

Iron requirements for dinitrogen- and ammonium-supported growth in cultures of *Trichodesmium* (IMS 101): Comparison with nitrogen fixation rates and iron:carbon ratios of field populations

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Abstract

We quantified the relationships among the specific growth rate, intracellular iron content, and steady state iron uptake rate for cultures of the marine diazotrophic cyanobacterium *Trichodesmium* (IMS 101) grown under differing conditions of Fe and N availability. The Fe quotas necessary to support a moderately Fe-limited growth rate (70% μ_{\max}) of 0.1 d⁻¹ under diazotrophy and ammonium were 38 and 8 $\mu\text{mol mol}^{-1}$, indicating a fivefold increased Fe cost for diazotrophy. This increased demand reflects the influences of both the ninefold lower marginal use efficiency and the greater maintenance Fe:C requirement at zero growth rate under diazotrophy (13.5 versus 5.2 $\mu\text{mol mol}^{-1}$). For diazotrophic growth at $\mu = 0.1 \text{ d}^{-1}$, we estimate that 19%–53% of the cellular Fe is bound in nitrogenase. *Trichodesmium* is capable of luxury uptake of at least 13-fold greater amounts of Fe than needed for moderately Fe-limited growth. At least half of the populations sampled from the continental shelf of northern Australia had Fe:C ratios within the range of iron limitation of laboratory cultures. Bottle incubations showed that, after 3–4 d with added iron, a low Fe:C population (21 $\mu\text{mol mol}^{-1}$) had higher N₂ fixation rates than controls, whereas a high Fe:C population (78 $\mu\text{mol mol}^{-1}$) showed no response. The cellular N:P quotas and N₂ fixation rates were compared to those previously reported for the Atlantic Ocean. Empirically, these data suggest a critical N:P ratio ~40–50 for the onset of P limitation of growth. Only 10% of the *Trichodesmium* populations from coastal Australia had N:P ratios within the putative P-limitation range (>40), whereas 40% the Atlantic populations fell within this range. These comparisons, along with those for N fixation versus colony Fe:C ratio, suggest that Fe limitation is more prevalent in the Australia *Trichodesmium* populations, while P limitation predominates in the Atlantic populations.

Trichodesmium spp. is a genus consisting of five species of nitrogen-fixing cyanobacteria that inhabit (sub)tropical oligotrophic waters. These plankton provide new N for up to 50% of primary production in these waters (Karl et al. 1997) and, thus, can play a prominent role in the biogeochemical cycling of C and N (Carpenter and Romans 1991). N₂ fixation facilitates net C fixation and export from the euphotic zone (Karl et al. 1995) and may have pronounced global climate implications (Falkowski 1997). Iron has been proposed to be a primary factor limiting N₂ fixation and growth of *Trichodesmium* due to the high iron requirement for diazotrophic growth and the low availability of iron in oceanic

waters (Rueter 1983). Several lines of qualitative evidence of iron limitation in *Trichodesmium* and other phototrophic diazotrophs are derived from culture work, correlative field observations, and theoretical considerations (Raven 1988; Rueter 1988; Rueter et al. 1990). While each approach has offered valuable insight, quantifying the Fe requirement for N₂-supported growth is necessary to determine the relationship between iron flux (e.g., from aeolian dust deposition) and marine N₂ fixation in global climate models. Recent results (Berman-Frank et al. 2001) directly demonstrate a high Fe requirement for N₂ fixation by *Trichodesmium*.

We compared the cellular Fe demands for growth supported by N₂ and NH₄⁺ in cultures of *Trichodesmium* (IMS 101). The relationships among specific growth rates, Fe:C quotas, and chlorophyll *a* (Chl *a*):C ratios were quantified to determine empirical iron use efficiencies for these modes of growth. In addition, these relationships allowed us to approximate the partitioning of intracellular Fe between the Fe intensive processes of N₂ fixation and photosynthesis, thus providing physiological insight into the high Fe demand of diazotrophic growth. We evaluated the capacity of *Trichodesmium* IMS 101 for luxury uptake of Fe and discuss the

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significance of such uptake in the context of episodic iron deposition from aeolian sources in oligotrophic oceanic waters. Relationships between nitrogen fixation rates and Fe:C ratios measured in field populations were compared to our culture results to help assess the Fe status of the field populations. In addition, on-deck Fe incubation experiments were conducted with field populations to further assess the response of natural populations to Fe addition.

Methods

Culture experiments—Maintenance cultures of *Trichodesmium* spp. (IMS 101) were grown in the medium of Chen et al. (1996) at 27°C and ~80 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ (12h:12h light:dark cycle). These were maintained in exponential growth phase to prevent accumulation of bacterial biomass in this nonaxenic culture. Prior to experiments, the cultures were transferred to media made up in filtered, salinity 36 Gulf Stream water (Sunda and Huntsman 1995). Cobalt, copper, zinc, nickel, and manganese were added to achieve total metal concentrations of 4, 4, 10, 10, and 9 nmol L^{-1} , respectively. The media contained 10 $\mu\text{mol L}^{-1}$ ethylenediaminetetraacetic acid (EDTA), a metal ion buffering agent. At this concentration of EDTA, these metal concentrations yield approximate free ion concentrations of cobalt, copper, zinc, and manganese of $10^{-11.03}$, $10^{-13.52}$, $10^{-10.99}$, and $10^{-8.54}$ mol L^{-1} , respectively, similar to free ion concentrations used in previous studies (Sunda and Huntsman 1995). The media were enriched with 2 $\mu\text{mol L}^{-1}$ NaH_2PO_4 , 10 nmol L^{-1} Na_2SeO_3 , and f/10 vitamins (thiamine HCl, biotin, and vitamin B₁₂ at 59, 0.041, and 0.070 nmol L^{-1} , respectively). For NH_4^+ -supported growth, the media were also enriched with 5 $\mu\text{mol L}^{-1}$ of NH_4OH (Trace Metal Grade, Fisher Scientific). Ammonium, rather than NO_3^- , was chosen as the source of fixed N because the uptake capacity for NH_4^+ far exceeds that for NO_3^- in field populations of *Trichodesmium* (Mulholland and Capone 1999). Total Fe concentrations ranged from 4.5 to 301 nmol L^{-1} , including an estimated background concentration in the medium of 1.5 nmol L^{-1} . For each treatment, media in duplicate bottles were amended with either ^{59}Fe -labeled FeEDTA or ^{14}C -labeled HCO_3^- . The added ^{14}C bicarbonate was used to measure concentrations of cellular fixed carbon as a function of time (Sunda and Huntsman 1995). Specific growth rates were then determined from linear regressions of \ln fixed C versus time. The cells were transferred from the maintenance media to the relatively low Fe treatments described. Before Fe:C was measured, cells were acclimated to low-Fe conditions for a minimum of 10 generations (two transfers). Cells were considered acclimated by an observed linear relationship between \ln (*Trichodesmium* C) and time over several generations. Preceding our Fe:C measurements, the regression coefficients, r^2 , were almost always greater than 0.98, indicating steady state growth dictated by iron availability.

In order to determine Fe:C ratios in lab populations, intracellular Fe:Chl *a* ratios were determined in one set of ^{59}Fe -labeled cultures, while cellular C:Chl *a* was determined in the second parallel set of cultures. For each Fe treatment, the ratio of the two values yielded Fe:C values. Intracellular

Fe and fixed carbon were measured radiometrically from the fraction of ^{59}Fe or ^{14}C bicarbonate assimilated by *Trichodesmium* cells (Sunda and Huntsman 1995). Chl *a* in the two parallel sets of cultures was measured fluorometrically after its extraction into a solution of dimethylsulfoxide and acetone (Sunda and Huntsman 1995). Samples were usually collected by restricted gravity filtration onto 5- μm polycarbonate (Poretics) filters, although occasionally it was necessary to apply a gentle vacuum. For intracellular Fe determination, cells were treated with a Ti-citrate-EDTA wash (0.05 mol L^{-1}) to remove iron hydroxides and iron bound to cell surfaces (Hudson and Morel 1989). In preliminary experiments, total Fe was also determined in cells without Ti treatment. This was done at concentrations of dissolved inorganic iron hydrolysis species (Fe') < 0.75 nmol L^{-1} , where Fe hydroxides do not precipitate. An average of $83\% \pm 8\%$ ($n = 12$) of the iron associated with the *Trichodesmium* was intracellular, similar to values observed for several eukaryotic marine phytoplankton (Sunda and Huntsman 1995). Ti treatment did not cause significant cell lysis, since $86\% \pm 10\%$ of the cellular fixed ^{14}C was retained after cell washing. Samples were collected at low cell densities (*Trichodesmium* C ~ 5–60 $\mu\text{mol L}^{-1}$ and always less than 30 $\mu\text{mol L}^{-1}$ for NH_4^+ experiments, except as in Fig. 1), to avoid NH_4^+ limitation in the ammonium experiments and to minimize growth on regenerated N in the diazotrophic growth experiments. Batch cultures under diazotrophic growth conditions exhibit biomass-specific N_2 fixation rates that progressively decline as biomass reaches excessive concentrations (i.e., 110–160 $\text{nmol Chl } a \text{ L}^{-1}$), thus becoming decoupled from C fixation (Chen et al. 1996; Mulholland and Capone 2001). Our median Chl *a* concentration before terminal sampling for Fe:C was 2.7 nmol L^{-1} . As our results indicate, the Fe cost of growth on ammonium is significantly lower than that for diazotrophy. Therefore, maintaining such low biomass helps ensure that the observed diazotrophic growth rates at measured Fe:C quotas are not compromised by uptake of regenerated N. These low biomass conditions also prevent pH-induced changes in Fe chemistry (Price et al. 1988/1989; Hudson et al. 1992) or dissolved inorganic carbon availability, which could influence iron availability (Hudson and Morel 1990) or cellular iron requirements (Raven and Lucas 1985).

