements of particulate carbon and nitrogen were completed in triplicate for each 1 L culture by vacuum filtering 25 mL onto a precombusted 25-mm GF/F filters. Filters were then dried overnight and analyzed for POC and PON using a Perkin Elmer 2400 CHNS Elemental analyzer following the procedure of Froelich (1980). Analyses were made in the Marine Sediments Research Lab at University of South Carolina.

Trace element measurements

Concentrations of P, Fe, Cu, Ni, Mn, Co, Zn, and Cd were measured in triplicate for each 1 L culture on an Element 2

ICP-MS. After removing 75 mL for the analysis of POC and PON, 60 mL of an oxalate-EDTA solution was added to the culture vessel to remove any extracellularly-bound elements (Tovar-Sanchez et al. 2003; Sanudo-Wilhelmy et al. 2004; Hassler and Schoemann 2009). Cultures were allowed to incubate with this solution for 30 min at room temperature before filtration. Triplicate 100 mL aliquots were vacuum-filtered onto trace-metal cleaned 47-mm 0.2 μm polycarbonate filters. Filters were removed from the filtration rig with plastic forceps and placed into a PFA vial. Each vial was amended with 20 ng of indium (In) to correct for analytical sensitivity and account for any material lost during the leaching process, then 1 mL each of concentrated ultrapure distilled HCl and HNO_3 were combined in the PFA vials to dissolve the cells. The vials were allowed to sit overnight at room temperature, venting to release the noxious gasses formed during the reaction of HCl, HNO_3, and organic material. The next day filters were removed from the vials with acid-rinsed tefzel tweezers, rinsed twice with UPW into the PFA vials to remove any extra liquid, and discarded. The remaining liquid in the PFA vials was evaporated to dryness overnight and then redissolved in 500 μL of 0.1 N HNO_3 for analysis. Samples were analyzed for elemental concentrations on a Thermo Element 2 ICP-MS at the Center for Elemental Mass Spectrometry at the University of South Carolina.

Modeling

The possible impact of Fe-limitation on global nutrient cycling was investigated using model output from the MIT biogeochemistry and ecosystem model (Dutkiewicz et al. 2015). Fe limitation was determined as in Dutkiewicz et al. (2012). This model is based on 3-dimensional ocean circulation from the MIT gcm coupled to an ecological model which includes two zooplankton functional groups and eight different phytoplankton groups including *Prochlorococcus* and *Synechococcus*, and two zooplankton groups. Model output includes the abundance of each phytoplankton functional group throughout the surface ocean, as well as the primary limiting nutrient for each group. The primary limiting nutrient is determined by assuming a fixed elemental stoichiometry for each group, and calculating the nutrient which is present in the dissolved phase at relative concentrations lower than required for additional growth. The extent of Fe limitation in this model is greater than some other models for which Fe-limitation occurs only in HNLC regions and limited parts of the South Pacific (Moore et al. 2002), although there is not enough known about the Fe-limitation of picophytoplankton to be sure which model better represents the reality of exactly where the boundary between different limiting nutrients lies for various species.

Model output was used to identify regions where Fe limitation may have a large impact on phytoplankton N : P. Using the model distributions of cyanobacteria and Fe-limitation we calculated the fraction of biomass which is made up of Fe-limited cyanobacteria, and we assumed that this biomass has the same N : P as measured in our Fe-deplete cultures. We similarly calculate the expected N : P in the absence of Fe-limitation based on our Fe-replete cultures. The difference between these two is used to identify the fraction of plankton N, which can be attributed to Fe limitation.

Data analysis

Statistical comparison of elemental composition of phytoplankton cells obtained throughout the experiments was performed using two-tailed *t*-tests and Kolmogorov–Smirnov tests for normality (SPSS Statistics v23.0, IBM Corporation).

Results

Physiological changes

Cells grown in Fe-deplete media exhibited physiological changes including reduced growth rate and, for *Synechococcus*, reduced cell size. Growth rates of *Synechococcus* WH7803 and *Prochlorococcus* MED4 were reduced by 38% and 24%, respectively, in Fe-deplete media (Table 1). Compared to other culture studies where growth rates may be reduced by over 90% under severe Fe limitation, the levels of Fe limitation observed here would be considered mild. Fe-limited *Synechococcus* WH7803 were noticeably smaller when grown in Fe-deplete media, based on forward scatter during flow cytometry (Fig. 1). The mode forward scatter is roughly an order of magnitude higher for Fe-replete compared to Fe-limited cells ($\sim$50 vs. $\sim$5). This should correspond roughly to a doubling in the mode cell diameter based on other studies with the Guava easyCyte, although our instrument was not calibrated with beads of a known diameter and thus this provides just a rough estimate. There were no such changes in cell size observed for *Prochlorococcus* MED4.

