



ELSEVIER

Research in Microbiology 153 (2002) 255–262

Research in
Microbiology
Established in 1887 as the *Annales de l'Institut Pasteur*

www.elsevier.com/locate/resmic

Mini-review

Iron and marine nitrogen fixation: progress and future directions

Adam Kustka^{a,*}, Edward J. Carpenter^b, Sergio A. Sañudo-Wilhelmy^a

^a Marine Sciences Research Center, State University of New York at Stony Brook, Stony Brook, NY 11794-5000, USA

^b Romberg Tiburon Center, San Francisco State University, 3152 Paradise Dr., Tiburon, CA 94920, USA

Received 11 February 2002; accepted 22 April 2002

First published online 14 May 2002

Abstract

A synthesis of the current understanding of potential iron limitation of pelagic nitrogen fixation is given, considering biochemical bases of Fe requirements and empirical observations of growth and Fe quotas of cultures and field populations of *Trichodesmium*. The potential for iron limitation of heterotrophic diazotrophy in the marine environment is also evaluated. © 2002 Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

Keywords: Nitrogen fixation; Iron metabolism; Growth efficiency; Cyanobacteria; Marine bacteria

1. Introduction

Biological nitrogen fixation serves to relieve nitrogen stress to phytoplankton assemblages in vast regions of the ocean where inorganic nitrogen species are depleted. Based on an analysis of the World Ocean Atlas 1998 database, 47% of the surface waters of the World Ocean are nitrate-depleted ($< 1 \mu\text{M}$) and have suitably warm surface temperatures ($> 20^\circ\text{C}$) for N_2 fixation by *Trichodesmium* during summer months [J.K. Moore, pers. comm]. As a result, moderate increases in primary productivity due to N_2 fixation by *Trichodesmium* (or other diazotrophs) in these regions may increase the oceanic sequestration of C and have a pronounced effect on global climate [3,9].

Research on nitrogen fixation in the open ocean had its beginnings in 1961, when Dugdale and co-workers [7] suggested that the warm water colonial cyanobacterium *Trichodesmium* spp. could fix dinitrogen. Although these results were initially viewed with some skepticism, there is now evidence that pelagic nitrogen fixation contributes $30 - 140 \times 10^{11}$ g N per annum to the biosphere [10] representing about 10–40% of nitrogen fixed from all sources. On the other hand, despite the selective advantage of the de novo synthesis of biologically available nitrogen in a nitrate-poor environment, it is enigmatic that *Trichodesmium* species do

not have a more widespread distribution among oligotrophic regions.

Our understanding of marine nitrogen fixation is rapidly progressing due to advances in three interrelated research areas. These include studies geared towards understanding the biochemical stoichiometry of diazotrophic growth, possible nutrient limitation of N_2 fixation by field populations of *Trichodesmium*, and the phylogenetic and metabolic diversity of organisms responsible for N_2 fixation. Here we limit our discussion of these three areas of research to the context of the trace nutrient iron. A more comprehensive review of marine nitrogen fixation is found elsewhere [14].

Iron is the most important potentially limiting nutrient metal for photoautotrophic growth in the marine environment. Although iron is quite insoluble in oxic seawater in the modern ocean, it is a required co-factor in the majority of photosynthetic and respiratory redox enzymes. As a result, in large regions of the ocean, the cellular iron demand to support optimal growth rates of autotrophic phytoplankton may exceed the supply. Diazotrophy imposes additional iron demands for growth beyond those attributed to photosynthesis and respiration.

2. Biochemistry of N_2 fixation

Despite the inferred iron limitation for diazotrophic growth in oceanic populations of *Trichodesmium* [9], the biochemical bases for such possible limitation have received

* Correspondence and reprints.

E-mail address: akustka@yahoo.com (A. Kustka).

surprisingly little attention. Some portion of the iron cost appears to be related to phylogeny rather than the mode of nitrogen nutrition, as nitrate grown cyanobacteria generally require greater Fe:C quotas than eukaryotic phytoplankton [2]. Diazotrophy imposes an additional Fe demand for growth, as NO_3^- or NH_4^+ grown diazotrophs (presumably downregulating nitrogenase) require less iron in culture media than those grown on N_2 [15,18].