The intracellular Fe:C and the calculated steady state Fe uptake rates were plotted as functions of the concentration of total Fe and of dissolved inorganic iron species (Fe') to assess the relationships among iron uptake, iron availability, and the capacity for luxury uptake of Fe. In fully acclimated, exponentially growing cultures, the steady state Fe uptake rate, V_{ss} , equals the product of the carbon specific growth rate and the intracellular Fe:C ratio. The iron uptake data are presented as a function of the concentration of biologically available Fe' in order to compare our results with those for other marine algae grown at different irradiances, temperatures, and EDTA concentrations (conditions that influence the $[\text{Fe}'] : [\text{Fe}_T]$ ratio). At an average pH of 8.12 under our experimental conditions, we calculate a ratio of $[\text{Fe}'] : [\text{Fe}_T]$ of 1:98 based on iron complexation data of Sunda and Huntsman (pers. comm.). The Fe' concentration in our cultures was computed by dividing the total iron concentra-

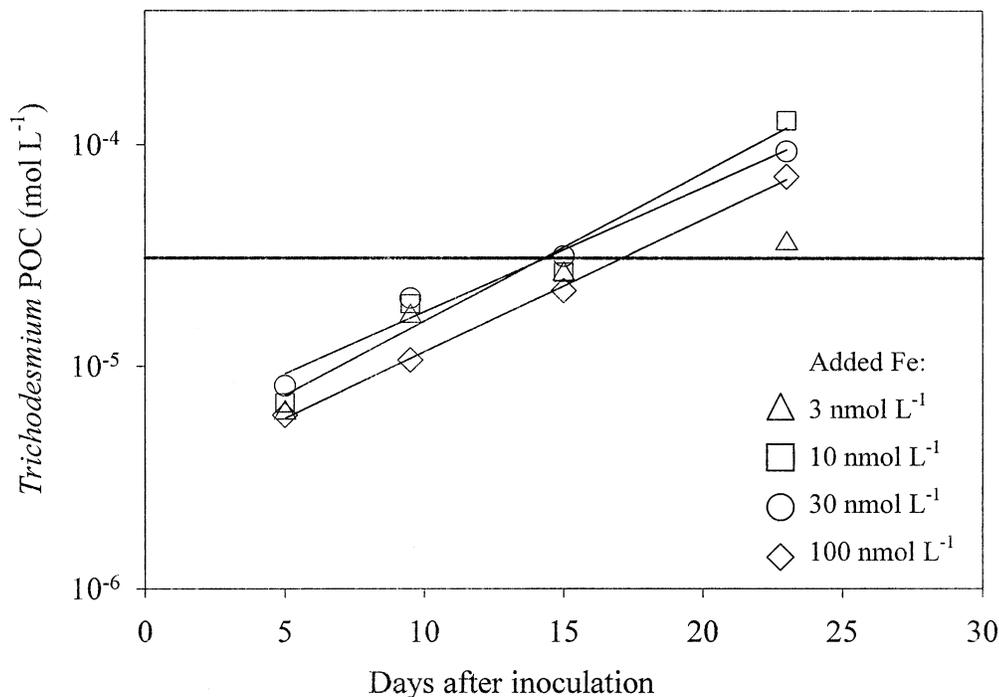


Fig. 1. Effect of added iron concentration (3, 10, 30, 100 nmol L⁻¹) on growth in acclimation cultures grown in media containing 5 μ mol L⁻¹ ammonium. All cultures showed similar growth rates (0.140 ± 0.012 d⁻¹) at low biomass, but after exceeding 31 μ mol L⁻¹ particulate organic carbon, cells in the 3 nmol L⁻¹ Fe treatment exhibited a dramatic decline in growth.

tion by this ratio. Estimates of [Fe'] could only be computed this way for Fe' concentrations at or below the solubility of 0.75 nmol L⁻¹ (Sunda and Huntsman 1995), as additional Fe_r in Fe' saturated media is expected to yield Fe hydroxide precipitates, not increases in [Fe'].

Elemental ratios and N₂ fixation in field populations—We measured the Fe:C ratios of field-collected *Trichodesmium* colonies on a cruise to the continental shelf off the north coast of Australia (Table 2). Colonies were collected from a *Zodiac* skiff with an all plastic 102- μ m mesh plankton net (stored in 0.01 N HCl). The net was towed at \sim 1 knot for 5 min at a depth of 5 m, usually 3–4 h after sunrise. A brief description of the net deployment is as follows. With use of polyethylene gloves, the net was removed from the storage bucket aboard the *Zodiac* and the polypropylene bridle line was fastened to a polyvinyl chloride (PVC) ring that was fastened around the boom line. The boom line ran from the bow out to the end of a 10 cm \times 10 cm wooden boom; this boom extended from the *Zodiac* by \sim 1.3 m. The net was fitted with an acid-cleaned polyethylene codend. Depending on the specific wind and current conditions, the net was fished either by drifting or by constantly engaging and disengaging the motor to achieve a tow speed of about 1 knot. The net was fished outside of the influence of the *Zodiac* wake. Aboard the ship, individual colonies were removed from the codend with a plastic inoculating loop in a class 100 clean hood. Typically, 50–100 colonies were aliquoted into Teflon vials containing seawater for CHN determination and 150–300 colonies were placed in vials with Nano-Pure

(18.2 m Ω) water for iron and phosphorus analysis. Colonies for CHN analysis were filtered onto precombusted glass-fiber filters for subsequent analysis at the Marine Sciences Research Center (MSRC) analytical laboratory. For Fe and P analysis, the Teflon vials were sealed and transported back to MSRC for acid digestion. The digestion procedure followed that of Sañudo-Wilhelmy et al. (2001), except the volumes of *Q*-HCl + *Q*-HNO₃ and *Q*-HF in the sequential digestion were reduced to 250 + 250 and 50 μ l, sufficient amounts to digest the organic matter and dissolve any aluminosilicates. The samples were split for Fe analysis and P analysis. Fe contents were determined by graphite furnace atomic absorption with a Hitachi Z-8100 spectrophotometer (GFAAS). Aliquots (2–5 mg dry weight) of standard reference material (NIST 1515; Apple Leaves) were weighed into Teflon vials and digested by the same procedures as used for the *Trichodesmium* colonies. The carbon contents of this mass range of standard reference material were similar to the estimated carbon content present in the *Trichodesmium* samples for metal analysis (based on the C:colony data), yielding a similar bulk organic C:acid stoichiometry as the *Trichodesmium* samples. Each *Trichodesmium* sample was analyzed by the standard addition method. In addition, an aliquot of each sample was spiked with an aliquot of NIST 1515 standard reference material digest. These steps were taken in order to evaluate the recovery of Fe from NIST 1515 within a *Trichodesmium* standard curve, in accordance with the advice of Collier and Edmond (1984) on careful matrix matching for GFAAS analysis of marine particles. The average percent recovery of Fe from this spike (encom-

passing sample digestion through analysis) was $97\% \pm 10\%$. Blanks were determined from Teflon vials brought to sea, filled with Nano-Pure water in the clean hood on the ship, and sealed for subsequent analysis. These vials were subjected to the same digestion procedure and had an average iron content of $10 (\pm 8)$ pmol vial⁻¹. Samples were diluted in 1 N *Q*-HNO₃ prior to analysis. The P content of the digests was determined using medium-resolution inductively coupled plasma mass spectrometry (MR-ICP-MS, Element II, Finnigan) by measuring the abundant ³¹P isotope in the sample against a calibration of P standards in 1 N *Q*-HNO₃. The ³¹P in the final dilutions was at least two orders of magnitude above the detection limit of 0.4 ng g⁻¹.

We compared relationships between Fe:C contents and nitrogen fixation rates of field populations to those between intracellular Fe:C quotas and specific growth rates obtained in diazotrophic laboratory cultures. Nitrogen fixation rates from ambient populations were measured using the acetylene reduction method (Capone 1993). An acetylene reduction: N₂ fixation ratio of 3:1 was assumed, based on mean ratios of ¹⁵N₂ fixation: C₂H₂ reduction from both field and lab populations of *Trichodesmium* (Mulholland and Capone 2001; Orcutt et al. 2001). Acetylene reduction assays were initiated at 0830 h (± 0.8 h) and were run for 5 h. They were conducted in on-deck incubators at 55% of the ambient irradiance using neutral density screening. This irradiance corresponds to that at a depth of ~ 15 m, where maximal biomass-specific N₂ fixation rates are often observed. This depth is similar to that of *Trichodesmium* collections for Fe:C analysis. The resultant N₂ fixation rates are expressed as average C-specific hourly rates ($\mu\text{mol N fixed h}^{-1} \text{ mol C}^{-1}$). To estimate C-specific growth rates, these hourly rates were first converted to daily rates by multiplying them by 9.8 h d⁻¹, the average ratio (SD = ± 1.9 h) of daily to hourly rates calculated for the N₂ fixation period of our observations (from 2.75 to 7.75 h after sunrise), derived from four diel studies (Kustka et al. 2003). Specific growth rates were then estimated by multiplying the daily C-specific N₂ fixation rates by the average measured C:N ratio of the *Trichodesmium* colonies (6.1 ± 0.7 mol: mol). Previously published data from the Atlantic Ocean (Kustka et al. 2003) were also presented this way, except the conversion factor differed slightly (by 11%; due to a different average C:N and different timing of N₂ fixation measurements).

For *Trichodesmium*, neither the critical N:P (i.e., the threshold above which P limitation is found) nor the relationship between P-limited diazotrophic growth and the cellular P quota is known. It was beyond the scope of this study to conduct P-limitation experiments in lab cultures similar to those we conducted for Fe. Nonetheless, we may hypothesize the relative potential for P limitation of Australian populations as compared to our previous results obtained along a cruise transect in the western North Atlantic Ocean (Sañudo-Wilhelmy et al. 2001). To this end, the cellular N:P quotas and N₂ fixation rates of Atlantic and Australian populations were compared, and the evidence regarding either Fe or P limitation is discussed.

Field Fe enrichment experiments—Incubation experiments were performed on 6 and 8 November 1999 using

seawater collected from the *Zodiac* skiff using trace metal clean procedures. The seawater was collected from a depth of 5 m from the *Zodiac* using Teflon tubing coupled to 0.5-m acid-cleaned C-flex tubing (for the pump head). Before sample collection, the tubing was purged with 10 liters of seawater at a flow rate of 1 L min⁻¹. An acid-cleaned polypropylene cartridge filter (0.22 μm , MSI, Calyx®) was then fixed to the end of the C-flex tubing. The cartridge was purged, rinsed, and conditioned by passing at least 5 liters of seawater through it before collecting incubation water. An acid-cleaned, fluorinated high density polyethylene 20-liter carboy was rinsed three times with filtered seawater and subsequently filled. In the incubation experiments, precleaned 250-ml polycarbonate bottles were rinsed three times with this seawater and filled to the rim. There were three experimental treatments: (1) a no Fe addition control, (2) addition of 2.5 nmol L⁻¹ FeCl₃, and (3) a combined addition of 2.5, 2.5, and 0.5 nmol L⁻¹ FeCl₃, MnSO₄, and CoCl₂ (hereafter, referred to as the combined metal treatment), respectively. Colonies were removed from the codend of the plankton net, rinsed in clean seawater, and aliquoted to individual bottles. All these experimental manipulations were conducted in a class 100 clean hood. Polycarbonate bottles were tightly capped and sealed with parafilm and then placed in an ambient temperature flowing seawater incubator under 55% ambient irradiance.