Major element stoichiometry

Major elements in the cell were determined both on a per cell basis and normalized by P (Figs. 2, 3). *Synechococcus* WH7803, which exhibited the only noticeable change in cell size, also had roughly half as much P per cell under Fe-limited conditions. *Prochlorococcus* MED4 did not exhibit a significant change in P per cell at the 2σ confidence level. Similarly, the amount of C per cell was roughly half as much for *Synechococcus* WH7803 under Fe-limited conditions.

For both species there was an increase in N : P under Fe-limited conditions. The N : P increased by 58% and 67% for *Synechococcus* WH7803 and *Prochlorococcus* MED4, respectively. C : P increased by 13% and 90%, respectively. It is important to note that we did not observe any significant changes in C : N ratios among species, between treatments (Table 1). Also, the average C : N ratios of our cultures are similar to previously published cyanobacteria C : N ratios (Bertilsson et al. 2003).

Table 1. Average elemental stoichiometry (moles/moles) of individual species grown in various nutrient-replete and Fe-deplete media, including combined averages of picophytoplankton measured for this study. Error represents the pooled 1 σ SD for duplicate cultures analyzed in triplicate.

	Growth rate (d^{-1})	C : N	C : P	N : P	1000Fe:P	1000Zn:P	1000Cu:P	1000 Mn:P	1000Co:P	1000Ni:P	1000Cd:P
Synechococcus WH7803 high Fe	0.72 ± 0.04	3.2 ± 0.27	65 ± 3.9	20 ± 1.2	78.7 ± 0.24	1.42 ± 0.15	0.37 ± 0.02	2.79 ± 0.03	0.088 ± 0.02	0.61 ± 0.39	$2.29 \pm 0.31 \times 10^{-3}$
Synechococcus WH7803 low Fe	0.52 ± 0.03	2.3 ± 0.42	73 ± 8.8	32 ± 4.4	17.4 ± 4.1	2.41 ± 0.02	0.21 ± 0.02	5.23 ± 0.15	0.21 ± 0.04	0.31 ± 0.02	$4.18 \pm 0.27 \times 10^{-3}$
Prochlorococcus MED4 high Fe	0.98 ± 0.07	5.1 ± 0.11	89 ± 1.7	17 ± 0.17	26.4 ± 1.9	0.30 ± 0.15	0.42 ± 0.17	1.02 ± 0.41	$5.91 \pm 2.7 \times 10^{-3}$	0.20 ± 0.04	$1.51 \pm 0.51 \times 10^{-3}$
Prochlorococcus MED4 low Fe	0.75 ± 0.03	5.8 ± 0.7	169 ± 17	29 ± 1.8	6.97 ± 5.3	0.98 ± 0.72	0.62 ± 0.21	1.21 ± 0.41	0.014 ± 0.01	0.40 ± 0.16	$3.52 \pm 1.6 \times 10^{-3}$
Picophytoplankton avg. high Fe	0.82 ± 0.1	4.2 ± 1.4	80 ± 13	19 ± 1.5	37.4 ± 37	1.11 ± 0.7	0.49 ± 0.16	1.67 ± 0.97	0.04 ± 0.04	0.30 ± 0.28	$1.63 \pm 0.62 \times 10^{-3}$
Picophytoplankton avg. low Fe	0.60 ± 0.1	4.1 ± 0.2	121 ± 48	29 ± 3.2	9.51 ± 6.9	1.43 ± 0.85	0.59 ± 0.37	2.73 ± 2.2	0.11 ± 0.1	0.29 ± 0.12	$3.37 \pm 0.9 \times 10^{-3}$
Bacillariophyceae avg.*	0.59 ± 0.3	7.6 ± 1.7	62 ± 22	8 ± 3.6	2.95 ± 2.7	0.52 ± 0.43	0.17 ± 0.09	2.9 ± 1.6	0.09 ± 0.06	—	0.10 ± 0.07
Chlorophyceae avg.*	0.7 ± 0.02	6.4 ± 0.76	198 ± 35	32 ± 9.2	13.4 ± 2.1	1.55 ± 0.12	0.64 ± 0.03	3.6 ± 3.6	0.11 ± 0.12	—	0.25 ± 0.35
Prasinophyceae avg.*	0.53 ± 0.2	13.9 ± 11	288 ± 162	23.5 ± 5.1	10.6 ± 6	0.94 ± 0.52	0.55 ± 0.06	4.6 ± 2.8	0.15 ± 0.08	—	0.28 ± 0.33
Dinophyceae avg.*	0.39 ± 0.16	11.3 ± 4.7	127.5 ± 11	13 ± 5.3	7.03 ± 5.3	0.78 ± 0.48	0.56 ± 0.57	3.6 ± 2.4	0.29 ± 0.16	—	0.11 ± 0.31
Synechococcus sp.†	0.67	6 ± 0.12	52 ± 9.8	8.7 ± 1.8	16.8 ± 3.4	1.1 ± 0.19	0.65 ± 0.06	2.4 ± 0.33	0.086 ± 0.05	—	0.02 ± 0.004