A revised estimate of the iron cost of nitrogen fixation, with specific reference to *Trichodesmium*, was recently constructed to estimate the iron demand as a function of growth rate in field populations [17]. These calculations apply the logic of an earlier effort [25] to estimate the Fe demands for biochemical catalysts that can be related to cell growth rates in a meaningful way, such as those directly involved in biosynthesis and catabolism. In this regard, Fe costs due to photosynthetic, diazotrophic and respiratory metabolism were calculated based on the assumption that the electron transport chains operate at maximal reaction rates, as modified by diel variation in activity. To compute the Fe costs associated with processes that have a poorly understood relationship with growth rates, empirical observations were evaluated.

Specifically, these calculations considered the iron content and maximum reaction rates of the nitrogenase complex (derived from model heterotroph systems) and estimates or direct measurements of biochemical parameters from field populations of *Trichodesmium*. Empirical measurements used in the calculations included rates of Mehler activity (relative to linear photophosphorylation), N_2 fixation (acetylene reduction) activity over the diel cycle, and the superoxide dismutase (SOD) activity of crude cell extracts. All these parameters were measured in field populations of *Trichodesmium*. Photosystem I (PSI) to PSII ratios were estimated based on literature values for other cyanobacteria, and a range of values was considered.

From these data, the required ‘moles’ of iron containing catalysts for linear photophosphorylation, N_2 fixation and respiration to support diazotrophic growth were calculated. The resulting ‘iron use efficiency’ for diazotrophic growth was expressed as the marginal capacity for increased growth with marginal increases in the Fe:C quota. To calculate the required Fe at a given growth rate, the iron use efficiency was considered in conjunction with the ‘maintenance’ Fe quota of $13.5 \mu\text{mol}:\text{mol}$ (Fig. 1). This Fe quota is extrapolated to a net growth rate of zero, based on results from iron-limited diazotrophic culture experiments [18]. Fig. 1 indicates the estimated Fe cost to *Trichodesmium* at a net growth rate of 0.1 d^{-1} , partitioned among iron-requiring processes. In Fig. 1a, data from the most ‘Fe efficient’ heterotrophic nitrogenase preparation (Table 1 in Ref. [17]) was used in conjunction with a moderately high PSI:PSII ratio of 4:1 and 48% Mehler activity. Therefore, this depicts the lowest expected iron cost due to nitrogenase, relative to that for photosynthetic units. This lower relative estimate indicates that nitrogenase is responsible for a significant

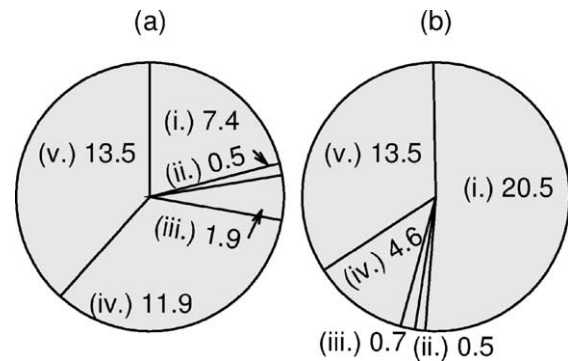


Fig. 1. Semi-empirical estimate of the iron requirement for field populations of *Trichodesmium* spp. with a net diazotrophic growth rate of 0.1 d^{-1} . The Fe:C cost for each process, shown as $\mu\text{mol}:\text{mol}$ Fe:C, was determined for two scenarios with variable PSI:PSII ratios and Fe efficiencies of the nitrogenase complex. 48% Mehler activity was assumed for each scenario [17]. (a) Calculations assuming a PSI:PSII ratio of 4:1 and the most Fe-efficient N_2 ase measured, reflecting the minimal relative Fe demand of N_2 ase. (b) Assuming a PSI:PSII ratio of 1:1 and the least efficient N_2 ase measured. (i) ‘Structural’ Fe associated with the nitrogenase complex. (ii) Fe required for respiration of C to fuel N_2 fixation. (iii) Fe required to fix C which is subsequently respired to fuel N_2 fixation. (iv) Fe required for C fixation, not directly related to N_2 fixation. (v) The maintenance Fe:C found in diazotrophic culture experiments with *Trichodesmium* [18].