For each incubation experiment, there were four to five replicate bottles for the control and +Fe treatments and three replicate bottles for the combined metal treatment. Each bottle received 15 colonies collected by the same trace metal clean procedures as described above. Fluorescence measurements of colonies were made for each treatment throughout the course of the experiments. For each time point, colonies from one bottle of a given treatment were sampled. Sampling was conducted without replacement of the bottles. Acetylene reduction rates were measured at the end of each experiment. These rates were determined from colonies removed from two bottles for each treatment. These colonies were transferred to gas-tight serum vials for the short-term acetylene reduction assay.

We surmised that any potential effect of iron additions would require considerable time for Fe assimilation and synthesis of metabolic catalysts before subsequent increases in growth processes might be observed. However, anecdotal reports suggest that long-term (>24 h) incubations are not feasible with *Trichodesmium* populations. Therefore, it was desirable to employ a rapid general diagnostic indicator of physiological competence. To assess this competence during the course of incubations, the ratio of variable to maximum fluorescence (F_v/F_m) was measured using a microscope-based pulse amplitude modulation (PAM) fluorometer (Walz, FRG). It was not our intent to compare our measurements of F_v/F_m to measurements acquired using fast repetition rate fluorometry (FRR), since to date we know of no intercalibrations between PAM and FRR fluorometric measurements, making such comparisons uncertain.

For F_v/F_m measurements, bottles from various treatments were sampled at night, between 0100 and 0400 h local time. F_v/F_m was measured at night to avoid potential influences of photoacclimation on F_v/F_m during the daytime (Campbell et

al. 1998). Single bottles from each treatment were removed from the incubator, and 10 colonies were isolated at random from each bottle for measurement with the PAM fluorometer. Control and +Fe bottles were sampled during the first sampling period. Owing to the restricted availability of trace metal clean collections of *Trichodesmium* colonies for experimentation, there were adequate replicates of the combined metal treatment for sampling only during the last sampling period. Therefore, during the last sampling period (~12 h prior to N₂ fixation measurements), F_v/F_m was measured in all treatments. During a given sampling period, single colonies from each treatment were measured sequentially on the PAM fluorometer to avoid any confounding differences among treatments due to shifts in F_v/F_m during the time required to measure all colonies (~2.5 h). However, no such time-dependent trend was observed. During PAM fluorometric measurements, the colonies were maintained in the dark, except briefly while mounting individual colonies on depression slides. After exposing each colony for 10 s to far-red illumination to oxidize photosystem II (PSII), each colony was exposed to weak actinic light (yielding F_0) followed by a pulse of 650 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ actinic light (yielding F_m). F_v/F_m was calculated as $(F_m - F_0)/F_m$.

After incubating for 3–4 d, two bottles from each treatment were opened and colonies were removed for short-term assays of acetylene reduction (Capone 1993). These acetylene reduction assays were started at 1300 h local time and ran for 3.5 h. Rates of acetylene reduction were normalized to Chl *a* for each serum vial. After acetylene reduction measurement, the contents of each vial were filtered onto glass-fiber filters, Chl *a* was extracted in dimethylformamide, and Chl *a* was measured with a Hitachi scanning fluorometer.

Clean versus conventional collection of Trichodesmium colonies—On select dates, *Trichodesmium* colonies were collected for Fe and C analysis with a 1-m² plankton net deployed from the hydrowire aboard the R/V *Ewing*, in addition to the clean net collections from the *Zodiac*. Other than this difference in collection method, the samples were treated identically to those collected aboard the *Zodiac* with the acid-washed all plastic plankton net. On 8 November, an Fe addition experiment was conducted with a control (no Fe added) and 5 nmol L⁻¹ FeCl₃ treatment, using water collected with more conventional methods (CTD rosette suspended from the ship's hydrowire). The objective was to compare the results with those of the parallel Fe addition experiment conducted using water collected from the *Zodiac* skiff by trace metal clean procedures. Water collected with the CTD was dispensed into acid-washed Pyrex bottles, whereas water collected aboard the *Zodiac* was dispensed into ultraclean polycarbonate bottles. The *Trichodesmium* colonies used in this intercomparison were collected from clean net tows, ensuring that the sources of seawater and incubation bottles were the only differences between the clean and conventional incubations.

Results

An elevated Fe demand for diazotrophy was apparent from the behavior of cultures during the acclimation to low-

Fe media. Upon transferring the inocula from the high-Fe maintenance media to the 5 $\mu\text{mol L}^{-1}$ NH₄⁺ treatments, we observed that a range of added iron concentrations (3–100 nmol L⁻¹) all yielded similar growth rates at low biomass. This average observed rate ($0.140 \pm 0.012 \text{ d}^{-1}$) is the maximum value reported by several investigators under these conditions (summarized in Mulholland and Capone 2001). However, for cells receiving 3 nmol L⁻¹ added Fe, once the biomass surpassed the carbon yield of 31 $\mu\text{mol L}^{-1}$ (the expected point of ammonium depletion, based on a C:N of 6.2:1 for *Trichodesmium*; Carpenter 1983), the growth rate dropped significantly, suggesting that cells had become nitrogen limited (Fig. 1). By contrast, the higher iron treatments continued to grow exponentially, reaching cell carbon concentrations that were threefold to fourfold higher, presumably due to the onset of diazotrophy. In subsequent experiments with acclimated cells, *Trichodesmium* cultures supplied with 3 nmol L⁻¹ added Fe, in the absence of added fixed N, were unable to sustain growth.

We evaluated the relationships between the Fe-limited growth rates and the Fe:C quotas for each mode of growth. Diazotrophic cultures required much higher cell Fe:C ratios to achieve a given growth rate than those grown on ammonium (Fig. 2A,B). The Fe:C ratio needed to support a moderately iron-limited growth rate of 0.1 d⁻¹ was 38 $\mu\text{mol mol}^{-1}$ under diazotrophy, similar to the value (48 $\mu\text{mol mol}^{-1}$) determined previously at this growth rate by Berman-Frank et al. (2001). For ammonium-supported growth, the cells needed an Fe:C of only 8 $\mu\text{mol mol}^{-1}$ (a fivefold lower value) to support the same growth rate. Curiously, ammonium-supported growth was inhibited at higher Fe concentrations ($\geq 100 \text{ nmol L}^{-1}$). To facilitate comparisons at a common growth rate, the quotas for N₂- and NH₄⁺-supported growth were interpolated for $\mu = 0.1 \text{ d}^{-1}$ from the linear relationships shown in Fig. 2A. These relationships were determined from the three observations obtained at the lowest Fe_T treatments, which yielded the lowest Fe:C ratios. The observed iron-limited growth rates were linearly related to the cellular Fe:C ratios and fit Eq. 1, as found previously for coastal diatoms and dinoflagellates (Sunda and Huntsman 1997).

$$\mu = \text{IUE} (\text{Fe:C} - m) \quad (1)$$

The data for NH₄⁺- and N₂-supported growth fit the above equation, at least up to an iron-limited growth rate of 0.10 d⁻¹, 70% of the maximum value (0.14 d⁻¹). The slope of the above equation is the marginal iron use efficiency (IUE) and equals the moles of additional carbon fixed per additional mole of intracellular iron per day (Raven 1988; Sunda and Huntsman 1997). The term *m* represents the maintenance iron, the intracellular Fe:C present in the cells at zero net growth rate. For growth on ammonium, the marginal iron use efficiency was 37,000 mol C mol Fe⁻¹ d⁻¹, while for diazotrophic growth it was ninefold lower at 4,100 mol C mol Fe⁻¹ d⁻¹. Likewise, the maintenance Fe:C ratio under diazotrophic growth (13.5 $\mu\text{mol mol}^{-1}$) was higher than that (5.2 $\mu\text{mol mol}^{-1}$) for growth on ammonium.

Above a growth rate of ~70% μ_{max} , the diazotrophic growth-quota relationship was no longer linear. This loss of linearity with increasing nutrient quota has been observed in

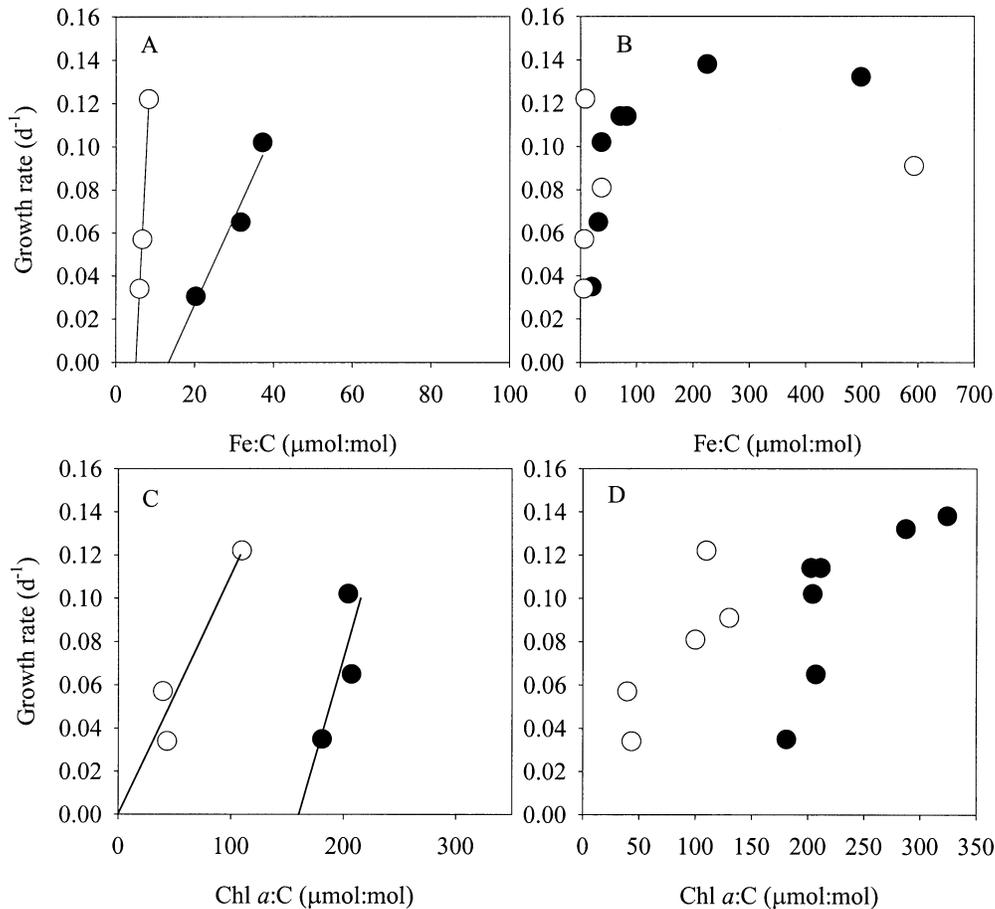


Fig. 2. Relationships among specific growth rate, intracellular Fe:C, and cellular Chl *a* for N₂- (closed symbol) and NH₄⁺- (open symbol) supported growth. (A) and (B) show the growth rate versus Fe:C relationships within the iron-limited region and the entire experimental range, respectively. Similarly, (C) and (D) show growth rate versus Chl *a*:C relationships. Linear regressions are shown in (A) and (C), within the iron-limited region of growth. Ammonium-supported growth was suppressed at Fe concentrations in excess of that just needed for maximum growth. However, within the iron-limited region, growth rate was linearly related to the intracellular iron pool.