*Averages calculated from Ho et al. (2003).
†Averages from Quigg et al. (2011).

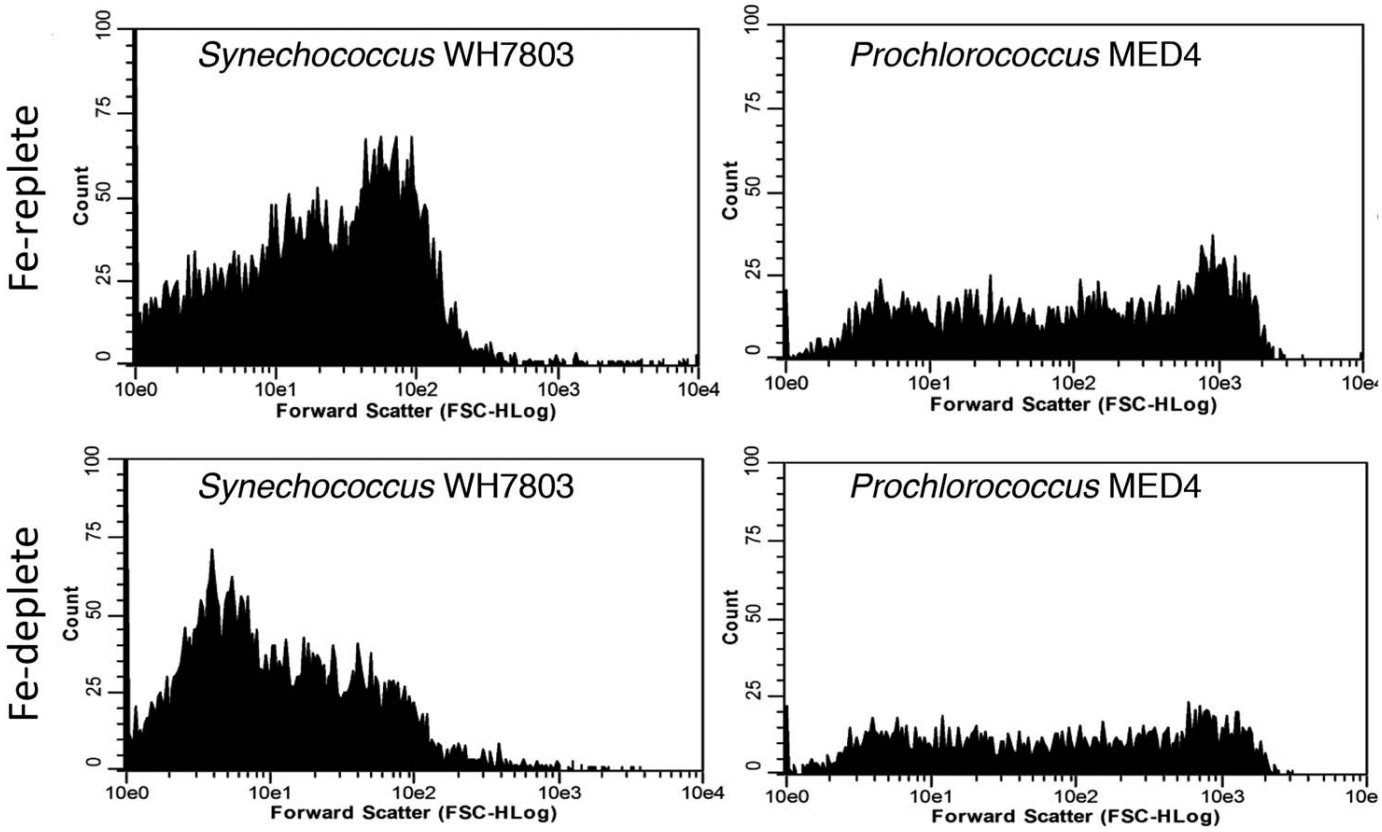

Fig. 1. Comparison of flow cytometry data for *Synechococcus* WH7803 and *Prochlorococcus* MED4 in Fe-replete and Fe-deplete media. Histograms show forward scatter vs. total number of cells. Greater forward scatter correlates with larger cell size.