Table 1

Estimates of the Fe costs for diazotrophic growth in heterotrophic bacteria

Enzyme or process	Minimum iron cost
Respiration [25]	$0.244 \text{ mol Fe} \cdot \text{mol C fixed}^{-1} \cdot \text{s}$
N_2 ase activity [17]	$10.7\text{--}29.4 \text{ mol Fe} \cdot \text{mol N fixed}^{-1} \cdot \text{s}$
Respiration for N_2 fixation [25]	$0.244 \text{ mol Fe} \cdot \text{mol C fixed}^{-1} \cdot \text{s}$
<i>Other parameters</i>	
C:N of marine heterotrophic bacteria ^a	3.99:1
Bacterial growth efficiency ^a	36%
Net growth rate	0.06 d^{-1}
Calculated minimum Fe:C requirements ($\mu\text{mol}:\text{mol}$) ^b	
Heterotrophy	0.30
Heterotrophic diazotrophy	2.5–5.7
Phototrophic diazotrophy	11–18
Phototrophic diazotrophy ^c	28

^a The bacterial growth efficiency and C:N ratio are grand average values for iron replete cultures of marine bacteria [33]. ^b These data are based on the calculations discussed in text, and are the marginal iron requirements for $\mu = 0.06 \text{ d}^{-1}$. ^c This value interpolated at $\mu = 0.06 \text{ d}^{-1}$ from diazotrophic culture work with *Trichodesmium* [18], and unlike other values reported here, inherently includes maintenance Fe:C costs of $13.5 \mu\text{mol}:\text{mol}$.

portion (at least 42%) of the marginal cellular iron burden. In addition, the maintenance iron represents a large portion of the total required iron for diazotrophic growth at 0.1 d^{-1} . The composition of this pool is poorly understood, but some fraction must be due to biosynthetic catalysts, and 3 of the $13.5 \mu\text{mol}:\text{mol}$ Fe:C may be due to Fe-SOD [17]. A minimal Fe-SOD quota of $3 \mu\text{mol Fe}:\text{mol C}$ would represent a significant iron cost to *Trichodesmium* field populations. This estimate of Fe in Fe-SOD alone exceeds the whole organism iron requirement for growth in the open ocean

diatom *Thalassiosira oceanica* by 50% [31]. Observations of elevated Fe-SOD quotas in *Trichodesmium* are consistent with results from other diazotrophs, and lends support to the hypothesis that Fe-SOD may protect nitrogenase in vivo, as “O₂” susceptibility of nitrogenase may be related to superoxide produced from O₂ [24].

These semi-empirical calculations and empirical observations are useful in understanding the biochemical bases for potential iron limitation of nitrogen fixation by *Trichodesmium*, but also make it clear that there is a dearth of specific biochemical in vivo data on iron and nitrogenase in marine diazotrophs. To our knowledge, the specific reaction rates of the nitrogenase complex are not known for any cyanobacterium, yet this information is the cornerstone of biochemical use efficiency calculations. Current models are based on in vitro maximum specific reaction rates observed in purified proteins prepared from heterotrophic diazotrophs, yet it is the in vivo specific reaction rates which determine the actualized enzyme-specific iron use efficiency. In addition, a 5:1 ratio of Fe to MoFe proteins is required for maximum MoFe-specific reaction rates in vitro [8], but an empirical, in vivo ratio of 3:1 is observed in the freshwater cyanobacterium *Gloeotheca* ATCC 27152 [26]. Since the absolute levels of proteins and achieved reaction rates were not quantified in this study (or in any study of cyanobacteria to our knowledge), heterotrophic data must continue to serve as approximations of in vivo specific reaction rates of nitrogenase in cyanobacteria. From a biochemical perspective, there is significant room for advancement towards understanding the bioenergetic bases for the increased iron costs of diazotrophy.

To date, two groups have quantitatively evaluated the Fe cost of diazotrophic growth in *Trichodesmium* by measuring the iron-limited growth response of cultures of *Trichodesmium* (IMS 101) as a function of the cellular iron quota [1,18]. In general, these groups made similar findings, as 38–48 $\mu\text{mol}:\text{mol}$ Fe:C were required to achieve a moderately iron-limited carbon specific, diazotrophic growth rate of 0.1 d^{-1} . Instantaneous N₂ fixation rates were dependent upon Fe:C within the range of iron limitation (Fig. 2a), beyond which luxury uptake of Fe did not increase fixation rates. Similarly, net carbon-specific growth rates were dependent upon Fe:C (Fig. 2b). Under ammonium-replete conditions, only 8 $\mu\text{mol}:\text{mol}$ Fe:C was required at a growth rate of 0.1 d^{-1} , a 5-fold reduction in cellular iron demand (Fig. 2b; [18]). Ammonium supported growth required about half the cellular investment in chl a as growth on N₂, consistent with the expectation that diazotrophic growth requires iron for additional photosynthetic units as well as for nitrogenase complexes.