other investigations of nutrient limitation and may reflect the saturation of growth processes with Fe as Fe repletion is approached. Therefore, it is little surprise that our data for specific growth rate versus Fe:C under diazotrophy also fit reasonably well to the empirically derived Droop equation (Droop 1968; Eq. 2), which takes such growth saturation behavior into account.

$$\mu = \mu_{\max} (1 - m/\text{Fe:C}) \quad (2)$$

The least squares fit of the data to this equation yielded an r^2 of 0.94 and a maintenance Fe:C of 14.2 $\mu\text{mol mol}^{-1}$, very similar to the value (13.5) obtained in our linear regression of μ versus Fe:C (Eq. 1). The Droop equation fit predicted a growth rate of 0.086 d^{-1} at 38 Fe:C ($\mu\text{mol mol}^{-1}$), again revealing the similar results of fitting either Eq. 1 or 2 to the empirical data. The Droop equation could not be fitted to the ammonium data, since high concentrations of Fe caused a decrease in growth rate under these conditions.

Diazotrophic growth imposes an additional photosynthetic

cost, due to the extra adenosine triphosphate (ATP) and reducing equivalents needed for N₂ fixation. We have compared the observed Chl *a*:C ratios between the two modes of growth to assess the Fe cost specifically attributed to diazotrophic metabolism. This rationale is based on the fact that the content of Chl *a* and other chromophores within photosynthetic units (which are Fe rich) is constrained by steric considerations of energy transfer from the light-harvesting complex to the reaction center and the rate of electron transport within the photosynthetic electron transport chain (Raven 1990). For consistency, we have plotted the Chl *a*:C from all observations (Fig. 2D), as well as those from the observations shown in Fig. 2A (Fig. 2C). At an Fe-limited specific growth rate of 0.1 d^{-1} , cells growing diazotrophically had a Chl *a*:C molar ratio (215 $\mu\text{mol mol}^{-1}$) that was 2.4-fold greater than that for ammonium-based growth (90 $\mu\text{mol mol}^{-1}$). To a first approximation, this increased Chl *a*:C suggests a more than doubling in the photosynthetic capacity and, therefore, in the photosynthetic iron demand for N₂- versus NH₄⁺-supported growth.

The high Fe cost of diazotrophy is also apparent when comparing Chl *a*:Fe values for diazotrophy and ammonium-supported growth. For ammonium-supported growth at 0.1 d⁻¹, the molar Chl *a*:Fe ratio was 11.6, very close to the average value (11.5) for coastal eukaryotic algae grown on nitrate at 20°C under saturating light (Sunda and Huntsman 1997). This agreement suggests a similar partitioning of Fe to photosystems in NH₄⁺-grown *Trichodesmium* and the NO₃⁻-grown eukaryotes. However, the Chl *a*:Fe ratio was 5.0 under diazotrophic growth at $\mu = 0.1$ d⁻¹, only 43% of the value observed for ammonium-supported growth. This lower Chl *a*:Fe ratio probably results from the additional metabolic iron present in nitrogenase complexes.

Nitrogenase Fe burden—The Fe burden structurally associated with the nitrogenase enzyme complex under N₂-supported growth in *Trichodesmium* has recently been estimated from a molecular iron use model (Kustka et al. 2003). Briefly, it was assumed that the nitrogenase complex in *Trichodesmium* fixes N₂ at the maximal specific rate observed in nitrogenase preparations from other diazotrophs, while considering observed diel variations in N₂ fixation rates in *Trichodesmium*. The maximum catalytic activity of in vitro nitrogenase assays varies by a factor of 2.75, depending on the species from which nitrogenase proteins are isolated. The maximum activity expressed in terms of the Fe content of the nitrogenase complex ranges from 0.034 to 0.094 mol N fixed s⁻¹ mol Fe⁻¹. Considering this range of values, a C:N ratio of 6.2 in *Trichodesmium*, and the diel activity of N₂ fixation in *Trichodesmium* (Kustka et al. 2003), a minimum cellular Fe:C burden of 7.3 to 20 $\mu\text{mol mol}^{-1}$ should be associated with nitrogenase complexes to support a specific growth rate of 0.1 d⁻¹. This represents 19%–53% of the cellular Fe:C ratio (38 $\mu\text{mol mol}^{-1}$) observed at that specific growth rate. During our experiments, acetylene reduction in ammonium-acclimated cultures was negligible. Therefore, we assumed there was no iron cost for nitrogenase in ammonium-grown cultures.

Photosynthetic Fe burden—Photosynthesis is dependent on a number of iron-containing proteins and protein complexes that comprise a significant portion of a phototroph's iron demand (Raven 1990). We estimated the iron content of the photosynthetic apparatus from our measured Chl *a*:C ratios and previously reported data for Chl *a* per photosynthetic reaction center (Berman-Frank et al. 2001). The iron burden associated with the photosynthetic apparatus under diazotrophic growth at $\mu = 0.1$ d⁻¹ was estimated using a PSI:PSII ratio (PSR) range of 0.96–1.05:1. These values were interpolated at $\mu = 0.1$ d⁻¹ from data presented in Berman-Frank et al. (2001), where *Trichodesmium* was grown under conditions nearly identical to ours. The range we calculated depends on whether the data for specific growth rate versus PSR from Berman-Frank and coworkers can be best fit to a linear equation [$\mu = 0.066(\text{PSR}) + 0.031$; $r^2 = 0.91$] or an exponential one [$\mu = 0.13(1 - 0.232^{\text{PSR}})$; $r^2 = 0.92$]. In our experiments, the Chl *a*:C ratio was 215 $\mu\text{mol mol}^{-1}$ and Fe:C was 38 $\mu\text{mol mol}^{-1}$ for diazotrophic growth at 0.1 d⁻¹. We estimate there should be ~23 Fe atoms per photosynthetic unit, based on a PSI:PSII ratio of ~1:1

and an assumed stoichiometry of 1:1:1 for PSI, cytochrome *c* (cyt *c*), and ferredoxin and 1:1 for PSII and cyt *b_{6-f}*-FeS (Raven et al. 1999). Based on this stoichiometry and a Chl *a*:PSI ratio of 341:1 (Berman-Frank et al. 2001), we calculate a total photosystem Fe content of 14.5 μmol per mol of cellular carbon, or 38% of the total intracellular Fe obtained in our experiments. Similar calculations for ammonium-grown *Trichodesmium* yield an estimate of photosystem bound Fe as 6.1 $\mu\text{mol mol}^{-1}$, or 77% of the measured intracellular Fe.

Iron quotas and uptake—In diazotrophic cultures, the log of the intracellular Fe:C content was linearly related to the log of the total Fe concentration in this EDTA-buffered medium (Fig. 3A) and can be described by Eq. 3.

$$\log \text{Fe:C} = 0.94(\log[\text{Fe}_T]) + 0.41 \quad (r^2 = 0.97) \quad (3)$$

Additional insight into the relationships between the Fe:C quota and external iron can be assessed from evaluating the steady state iron uptake rate. For diazotrophic cultures, a log–log plot of the computed steady state uptake rate versus the total iron concentration (Fig. 3B) fit the linear Eq. 4.

$$\log V_{\text{ss}} = 1.07(\log[\text{Fe}_T]) - 0.737 \quad (r^2 = 0.97) \quad (4)$$

A slope of unity (approximated by these data) indicates that the uptake rate is proportional to the iron concentration throughout the experimental range. At low Fe concentrations in EDTA-buffered culture media, many species exhibit Fe uptake rates that operate at the maximum velocities permitted by the physics of Fe diffusion toward the cell surface and the ligand exchange kinetics of iron binding to membrane transport sites (Hudson and Morel 1990; Sunda and Huntsman 1995). These limiting rates are usually proportional to the external concentration of available iron species. But at higher Fe concentrations, the slopes for cellular Fe uptake rate versus external iron concentration typically decline due to saturation of membrane uptake sites or down regulation of transport systems, effects that often vary among species (Harrison and Morel 1986; Sunda and Huntsman 1995). For example, the uptake rates for the oceanic species *Thalassiosira oceanica* and *Emiliania huxleyi* (Sunda and Huntsman 1995; Fig. 3B) are roughly proportional to [Fe'] below 20 pmol L⁻¹ but deviate substantially below proportionality at higher concentrations (slope = 0.39 for the plot of log *V* versus log [Fe']; Fig. 3B). By contrast, the iron uptake rate in *Trichodesmium* is proportional to the concentration of total iron or Fe' throughout the experimental range, indicating no apparent saturation or down regulation of the iron uptake system at high iron concentrations. In addition, iron uptake continues to increase with total Fe concentration well into the region of iron hydroxide precipitation, suggesting that colloidal Fe could be used in this genus. This behavior is similar to that observed in coastal diatoms, *T. weissflogii* and *T. pseudonana* (Sunda and Huntsman 1995).