Extended element stoichiometry

Overall, the average elemental composition of the *Synechococcus* and *Prochlorococcus* nutrient-replete cells average $(C_{65}N_{20}P_1)_{1000}$ $Fe_{78.7}Mn_{2.79}Zn_{1.42}Cu_{0.37}Ni_{0.61}Co_{0.088}Cd_{0.0023}$ and $(C_{89}N_{17}P_1)_{1000}$ $Fe_{26.4}Mn_{1.02}Zn_{0.30}Cu_{0.42}Ni_{0.20}Co_{0.006}Cd_{0.0015}$, respectively, while *Synechococcus* and *Prochlorococcus* Fe-deplete cells average $(C_{73}N_{32}P_1)_{1000}Fe_{17.4}Mn_{5.23}Zn_{2.41}Cu_{0.21}Ni_{0.31}Co_{0.21}Cd_{0.0042}$ and $(C_{169}N_{29}P_1)_{1000}Fe_{6.97}Mn_{1.21}Zn_{0.98}Cu_{0.62}Ni_{0.40}Co_{0.14}Cd_{0.0035}$, respectively. This corresponds to a significant ($p < 0.05$) element : P quota (moles cell^{-1}) increase in Mn, Co, and Cd and a significant decrease in Fe between cells grown in nutrient-replete compared to Fe-deplete media (Table 1). Also, it should be noted that when both species are averaged, there is no significant difference element : P quota in Zn between treatments. However, there is a significant increase ($p < 0.05$) in Zn in *Synechococcus* cells grown in low Fe (Fig. 3).

Discussion

Extended element stoichiometry

The extended elemental stoichiometry of picophytoplankton is similar to previous analyses of many eukaryotic phytoplankton and *Synechococcus* (Ho et al. 2003; Quigg et al. 2003, 2011) (Table 1). We find that our extended stoichiometric measurements of Fe-replete and Fe-limited picophytoplankton cultures fall within the elemental ranges measured for Bacillariophyceae, Chlorophyceae, Prasinophyceae, Dinophyceae, and a species of *Synechococcus* (Ho et al. 2003; Quigg et al. 2003, 2011). We observe higher concentrations of Fe in the cyanobacteria compared to previously reported eukaryotes, similar to other culture studies (Brand 1991; Wilhelm et al. 1996; Twining and Baines 2013). Both species had higher Fe : P in Fe-replete compared to Fe-deplete media, consistent with many previous studies demonstrating "luxury uptake" of Fe under Fe sufficient conditions (Palenik et al. 2006; Twining and Baines 2013; Palenik 2015). Another open ocean strain of *Synechococcus* (WH8102) was shown to have a more robust Fe luxury uptake mechanism than a coastal strain (WH8020) (Mackey et al. 2015), consistent with the particularly high levels of luxury uptake we observe here for WH7803 which is also an oceanic strain (Carr and Mann 2004).

Other metals showed a variety of different responses to Fe-limitation. For both species, the element : P ratios of Cd, Co, and Mn increased in Fe-deplete media. Fe-limitation has been previously linked to higher Cd uptake by diatoms because the divalent ion transporters they upregulate to facilitate Fe^{2+} uptake can also transport divalent Cd^{2+}