3. Field populations

How do the relationships between growth and iron quotas observed in iron-limited cultures or predicted by the

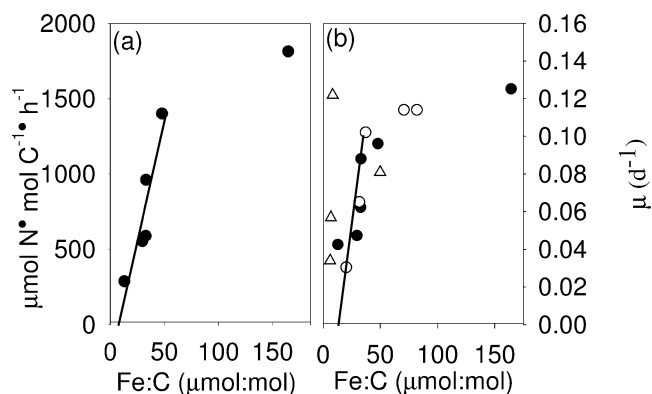


Fig. 2. Carbon-specific N₂ fixation rates and net growth versus cellular Fe:C content. (a) Instantaneous N₂ fixation rates from cultures of *Trichodesmium* [1], shown here as C-specific rates from reported C:chl a ratios and chl a specific rates. The solid line depicts the linear relationship between Fe:C and Fe-limited N₂ fixation. (b) Net growth and Fe:C for N₂ supported (closed circles [1], open circles [18]) and NH₄⁺ supported (open triangles [18]) cultures of *Trichodesmium*. The line indicates the predicted Fe-growth relationship for diazotrophy, as in Fig. 1a.

iron cost model compare to the iron status of field populations? Oceanographic approaches to assess iron limitation of phytoplankton growth have included assessments of cellular iron quotas and growth rates of ambient populations, as well as evaluation of physiological responses and geochemical parameters during iron addition experiments. Elemental (C:N:Fe) quotas have been measured in a few *Trichodesmium* field populations (Fig. 3). These data suggest a wide range of iron contents (from 20 to > 500 $\mu\text{mol}:\text{mol}$ Fe:C; Fig. 3) both within and among regions. Many of these values exceed the Fe requirements for near maximal growth of laboratory populations (38–48 $\mu\text{mol}:\text{mol}$ Fe:C). However, it is difficult to interpret these observations without knowledge of in situ N₂ fixation or growth rates. Therefore, we compiled the published data on Fe:C content and C specific N₂ fixation rates of field populations of *Trichodesmium* (Fig. 4). The first available data set, from Caribbean populations [28], apparently corroborated earlier speculations about elevated iron requirements for diazotrophic growth of *Trichodesmium*. Indeed, the relationship for these populations loosely resembles the Droop equation for nutrient-limited algal growth [6]. Subsequent observations of similar nitrogen fixation rates at lower Fe:C contents for North Atlantic [29] and the Bermuda-Atlantic Time Series (BATS) station [21] populations show the earlier Fe:C contents may not necessarily reflect a high demand. Under steady state conditions, the relationship between Fe:C and growth rate is described by equation (1). If factors other than iron are growth-limiting, low growth rates combined with iron uptake in excess of growth requirements may result in high cellular Fe:C quotas.

$$\text{Fe} : \text{C} = V_{\text{ss}} \cdot \mu \quad (1)$$

In Fig. 4, the regression of iron-limited N₂ fixation against the cellular Fe quotas in cultures (arrow, as in Fig. 2a) suggests most of the in situ iron quotas are in excess of