The continuing increase in iron uptake rates at high iron concentrations results in an accumulation of intracellular iron in substantial excess of that needed to support algal growth (Fig. 3B). At 300 nmol L⁻¹ total Fe (the highest Fe concentration tested), N₂-supported *Trichodesmium* accu-

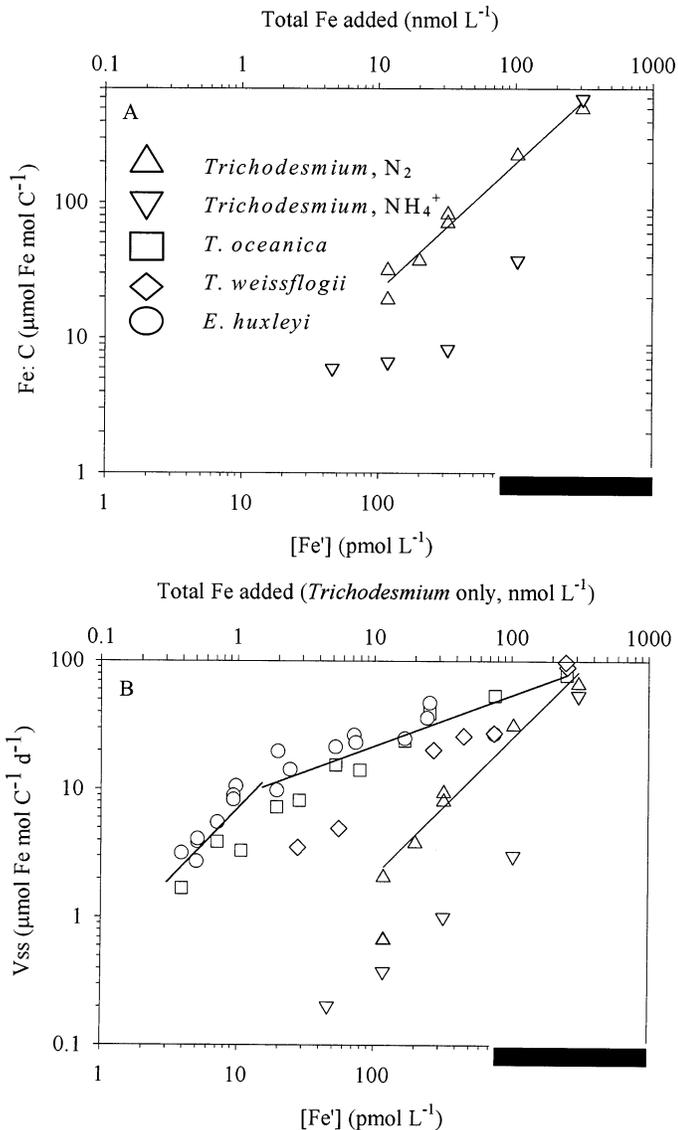


Fig. 3. Relationships among (A) intracellular Fe:C and (B) steady state iron uptake rates and the concentrations of total added iron and dissolved inorganic iron species for *Trichodesmium* grown on N_2 and NH_4^+ . In (B), the steady state iron uptake rates (V_{ss}) for *Trichodesmium* are compared with rates for nitrate-supported *Thalassiosira oceanica*, *Emiliania huxleyi*, and *Thalassiosira weissflogii* taken from Sunda and Huntsman (1995). Uptake rate data for these eukaryotes were obtained in media containing $100 \mu\text{mol L}^{-1}$ EDTA, 10 times the EDTA in the *Trichodesmium* cultures. Therefore, these rates are only plotted against $[\text{Fe}']$ (bottom scale) to facilitate comparison with *Trichodesmium* uptake data. In (A) and (B), the region of expected oxyhydroxide precipitation is indicated by the black bar on the $[\text{Fe}']$ axis. One calculated uptake rate ($V_{ss} = 0.67 \mu\text{mol Fe mol C}^{-1} \text{d}^{-1}$) for N_2 -supported *Trichodesmium* at 11.5 nmol L^{-1} total Fe was a statistical outlier ($p < 0.05$) and is shown in the figure but not included in the linear fit to Eq. 4.

culated $498 \mu\text{mol Fe per mol C}$, 13-fold higher than the value required to support a specific growth rate of 0.1 d^{-1} . Such luxury iron uptake and storage can be highly beneficial during periods of episodic iron input. The relationship between iron uptake and total iron in the medium was not as straightforward in NH_4^+ -grown cultures of *Trichodesmium* (Fig. 3). The steady state iron uptake rate was depressed when compared to cells grown on N_2 , except at 300 nmol L^{-1} total Fe, within the range of iron hydroxide precipitation. It is unknown whether this reflects a down regulation of Fe uptake or other phenomena.

Field measurements—Field collections of *Trichodesmium* from the northern coast of Australia had an average Fe:C ratio of $35 \pm 21 \mu\text{mol mol}^{-1}$ ($\pm\text{SE}$; Table 2). Most of these ratios fall within the range for iron-limited diazotrophic cultures (Fig. 2A,B), suggesting that many of these populations may be iron limited. However, for a more accurate assessment of iron limitation, we compared the relationships between *Trichodesmium* N_2 fixation rates and colony Fe:C ratios in field populations with our laboratory culture data for Fe-limited growth rate versus intracellular Fe:C. To make this comparison, we calculated daily rates of N_2 fixation from the hourly N_2 fixation rates in the field populations (see *Materials and methods*) and assumed that the specific growth rate equals the daily integrated C-specific N_2 fixation rate times the average molar C:N ratio of 6.1 (Fig. 4A). Superposed on these field data is the relationship between iron-limited growth and intracellular Fe:C from our diazotrophic cultures (solid line). The culture measurements reflect intracellular iron only, whereas total Fe:C contents were determined in the field *Trichodesmium* collections. Therefore, for a more accurate comparison, we converted the intracellular Fe:C values from the diazotrophic cultures to total cellular values (dashed line, Fig. 4A,B) by dividing them by the average measured ratio of intracellular to total cellular Fe (0.83). If the laboratory results are applicable to the field observations, field data that coincide with the dashed line indicate iron limitation of N_2 fixation, whereas observations to the right of the line would suggest iron in excess of that required to support diazotrophic growth. Many of the field data for N_2 fixation versus Fe:C plotted in Fig. 4A fall near the relationship estimated for our iron-limited *Trichodesmium* cultures, suggesting that growth rate in a majority of the Australian populations may be Fe limited. In Fig. 4B we have depicted the empirical data from our earlier Atlantic Ocean cruise. Only three of eleven observations of Fe:C and fixation rates were indistinguishable from the relationship obtained from our Fe-limited N_2 -fixing cultures. To facilitate a ready comparison of the likelihood of P versus Fe limitation of populations from each ocean basin, we have made the distinction between populations with N:P ratios above and below 40 mol:mol in Fig. 4A and 4B. This is discussed below.

The C:P molar ratios in the Australian populations ranged from 79 to 310, while the N:P ratios ranged from 14 to 52 (Table 2). In Fig. 5 we have plotted the C-specific N_2 fixation rate versus the *Trichodesmium* N:P from Australian populations, as well as those from a cruise transect in the equatorial North Atlantic (Sañudo-Wilhelmy et al. 2001). While

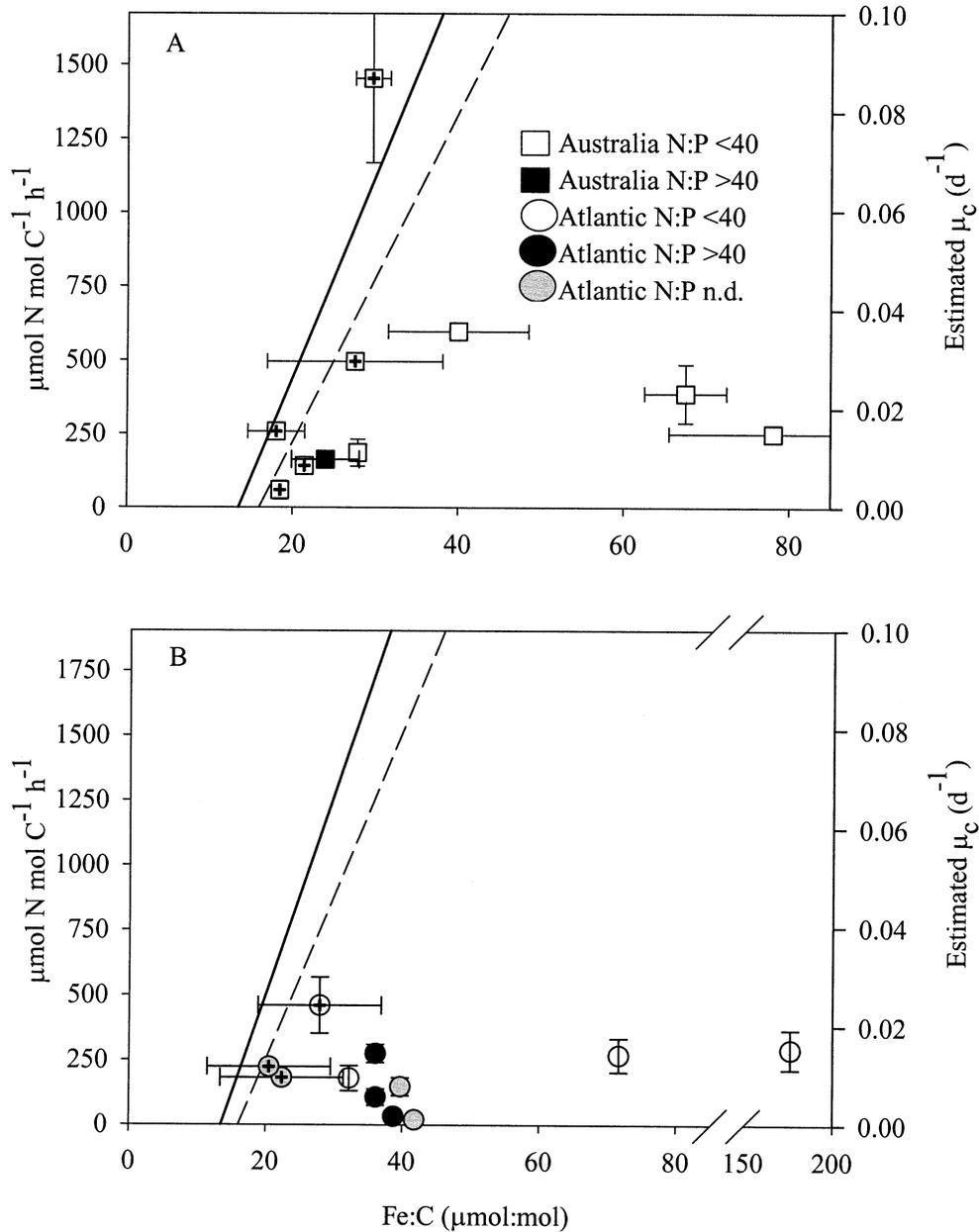


Fig. 4. Relationships among C-specific rates of N_2 fixation, estimated daily specific growth rates, and Fe:C ratios in *Trichodesmium* colonies collected from (A) coastal waters off northern Australia and (B) the North Atlantic Ocean. The solid line gives the relationship between the specific growth rate and intracellular Fe:C observed in iron-limited cultures of *Trichodesmium* based on the data in Fig. 2A. The dashed line gives the relationship between specific growth rate and the estimated total cellular iron for the same cultures. In both panels, open, closed, and gray symbols represent observations of cellular N:P below 40, above 40, and not determined, respectively. Symbols with enclosed cross-hatches are identified as putatively Fe limited, based on the observed N_2 fixation–Fe:C relationship.