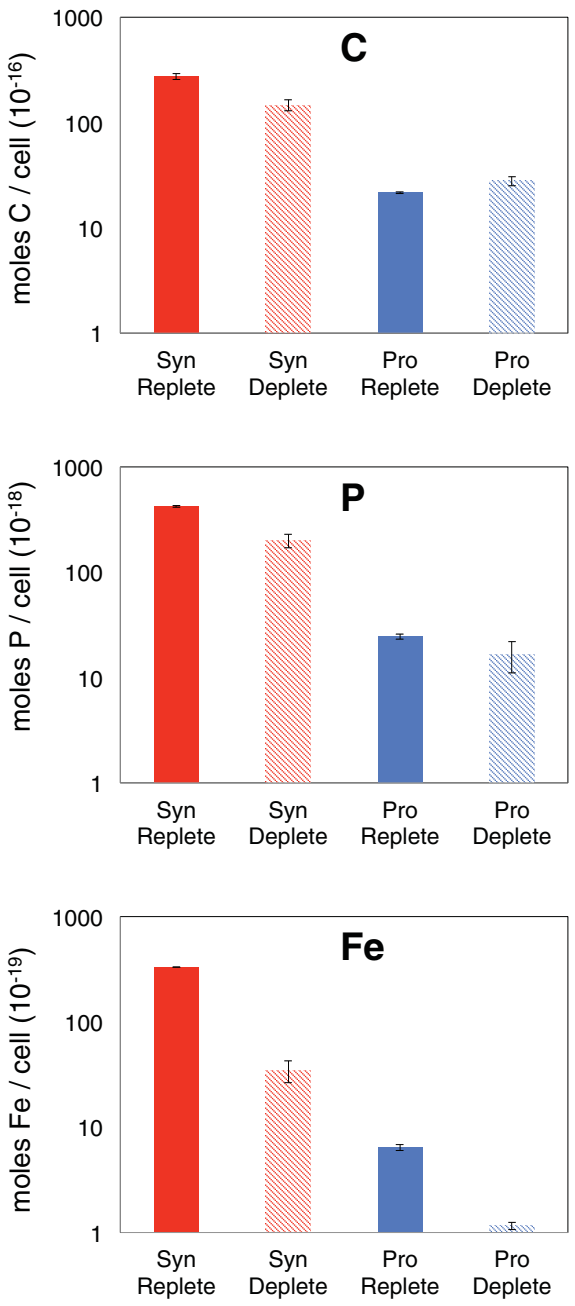

Fig. 2. Average cellular P and Fe concentration per cell for *Synechococcus* WH7803 (red bars) and *Prochlorococcus* MED4 (blue bars), under Fe-replete (solid) and Fe-deplete (hashed) conditions. Error bars represent the pooled 1 σ SD for duplicate cultures analyzed in triplicate.

(Cullen 2006; Lane et al. 2009). Reduced Co and Mn are also stable in seawater as divalent cations for short amounts of time, so perhaps their preferential uptake by Fe-limited cells also occurs through Fe-transporters. Fe-stressed phytoplankton may also be more susceptible to intracellular production of reactive oxygen species during photosynthesis, necessitating the production of superoxide dismutase enzymes to prevent cell damage (Apel and Hirt 2004; Wolfe-Simon et al. 2005). Superoxide dismutases occur in four metalloforms with Fe, Mn, CuZn, or Ni at the reactive site. Cyanobacteria (such as *Synechococcus* and *Prochlorococcus*) have been shown to contain Fe, CuZn, and Ni metalloforms (Chadd et al. 1996; Eitinger 2004; Wolfe-Simon et al. 2005; Dupont et al. 2008). Perhaps some of the variability in metal : P stoichiometry between Fe-replete and Fe-limited cells is due to changes in the quantity and metalloforms of superoxide dismutases. However, it is also possible that other cellular processes, such as carbonic anhydrase transcriptional regulators, oxygenic photosynthesis electron transfer, or ATPases, are also influencing the changes we observe in Cd, Co, and Mn (Cavet et al. 2003; Shcolnick and Keren 2006; Waldron and Robinson 2009).

Interestingly, we did notice a significant increase in Zn, but only in the low Fe treatment for *Synechococcus* cells (Fig. 3). It is not entirely sure why there is a large increase for Zn, however, Zn is known to easily bind onto biological detritus (e.g., John and Conway 2014). Therefore, it is possible that some of the Zn signal is not true biological Zn, but just Zn adsorbed onto cell surfaces.

Major element stoichiometry

The observed increase in cyanobacteria N : P under conditions of Fe-limitation is opposite of prior work with large eukaryotic phytoplankton. Previous studies of how Fe-limitation affects phytoplankton N : P have consistently found either no effect or a decrease in N : P, both in the field (Martin et al. 1989; Hutchins et al. 2002; Hoffmann et al. 2007; Mills et al. 2012; Lasbleiz et al. 2014) and in culture (Greene et al. 1991; La Roche et al. 1993; Price 2005; Hoffmann et al. 2007; Timmermans and van der Wagt 2010; Sugie and Yoshimura 2013). However, all of this work has either been done in culture with large eukaryotic phytoplankton such as diatoms, or in HNLC regions of the ocean where large eukaryotic phytoplankton dominate. We are aware of no previous experiments which have directly examined the effect of Fe-limitation on the N : P of non-diazotrophic cyanobacteria.

Although our results stand in contrast to prior work with large eukaryotes, they are consistent with theories of resource allocation which generally suggest that slower growing cells should have higher N : P (Sterner and Elser 2002; Klausmeier et al. 2004; Arrigo 2005; Weber and Deutsch 2010, 2012). Fe-limited picophytoplankton would be expected to increase their N : P as they devote less of their cellular resources to growth and division, and more toward the proteins involved in Fe acquisition. Our study cannot shed light on the particular mechanism by which drives changes in N : P, for example whether it is due to changes in the expression of particular Fe-containing proteins, or if it simply reflects a general response to growth-rate limitation.