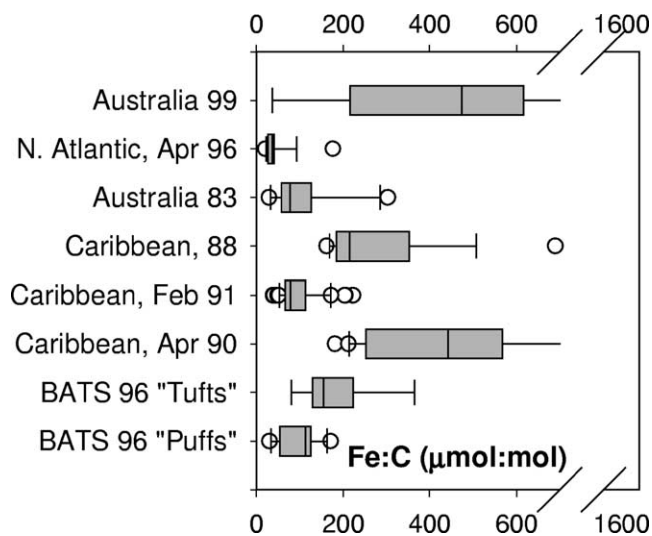


Fig. 3. Summary of *Trichodesmium* Fe:C from various oceanographic regimes. Australia 1983 *Trichodesmium* data originally reported as $\mu\text{g/gdw}$ [13], and we excluded an observation of Fe = 5% of *Trichodesmium* by weight. To derive Fe:C, we assumed 1 gdw cyanobacteria = 0.45 g C. Australia 1999 [1] ("picked" colonies), BATS 1996 [21], and N. Atlantic 1996 [29] Fe:C were determined directly. Caribbean 1988 [28] data reported as protein N:colony and Fe:colony, and Caribbean 1991 and 1990 [21] data as N:colony and Fe:colony. We assumed protein N = 0.71 total N and a *Trichodesmium* C:N of 6.2:1.

those required by cultures to attain identical rates of N_2 fixation. Comparing the field results to the culture and model data suggests at least three possibilities. One possibility is that field populations in these study areas are not limited by iron. Alternatively, the iron cost for growth is greater in field populations than anticipated by models or actualized under steady state laboratory conditions. It is also possible that some fraction of field "quotas" may reflect extracellular iron scavenged onto cell surfaces. In this regard, there is a need to discriminate between extracellular bio-unavailable Fe and cellular iron to adequately understand the relationships between cellular iron and N_2 fixation rates of field populations.

Some observations of *Trichodesmium* responses to natural iron input events, as well as results from planned iron incubation experiments, suggest field populations may be Fe limited. The ocean-basin scale correlation between *Trichodesmium* abundance and aeolian dust flux has been advanced as an argument for a relatively high Fe demand for *Trichodesmium*. Aeolian dust supplies most of the iron to oligotrophic open ocean regions removed from coastal input processes [12]. Two time series studies have since evaluated this suspected relationship. In many regards, these can be viewed as "natural" iron addition experiments. One such study on the continental shelf of the west coast of Florida revealed that the input of aeolian dust from Africa was followed by an increase in water column dissolved iron concentrations and a massive *Trichodesmium* bloom [19]. Similarly, bloom concentrations of *Trichodesmium* developed at the Bermuda-Atlantic Time Series station after dust in-

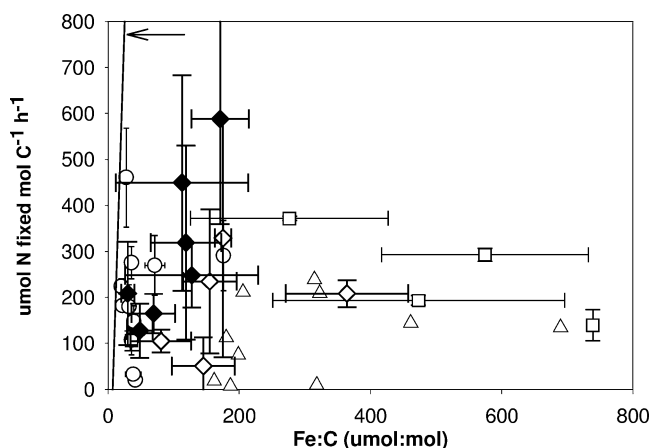


Fig. 4. N_2 fixation and Fe:C from field populations of *Trichodesmium*. Fe:C ratios were calculated as in Fig. 3. C-specific N_2 fixation rates calculated from original references or (for [1]) from rates and C · colony⁻¹ provided by D. Capone. Data are from North Atlantic (open circles, [29]), Australia (open squares, [1]), Caribbean Sea 1988 (open triangles, [28]), "puff" (closed diamonds) and "tuft" colonies (open diamonds) from BATS [21]. A 4:1 ratio of acetylene reduction: N_2 fixation was assumed. Arrow denotes Fe requirement in cultures (see text).

put events [21]. Data from this latter study reveal that the biomass-specific rates of nitrogen fixation increased with Fe:C content of colonies of the "puff" morphotype (Fig. 4; $r^2 = 0.68$, regression not shown). The increase in "puff" Fe:C quotas and N_2 fixation rates coincided with increased atmospheric dust concentrations, which started around Julian day 190 (Fig. 5a; see [21]). Tuft colonies were not sampled before the arrival of atmospheric dust. The Fe:C contents of tufts are statistically indistinguishable from that of puffs from 4 of 5 sampling events. Curiously, about 90% of the *Trichodesmium* biomass at the Hawaii Ocean Time series station (HOT) exists as free filaments [14], which might be expected to have greater carbon specific iron uptake rates, due to an increased exchangeable cell surface area:volume ratio. Nothing is known about the iron uptake or quotas of free filaments.