the critical N:P is unknown for *Trichodesmium*, we make the following empirical observations. From the data shown in Fig. 5, the greatest ratio of explained to unexplained sum of squares was obtained by considering those observations with N:P above and below 35 mol:mol ($n = 6$ and 11 observations, respectively). Thus, the combined observations from both cruises can be described by two linear regressions

for observations with N:P ratios above and below 35 mol:mol. At N:P ratios above 35, most of the variability in N_2 fixation can be explained by the N:P ratio ($R^2 = 0.66$), and the negative relationship between the N:P ratio and N_2 fixation is significant ($\alpha < 0.05$), consistent with our earlier findings. Second, populations with N:P ratios less than 35 had fixation rates that increased with the N:P ratio. How-

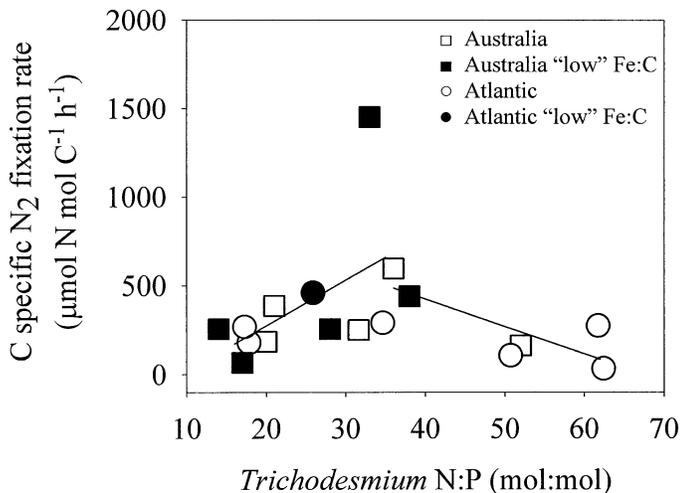


Fig. 5. N_2 fixation rates and cellular N:P ratios of coastal Australia and North Atlantic populations of *Trichodesmium*. Closed symbols indicate observations with putative Fe limitation, as shown by cross-hatched data points in Fig. 4. N_2 fixation rates of populations with N:P ratios below and above 35 mol:mol can be described by the linear equations (1) N_2 fixation = $25.6(N:P) - 236$ (below N:P 35; $R^2 = 0.25$) and (2) N_2 fixation = $-15.4(N:P) + 1,043$ (above N:P 35; $R_2 = 0.66$). These data suggest a "critical" cellular N:P greater than 35 mol:mol, probably between 40 and 50 mol:mol. A putative value of 40 mol:mol is chosen here to discuss the relative potential of P limitation in each region. Due to the lack of observations between 40 and 50 mol:mol N:P, a designation of the critical N:P as 50 mol:mol would be equally valid with these data.

ever, this positive relationship was not significant. In Fig. 5, we have made the distinction between putatively Fe-limited and Fe-replete populations from each ocean basin (as determined from the comparisons indicated in Fig. 4A and 4B).

Field iron addition experiments—Results of the field iron addition experiments varied with the Fe:C in the colonies and the dissolved iron concentration in the seawater. For the population collected on 6 November 1999, there were no differences among treatments in N_2 fixation rates measured at $t = 3$ d (Fig. 6A). The grand average for all treatments was $15 \text{ mol N mol Chl } a^{-1} \text{ h}^{-1}$. Variable to maximum fluorescence was measured among treatments during the first, second, and third nights of incubation (after 0.6, 1.5, and 2.5 d of incubation). There were no significant differences among treatments or among nights, and the grand average F_v/F_m was 0.557 ± 0.019 (\pm SE), suggesting that these populations remained viable in the bottles for the duration of the experiment. The ambient population from 6 November 1999 had a measured Fe:C ratio of $78 \pm 12 \text{ } \mu\text{mol mol}^{-1}$ (\pm SD), the highest value observed in this region from our ultraclean collections. Moreover, dissolved Fe at this station was 910 pmol L^{-1} , among the highest values observed on this cruise (Kustka et al. unpubl. data). Briefly, dissolved Fe samples were collected and measured as follows: 1 liter of filtered seawater was collected just prior to filling the 20-liter carboy for incubation water (see Methods). At the clean lab at Stony Brook, samples were acidified and extracted

using the method of Bruland et al. (1979) for subsequent analysis on a graphite furnace atomic absorption spectrophotometer. Blank extractions of lab Milli-Q yielded an average Fe concentration of 0.06 nmol L^{-1} (SD = 0.07 nmol L^{-1}).

For the *Trichodesmium* populations collected on 8 November 1999, there was no significant difference between N_2 fixation rates after 4 d incubation in the Fe and combined metal treatments. However, the average rates for these two treatments combined was about double that of the control (34 ± 10 versus $16 \pm 8 \text{ mol N mol Chl } a^{-1} \text{ h}^{-1}$). A one-tailed Student's t -test revealed this difference was significant ($p = 0.028$). Since less *Trichodesmium* biomass was available for this incubation, there were fewer F_v/F_m sampling events than in the first incubation. At $t = 1.5$ d, the F_v/F_m was not significantly different among treatments, and the grand average was 0.493 ± 0.008 . However, at $t = 3.5$ d, the F_v/F_m in control treatments (0.388 ± 0.049) showed a statistically significant decline compared to that in treatments with added Fe (0.488 ± 0.061 ; t -test, $p = 0.0002$). The ambient population had an Fe:C ratio of $21 \text{ } \mu\text{mol mol}^{-1}$, close to the lower limit observed for our Fe-limited *Trichodesmium* cultures (Fig. 4). Dissolved iron at this station (0.29 nmol L^{-1} , unpubl. data) was also substantially lower than that at the previous station.

In the two incubation experiments, measurements of F_v/F_m after 37 h incubation yield similar values for the 6 and 8 November 1999 populations (0.551 ± 0.06 and 0.499 ± 0.03 , respectively). Since 37 h was the earliest time point of F_v/F_m measurements common to both incubation experiments, this suggests both in situ populations were physiologically similar, and competent, after 1.5 d in the bottles. This observation has important implications (discussed below) for the interpretation of these incubation experiments.

Clean versus standard techniques for field sampling and incubation—Our shipboard collections of *Trichodesmium* yielded Fe:C ratios of 143 and $222 \text{ } \mu\text{mol mol}^{-1}$, respectively, for the 2 November and 6 November collections. These values exceed all our measured Fe:C ratios from ultraclean collection from a *Zodiac* skiff on this cruise (Table 2). This is not surprising, since we used the conventional oceanographic gear (see Methods), which may be expected as a source of Fe contamination. However, trace element clean collections can be achieved from shipboard collections when adequate precautions are taken (Collier and Edmond 1984).

For the 8 November experiment described above, a set of comparison incubations was performed with water collected from the ship, the traditional method formerly used in such incubations. We attempted to measure F_v/F_m during the fourth night of incubation in this experiment, which would have been followed by N_2 fixation measurements the following day. This would provide concurrent N_2 fixation measurements for comparison with measurements from the clean incubation treatments (Fig. 6C,D). However, we were only able to isolate a single colony from the 60 colonies originally placed in four Pyrex bottles. This colony was physiologically incompetent since it had an F_v/F_m of 0.027 (Fig. 6D). Therefore, under these conditions, colonies could not be main-