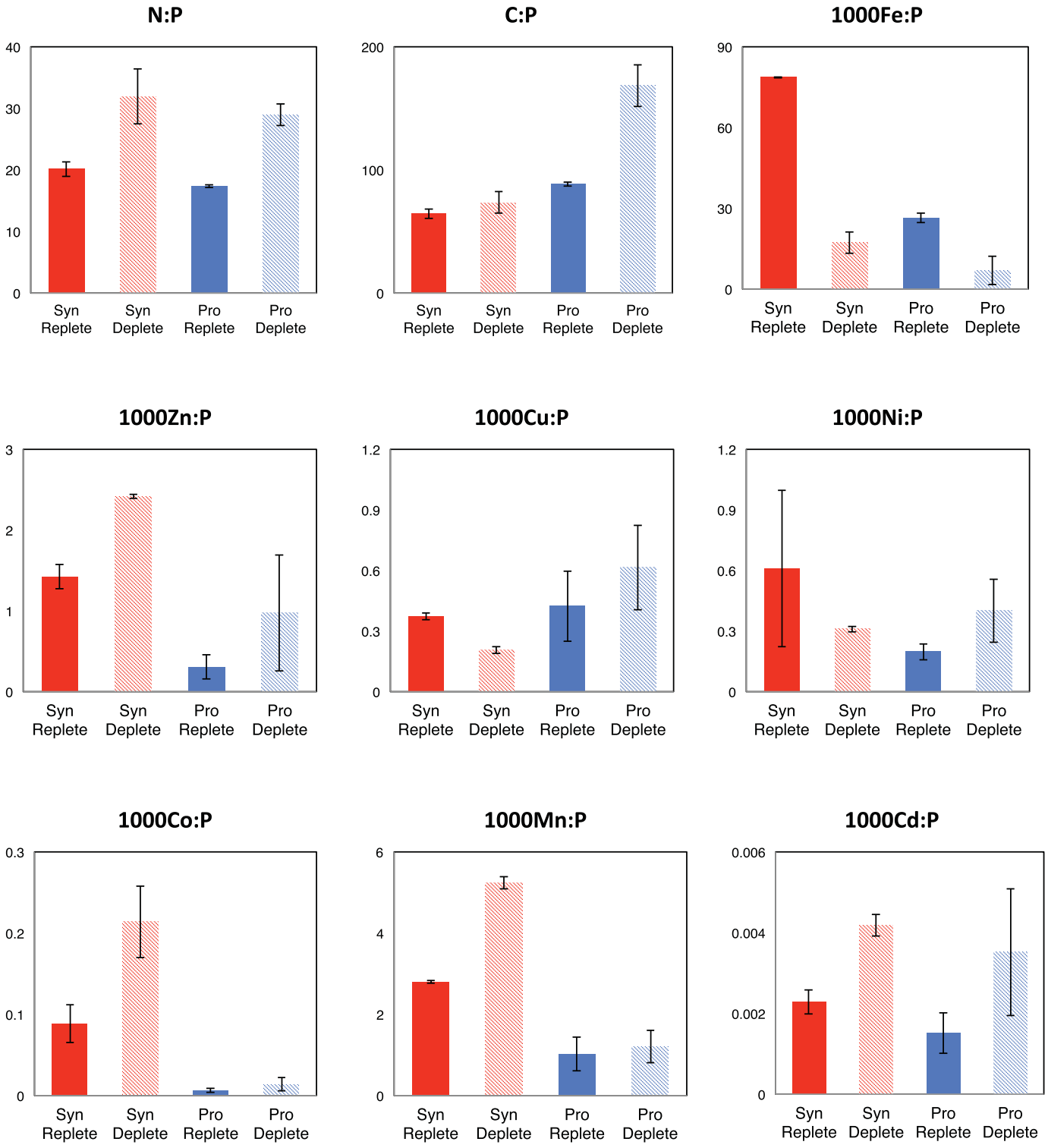

Fig. 3. Comparison of average elemental quotas of *Synechococcus* WH7803 (red bars) and *Prochlorococcus* MED4 (blue bars), under Fe-replete (solid) and Fe-deplete (hashed) conditions. All elements are normalized to P (mol/mol or mmol/mol). Error bars represent the pooled 1σ SD for duplicate cultures analyzed in triplicate.