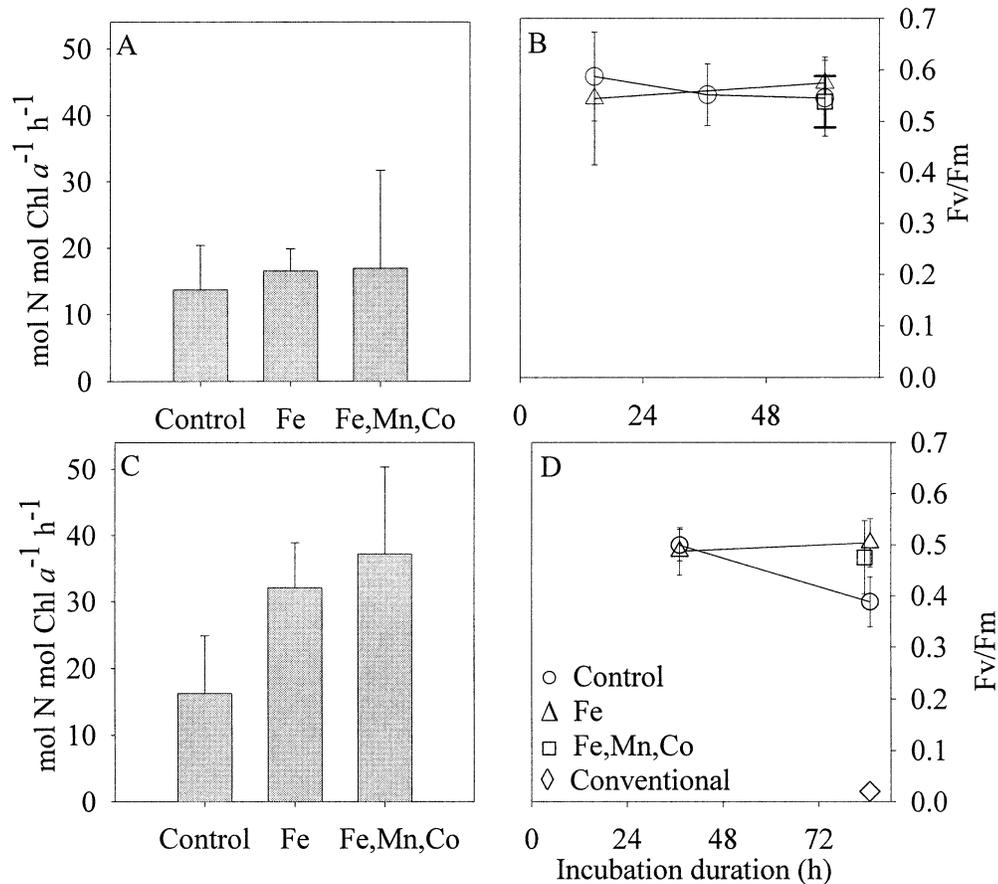


Fig. 6. Nitrogen fixation rate and nighttime variable to maximum fluorescence for incubation experiments with field populations of *Trichodesmium*. N_2 fixation measurements were made in control, Fe, and combined metal treatments during the (A) 6 November 1999 and (C) 8 November 1999 incubation experiments. Fluorescence measurements from the (B) 6 November and (D) 8 November experiments are also shown. Ambient populations from 6 November and 8 November had measured Fe:C ratios of 78 ± 13 and $21.4 \mu\text{mol mol}^{-1}$ (Table 2). Also in panel D, F_v/F_m measured from the conventional technique incubation experiment is plotted. N_2 fixation was assayed after 3.0 and 4.0 d, respectively, for the 6 and 8 November experiments. Bottles were measured for F_v/F_m at various times throughout the incubations; the last sampling occurred 12–15 h before N_2 fixation assays.

tained for this duration in either the control or Fe treatments. These results are consistent with anecdotal reports of the difficulty in maintaining field populations of *Trichodesmium* for extended periods.

Discussion

In *Trichodesmium* cultures, diazotrophy required 2.5-fold to fivefold higher cellular Fe:C ratios than needed for equivalent growth on ammonium (Fig. 2A), indicating a substantial increase in metabolic iron demand for N_2 fixation. The magnitude of this difference depends on the growth rate under consideration due to the combined effects of different maintenance Fe:C quotas and marginal iron use efficiencies for each mode of growth. We compared the daily marginal iron use efficiencies of *Trichodesmium* to predictions and empirical observations for other taxa and growth modalities. When appropriate, we scaled these other

data to reflect a 12-h daily light period for this comparison (Table 1). Metabolic biochemical models (Raven 1988; Raven et al. 1999) indicate that algae growing at saturating light on ammonium and nitrate at 20°C should have iron use efficiencies of 60,500 and 36,000 $\text{mol C mol Fe}^{-1} \text{ d}^{-1}$, respectively, for a 12-h daily photoperiod. The lower computed value for nitrate- versus ammonium-based growth results from additional iron needed for synthesis of nitrate and nitrite reductases (both iron-containing enzymes) and for synthesis of additional photosynthetic capacity to produce the ATP and reducing equivalents needed for nitrate reduction. Likewise, the large decrease in the experimentally measured iron use efficiency for diazotrophic versus ammonium-based growth in *Trichodesmium* (4,055 vs. 37,350 $\text{mol C mol Fe}^{-1} \text{ d}^{-1}$; Table 1) results from both direct iron requirements in the nitrogenase enzyme complex and the increased demand for reducing equivalents and ATP. The observed iron use efficiency for diazotrophic

Table 1. Predicted and empirical iron use efficiencies for phototrophic growth on various sources of N. For all growth modalities, the required Fe : C is derived from the empirical iron use efficiency (IUE) as well as the empirically determined maintenance Fe cost for growth, and is calculated for $\mu = 0.1 \text{ d}^{-1}$.

Species and N source	Marginal IUE*	Fe : C ($\mu = 0.1 \text{ d}^{-1}$)	Source
<i>Trichodesmium</i> (IMS 101), NH_4^+	37,000	8	These data
<i>Trichodesmium</i> (IMS 101), N_2	4,100	38	These data
Coastal diatoms, NO_3^-	42,000	5.4†	Sunda and Huntsman (1997)
Predicted for phototrophs, NH_4^+	61,000	n/a‡	Raven (1988)
Predicted for phototrophs, NO_3^- (generic PS stoichiometry)	36,000	n/a	Raven et al. (1999)
Predicted for phototrophs, N_2 (PSI:PSII=1:1, 48% Mehler activity)	3,800–7,700	28–40§	Kustka et al. (2003)

*All values are scaled to a 12 h photoperiod from the reported efficiencies and photoperiods.

†Value calculated for the composite coastal diatom, based on Eq. 1 and data in Sunda and Huntsman (1997).

‡Not applicable, as these models do not consider maintenance metabolic costs.

§Calculated according to Eq. 1 with the maintenance Fe : C of $13.5 \mu\text{mol} : \text{mol}^{-1}$.

growth in *Trichodesmium* is at the lower end of the range of 3,800–7,700 mol C mol $\text{Fe}^{-1} \text{ d}^{-1}$, as calculated for a diazotrophic phototroph growing with a 1:1 PSI to PSII ratio and 48% Mehler activity (Table 1). The range of predicted values is attributed to differences in maximum catalytic activity of nitrogenase, which varies depending on the species from which it was isolated.

For diazotrophy, we calculated that the structural iron bound in nitrogenase may range from 19% to 53% of the overall metabolic iron, with an additional 38% present within the photosynthetic apparatus. Thus, ~60%–90% of the intracellular iron can be accounted for in these Fe expensive metabolic processes. Much of the remainder of the intracellular iron in N_2 -fixing *Trichodesmium* may be required to support increased respiratory activity and/or an increase in Fe-containing antioxidant molecules. Nitrogenase activity is readily inhibited by molecular oxygen, and high rates of respiration may be needed to prevent oxygen toxicity. This is a common strategy for nonheterocystous daytime N_2 fixers (Gallon 1992) and is suggested in *Trichodesmium* by the colocalization of cytochrome oxidase and nitrogenase among cells within colonies in this genus (Bergman et al. 1993). Iron-containing antioxidant enzymes may protect nitrogenase from reactive oxygen species, as suggested by Puppo and Rigaud (1986), and, therefore, may also contribute to an elevated diazotrophic iron cost. Another Fe cost under diazotrophy is that related to the bioenergetically expensive daily cycle of nitrogenase synthesis and destruction. In *Trichodesmium*, the Fe protein is degraded shortly after dark and resynthesized in the predawn hours with concomitant variation in rates of acetylene reduction (Capone et al. 1990; Wyman et al. 1996). Our understanding of the bioenergetic costs of amino acid degradation and synthesis is only recently coming into focus. The ATP cost of polypeptide breakdown and resynthesis is estimated to be in the range of 11–21 ATP per amino acid residue, depending on the assumptions about the mechanism of protein breakdown and the costs of “tool maintenance” and error detection and correction in protein synthesis (Raven et al. 2000).

The marginal iron use efficiency for ammonium-based growth in *Trichodesmium* (37,350 mol C mol $\text{Fe}^{-1} \text{ d}^{-1}$) is

close to the measured value (42,300 mol C mol $\text{Fe}^{-1} \text{ d}^{-1}$) observed for growth of coastal diatoms and dinoflagellates on nitrate at 20°C and saturating light (Sunda and Huntsman 1995). Molecular models and empirical data (*see Results section*) indicate that the majority of the metabolic Fe in cells grown on ammonium is Fe bound within functional components of the photosynthetic apparatus. Our values of the IUE are not tightly constrained, due to the relatively low number of observations within the region of Fe limitation for each mode of growth. Despite this, the observed values obtained from our lab data are in general agreement with previous predictions of the IUE for NH_4^+ - and N_2 -supported growth (Raven 1988; Kustka et al. 2003). Perhaps the most meaningful comparisons to emphasize the differences in the Fe cost for net growth under NH_4^+ and N_2 (i.e., outside of the context of discussions of catalyst stoichiometry) are of the Fe : C quotas interpolated at $\mu = 0.1 \text{ d}^{-1}$, as well as those extrapolated for the maintenance requirement. Thus, these data suggest diazotrophy requires 2.5-fold to fivefold more Fe.

Cells grown on N_2 had greater iron requirements for growth (Fig. 2A) but also had greater iron uptake rates at a given total iron concentration (Fig. 3B). These results are analogous to the elevated iron requirements and elevated iron uptake rates in NO_3^- (vs. NH_4^+) supported diatoms (Maldonado and Price 1996). The cellular iron uptake rate in diazotrophic cultures was proportional to the iron concentration in the medium, indicating no saturation or down regulation of the Fe uptake system at high Fe concentrations. At dissolved inorganic iron concentrations (Fe') above 0.75 nmol L^{-1} , iron hydroxides should precipitate and, thus, any additional iron would not increase $[\text{Fe}']$ or $[\text{Fe-EDTA}]$ but would only increase the concentration of iron hydroxides, based on earlier results (Sunda and Huntsman 1995). Continued uptake of iron within this region suggests that the cells are able to take up the iron from these hydroxides. This may occur via bioreduction of inorganic iron species, although some evidence suggests that *Trichodesmium* does not have this capacity (Moffett, Barbeau, Waterbury, and Averyt unpubl.).

Trichodesmium was able to take up and store substantial

Table 2. Fe:C:N:P for *Trichodesmium* from the north coast of Australia. All samples collected in November 1999; date indicates day of month. Paired samples for Fe:P and C:N were collected (*see text*) in duplicate in most cases, except on 17 November 1999 ($n = 3$) and where no standard deviation is reported ($n = 1$).