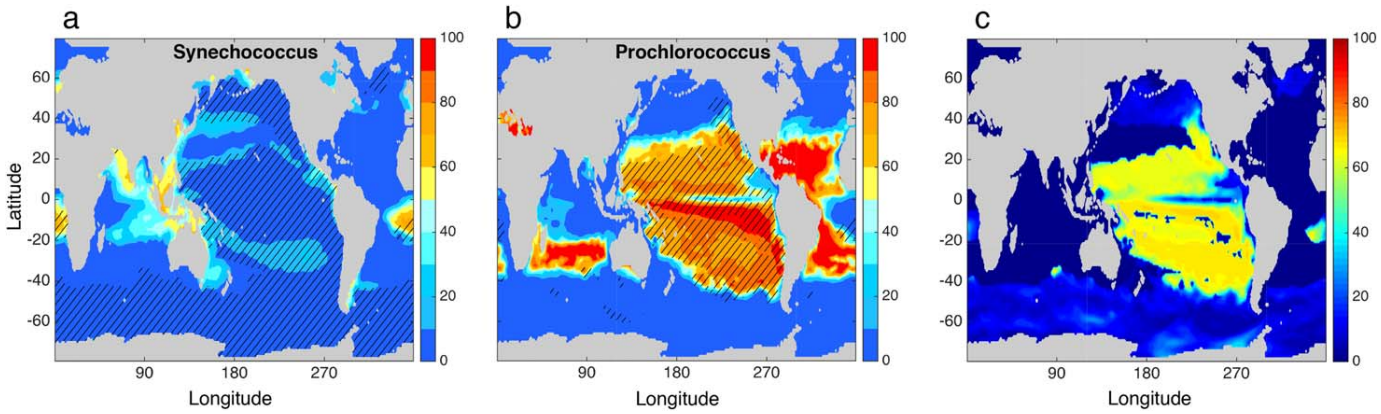

Fig. 4. Modeled distributions of *Synechococcus* (**a**) and *Prochlorococcus* (**b**) are plotted as the percentage of total phytoplankton biomass, the hashed area represents the regions in which each of these species is limited by Fe (Dutkiewicz et al. 2015). Distributions of Fe-limited cyanobacteria are combined with data on N : P from Fe-replete and Fe-deplete media to estimate the fraction of total phytoplankton particulate N which can be attributed to excess N uptake caused by Fe-limitation of cyanobacteria (**c**).

Global implications

The factors which control the nutrient stoichiometry of phytoplankton have been extensively studied and include taxonomy (e.g., Lynn et al. 2000; Sañudo-Wilhelmy et al. 2001; Bertilsson et al. 2003; Twining et al. 2003, 2010; Quigg et al. 2003; Twining and Baines 2013), growth rate (e.g., Sterner and Elser 2002; Klausmeier et al. 2004; Arrigo 2005; Weber and Deutsch 2010, 2012), and nutrient supply ratios (e.g., Mills and Arrigo 2010; Martiny et al. 2013; Karl 2014; Teng et al. 2014; Galbraith and Martiny 2015; Mouginot et al. 2015). Our findings suggest that there may be instances where Fe-limitation also influences nutrient stoichiometry in natural communities.

The cyanobacterial Fe : P observed in our cultures are consistent with Fe-limitation of cyanobacteria in much of the global ocean. For example, the MIT biogeochemistry and ecosystem model predicts that both *Synechococcus* and *Prochlorococcus* are Fe-limited throughout the Pacific by assuming a Fe : P of 1 : 1000 (Dutkiewicz et al. 2012, 2015). In our cultures even the Fe-limited *Synechococcus* and *Prochlorococcus* have higher Fe : P of 17 : 1000 and 6 : 1000, respectively. Similarly, typical soluble reactive phosphorous concentrations in the oligotrophic Pacific at Station ALOHA are ~10–100 nM (Karl 2014), while Fe concentrations are typically ~0.1–0.2 nM (Boyle et al. 2005; Fitzsimmons et al. 2014, 2015; Hayes et al. 2015), meaning that the supply ratio of Fe : P to surface phytoplankton will often be lower than the biological demands we observe in culture. On the other hand, cyanobacteria are known to respond much less than diatoms to Fe addition in Fe-limited regions of the ocean, with some studies showing limited growth enhancement (e.g., Landry et al. 2000) and others showing no growth enhancement (e.g., Cavender-Bares et al. 1999). It may be that the Fe requirements of natural cyanobacteria are lower than for the cultured species studied here, which were isolated from the relatively Fe-enriched North Atlantic (*Synechococcus* WH7803) and Mediterranean (*Prochlorococcus* MED4) oceans. Indeed, *Prochlorococcus* from Fe-limited regions have been shown to contain a reduced complement of Fe-containing proteins, suggesting a lower Fe : P requirement (Rusch et al. 2010).

Output from the MIT biogeochemistry and ecosystem model was used to identify the regions of the ocean where Fe-limitation of cyanobacteria is most likely to affect N : P. The model predicts Fe limitation of *Synechococcus* throughout the Southern Ocean and much of the Pacific, and Fe limitation of *Prochlorococcus* in the tropical and subtropical Pacific (Fig. 4). Combining this data with the differences in N : P measured here for Fe-replete and Fe-deplete cultures, we calculate that up to 40% of the particulate organic nitrogen in the surface ocean might be associated with Fe-limited waters. The greatest impact is observed in the subtropical Pacific dominated by *Prochlorococcus*. This exercise probably represents the maximum impact that Fe-limitation may have on N : P in natural conditions because our culture data are from conditions with abundant macronutrients and abundant light, so that Fe is the only factor limiting growth and N : P. In the field Fe-limitation is just one of many factors which are known to impact N : P stoichiometry (Sterner and Elser 2002; Klausmeier et al. 2004; Arrigo 2005; Weber and Deutsch 2010, 2012).

In the oceans, we expect the greatest impact of Fe on nutrient stoichiometry in upwelling conditions near the base of the mixed layer. Upwelling from the thermocline would inject waters into the photic zone, which contain relatively high concentrations of N and P (compared to Fe) in approximately a 16 : 1 ratio. Then, under conditions of Fe-limitation, cyanobacteria would deplete N from the upwelled waters more quickly than P leaving behind a relative excess of P. Subsequently, the excess of dissolved P in the mixed

layer could stimulate growth of diazotrophs, which have a high P requirement but which can fix their own N. It may also favor the growth of picoeukaryotes since they require less Fe than prokaryotes, and they have a larger genome which requires more P for DNA replication (Brand 1991; Wilhelm et al. 1996; Palenik et al. 2007; Twining and Baines 2013; Zubkov 2014). Crucially, because C : N ratios are typically much less variable than N : P (Klausmeier et al. 2004; Arrigo 2005; Martiny et al. 2014), this Fe-limited scenario would results in a greater total growth of biomass and a greater net carbon fixation. Conversely, we would predict that increases in Fe supply to ocean regions where cyanobacteria dominate would lead to lower N-fixation and carbon fixation.

The observations of a higher N : P ratio of phytoplankton in oligotrophic subtropical regions has traditionally been attributed to the high concentrations of cyanobacteria and diazotrophs (Mills and Arrigo 2010; Weber and Deutsch 2010, 2012; Martiny et al. 2013). Our results are consistent with a high N : P driven by these species, but they suggest that in some cases Fe-limitation may be a contributing underlying factor for why these species exhibit high N : P. During glacial periods this would have been a negative feedback on climate change, with greater aridity and dust flux during glacial periods relieving Fe-limitation in the subtropics, leading to lower N : P and therefore lower carbon fixation and export from the surface ocean. Similarly, future anthropogenic climate change may alter Fe delivery to the oceans and thus impact the carbon cycle through changes in N : P. Models suggest an increase in surface ocean Fe concentrations in the high latitudes and the North Atlantic (Boyd et al. 2015), but a decrease in Fe in the South Atlantic and South Pacific (Yool et al. 2013).

Conclusion

In many ways, the extended elemental stoichiometry of picophytoplankton is similar to that of large eukaryotic phytoplankton such as diatoms, which have been much more extensively studied. Yet, quantifying the extended elemental stoichiometry of cyanobacteria is important for understanding the global biogeochemical cycling of trace-metal micronutrients and incorporating these processes into models. In contrast to studies of large eukaryotes, we found that cyanobacterial N : P was 58–67% higher in Fe-limited cultures. Understanding whether Fe-limitation impacts cyanobacterial nutrient stoichiometry in natural environments will require further studies, both in vitro and in situ, which more closely replicate natural ocean conditions. If Fe-limitation of cyanobacteria is widespread, and if this leads to increased N : P in natural populations, it would have a large impact on community structure and global carbon cycling.

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Acknowledgments

Thanks to Stephanie Dutkiewicz and Mick Follows for providing limitation and species map output from the MIT GCM. Thanks to Amy Zimmerman for useful discussions about the manuscript. Thanks to Eric Tappa, Bob Thunnell and the USC Marine Sediments Research Lab for assistance with particulate N and C analyses. This publication was funded by Gordon and Betty Moore Foundation grant GBMF3305 to John, and a grant from the Simons Foundation (SCOPE Award 329108, John and Follows).

Conflict of Interest

None declared.

Submitted 06 June 2016
Revised 22 September 2016
Accepted 26 October 2016

Associate editor: Maren Voss