Date	Latitude collections	Longitude	Fe:C (SD) ($\mu\text{mol}:\text{mol}$)	Fe:P (SD) ($\text{mmol}:\text{mol}$)	C:P (SD) ($\text{mol}:\text{mol}$)	N:P (SD) ($\text{mol}:\text{mol}$)
<i>Zodiac</i>						
1	11°12.75'S	139°19.47'E	67.5 (5.0)	1.06 (0.09)	158 (25)	21 (7)
2	10°32.73'S	136°34.15'E	29.6 (2.1)	0.69 (0.07)	235 (7)	33 (1)
5	11°49.82'S	128°42.64'E	28 (11)	0.51	253	38
6	12°49.22'S	125°53.54'E	78 (13)	1.58 (0.19)	203 (8)	31.6 (0.5)
8	14°06.22'S	123°09.46'E	21.4	0.23	105	14
12	14°47.22'S	122°10.31'E	27.9	0.32	115	20
13	13°33.57'S	124°31.13'E	18.5	0.14	79	17
14	12°43.43'S	127°33.64'E	18.0 (3.4)	0.29 (0.06)	159 (2)	28 (1)
17	12°04.65'S	128°56.72'E	24.0 (4.1)	0.74 (0.13)	310 (20)	52 (7)
19	9°49.60'S	130°06.69'E	40.0 (8.5)	0.79	232	36
		Mean (SE)	35 (21)	0.64 (0.44)	185 (74)	29 (12)
<i>R/V Ewing</i> collections						
2	10°32.73'S	136°34.15'E	143 (82)			
6	12°49.22'S	125°53.54'E	222			

quantities of cellular iron in excess of that needed to support maximum growth (Figs. 2B, 3A). This ability for luxury uptake may be an important adaptive strategy for *Trichodesmium* in near-surface oceanic waters, where iron inputs are often sporadic. The majority of the Fe flux to the open ocean can be supplied by aeolian dust deposition (i.e., calculated as 95% at a station in the N. Pacific Gyre; Martin and Gordon 1988), which is highly seasonal in the North Pacific and North Atlantic Oceans. Superposed on this broad seasonality are episodic dust deposition events. For example, at Midway Island, about 50% of the annual deposition of iron to the mixed layer occurred during a 3-week period (Uematsu et al. 1985). In contrast, the flux of iron due to upwelling in high nutrient low chlorophyll (HNLC) areas is believed to be less sporadic. In oligotrophic waters, luxury uptake of Fe may confer a selective advantage to *Trichodesmium* to help offset the high Fe demand of diazotrophy.

All of the Fe:C ratios measured from the north coast of Australia were at least $18 \mu\text{mol mol}^{-1}$ (Table 2), in concordance with our diazotrophic culture data showing a maintenance total cellular Fe:C of $\sim 16 \mu\text{mol mol}^{-1}$ (Fig. 4). This quantity is estimated from the intracellular iron value divided by the ratio of intracellular to total cellular iron (i.e., $13.5 \mu\text{mol}:\text{mol}$ divided by 0.83). About half of the populations sampled on this cruise had Fe:C contents below the $38 \mu\text{mol mol}^{-1}$ Fe:C required for a moderately Fe-limited diazotrophic growth rate in cultures of 0.1 d^{-1} (Fig. 4). A comparison of the growth rate versus Fe:C relationship measured in our diazotrophic cultures with the observed relationship between N_2 fixation rates and colony Fe:C in field populations suggests that iron may be limiting in these populations. Some in situ *Trichodesmium* Fe:C values exceed those required by cultures to attain identical rates of N_2 fixation. This observation has at least two possible explanations. The field populations may have stored excess Fe and were not limited by this nutrient at the time of collection. The Fe:C ratio is regulated by the balance between iron

uptake and growth, and, thus, populations can achieve high Fe:C ratios during periods of low specific growth rate due to other limiting factors. This additional iron can then be used to support diazotrophy once other limitations subside. Alternately, the extracellular contributions of Fe toward the field Fe:C contents are unknown, suggesting that there may be physiological limitation despite the apparent excess of measured Fe.

The Fe:C ratios in *Trichodesmium* reported here are considerably lower than previous reports for this region (Berman-Frank et al. 2001). Although we cannot offer a conclusive explanation for these differences, the higher previously measured values may have resulted from iron contamination, a potential problem in any iron analyses of natural marine samples (Martin and Knauer 1973; Collier and Edmond 1984). The clean techniques we used should have decreased, and hopefully eliminated, contamination by Fe. The results from our shipboard collections (during which we did not employ any special precautions, except that colonies were picked in the clean hood and digested in our clean lab) support this supposition.

There is a general consistency between the effects of added iron in our field incubation experiments and the measured Fe:C of natural populations. The enhancement of N_2 fixation in Fe-amended treatments (Fig. 6C) relative to controls from the 8 November 1999 incubations is consistent with the low *Trichodesmium* Fe:C ratio (Table 2) and dissolved Fe at this station. The lack of an iron effect in the 6 November 1999 incubations (Fig. 6A) is also consistent with the high colony Fe:C content and dissolved Fe at this station. However, these incubation results do not prove iron limitation in the ambient 8 November population. First, F_v/F_m at $t = 1.5 \text{ d}$ was not significantly different among treatments; only after an additional 2 d did the control (no Fe added) incubations show a decline in F_v/F_m (Fig. 6D). These results could suggest that iron was not initially limiting in the control but rather became limiting with time

due to Fe adsorption to vessel walls or to increased biological uptake with exclusion of grazers from the bottles. Potential difficulties in interpreting data from iron addition experiments in bottles are well known from extensive work in HNLC areas. These problems may be exacerbated in a slow-growing phytoplankton such as *Trichodesmium*. With this in mind, the need for molecular probes of iron status of in situ populations to complement these approaches cannot be understated.

Diazotrophic growth in *Trichodesmium* cultures required 2.5 to 5 times more intracellular iron than growth on ammonium. Based on molecular models, the relatively high cellular iron requirements could be accounted for largely by the metabolic iron present in nitrogenase complexes and the iron needed for increased photosynthetic capacity to support the high energetic cost of N_2 fixation. About half the populations from the north coast of Australia have Fe:C values suggestive of Fe limitation of N_2 fixation rates. It is important to understand the distinction between the concepts of Fe limitation of N_2 fixation/growth rates and of limitation of achieved biomass. We have employed the former approach in our discussion of the potential for biomass-specific N_2 fixation rates of field populations. This should not be confused with discussions of "limitation" of biomass or sea surface area-integrated N_2 fixation rates, which can be fraught with additional uncertainties.

This study represents an attempt to understand the Fe demand for diazotrophy in laboratory-cultured *Trichodesmium* and to compare such findings to observations of field populations. However, phosphorus has received increasing attention over the last few years as a potentially limiting nutrient for *Trichodesmium* (Wu et al. 2000; Sañudo-Wilhelmy et al. 2001), since a lack of P has been long known to limit freshwater diazotrophs in Fe-rich lakes. With our auxiliary data on the C:N:P content of *Trichodesmium* and published data from other regions, we can hypothesize on the relative likelihood of P versus Fe limitation off the north coast of Australia. Most of the observed N:P quotas were above the Redfield ratio of 16 (Table 2), but it is difficult to interpret ratios such as these in terms of the potential for P limitation without an appropriate critical N:P above which P limitation can be inferred. However, our empirical observations suggest a critical N:P somewhere around 40 mol: mol. If this was the case, it would suggest that N_2 fixation in about half the populations measured from the Atlantic cruise transect (Sañudo-Wilhelmy et al. 2001) were indeed limited by phosphorus. Similarly, with this conditional value for the critical N:P, only one population on the Australia transect had an N:P that may be considered P limited.

The empirical observations shown in Fig. 5 agree with the calculations reported by Geider and LaRoche (2002). These authors compiled empirical C:N:P data from lab cultures of other phytoplankton under P satiation and limitation. They also provided theoretical estimates of the range of cellular P requirements among microalgae based on the C:N:P stoichiometries and relative proportions of N- and P-rich biomolecules. They concluded that the critical N:P ratio for a given species is most likely between 20 and 50 (notably above Redfield), with the calculated critical ratio for a given species dependent on the biomol-

ecule stoichiometry. Species with high protein content (as can be expected for diazotrophic cyanobacteria) and those with relatively slow growth rates (as observed in *Trichodesmium*, Carpenter 1983) may be expected to have a critical N:P toward the higher end of this range (Geider and LaRoche 2002), consistent with our empirical observations of a significant negative relationship between N:P and N_2 fixation rates at $N:P > 35$ (Table 2; Fig. 5).

Geider and LaRoche (2002) calculate that marine microalgae may have an expected range of critical N:P ratios from 20 to 50. This implies that *Trichodesmium* populations with $N:P < 20$ (and probably those with $N:P$ below some greater value, or below the actual critical N:P for *Trichodesmium*) are participating in luxury uptake and subsequent storage of P. Therefore, putative luxury uptake of P would be parsimonious with low, resource-limited N_2 fixation rates (and thus low biodilution rates) observed at N:P ratios below the critical N:P. This is a logical extension of our discussion of luxury uptake of Fe during periods of low growth rate. However, if this phenomenon of luxury uptake of P exists under Fe limitation, it would seem to explain only a portion of the empirical observations presented here. First, while it is true that the other limiting nutrient in *some* of these near-Redfield N:P populations appeared to be Fe, as identified by the data plotted in Figs. 4A and 5, there is no clear distinction between the N:P ratios of the putative Fe-limited and Fe-replete populations. Moreover, the slope of N_2 fixation versus N:P ratio at low N:P (Fig. 5) was not significantly different from a slope of zero. A more detailed study is necessary to adequately define the relationships between luxury uptake of P, cellular N:P ratios, and resource-limited growth.

This study has provided a benchmark for the relationship between Fe:C quotas and iron-limited growth rates for lab cultures of *Trichodesmium*. With the assumption that field Fe:C requirements are similar to those of diazotrophic lab populations, the potential for Fe limitation in field populations from the north coast of Australia and the Atlantic Ocean has been estimated. These results are generally consistent with our incubation results from Australian field populations. That said, it is important to recognize that employing this simple cell quota approach has identified only a fraction of field populations as being either putatively Fe or P limited. In several of the populations from each study region, neither nutrient appears to be limiting (despite low C-specific N_2 fixation rates). Given the myriad of complicating issues surrounding attempts to assess nutrient limitation in field populations, the task may be simplified by including molecular approaches in our arsenal of geochemical and physiological tools. For example, a promising probe for iron limitation has been developed and tested in cultures of three open ocean cyanobacteria, including *Trichodesmium* (Webb et al. 2001). The IdiA protein, involved in iron scavenging in the freshwater cyanobacterium *Synechococcus* PCC 6801, is expressed in high quantities under iron limitation in cultures of *Crocosphaera* spp., *Trichodesmium*, and *Synechococcus* WH7803. In addition, within 3 d of iron addition to iron-stressed cultures of *Synechococcus* WH7803, cellular IdiA levels decreased significantly. This should be a useful tool to assess the iron

status of ambient populations and perhaps in field iron addition experiments as well. Coupling a diagnostic indicator such as this with measurements of cellular iron quota and growth processes should help us further refine our quantitative understanding of the biochemical control of Fe on C-specific rates of nitrogen fixation in various oceanographic regimes.

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