Iron incubation experiments provide another approach to assessing iron limitation of field populations. The classical hypothesis that Fe limits growth of (non-diazotrophic) phytoplankton in macronutrient-rich upwelling regions of the ocean (so called HNLC, or high nutrient low chlorophyll, regions) was formulated in the 1930s by Gran [5,12]. However, it was not until the 1980s that the Fe hypothesis was adequately tested; with the advent of clean techniques, Martin and Fitzwater provided the first credible evidence of iron limitation using bottle incubation experiments at sea. Martin's group and others subsequently demonstrated iron limitation of phytoplankton growth in several HNLC areas [5,12], yet considerably less work has been done in oligotrophic regions suitable for N_2 fixation.

Incubation experiments with populations of *Trichodesmium* isolated from offshore waters of Barbados have shown iron-induced stimulation of N_2 fixation rates [27]. In these experiments, micromolar iron additions were required to

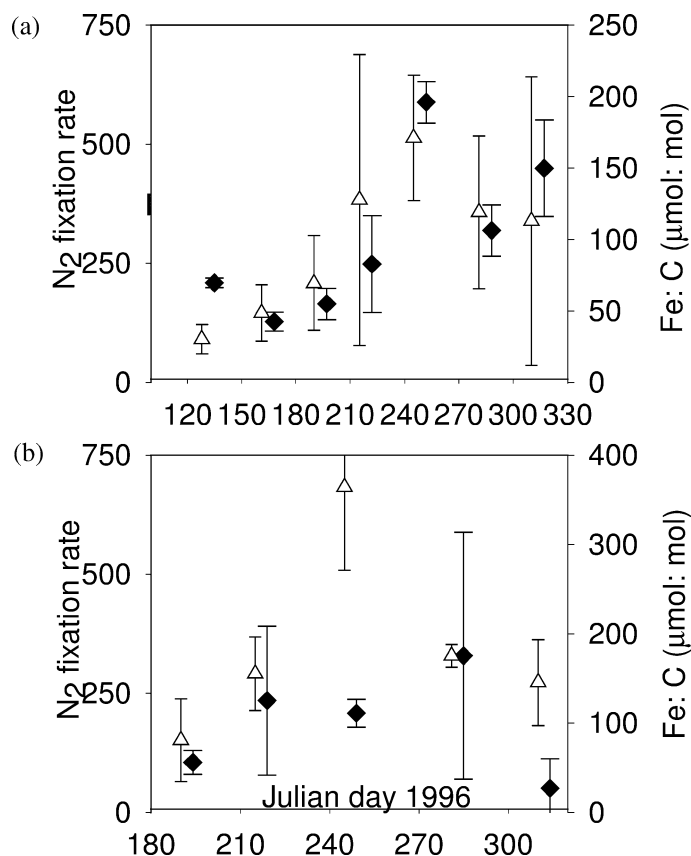


Fig. 5. Seasonal progression of N_2 fixation and Fe:C of two morphotypes at BATS [Fig. 8 in [21] and $C \cdot col^{-1}$ provided by Orcutt]. (a) N_2 fixation rates and Fe:C contents of “puff” morphology colonies collected at BATS from Julian days 128–310. N_2 fixation rates ($\mu mol N \cdot mol C^{-1} \cdot h^{-1}$; diamonds) are offset +7 Julian days to distinguish from Fe:C contents (triangles). (b) As (a) except “tuft” colonies shown. Dust concentrations were elevated starting \sim Julian day 190.

elicit an effect. These results are difficult to interpret, as this concentration of iron exceeds the solubility of iron in seawater by several orders of magnitude [12]. Similarly, bottle incubations of a North Carolina coastal population (12 km offshore) indicated an 8-fold increase in acetylene reduction rates from iron treatments compared to control, molybdenum and phosphorus treatments [22]. These results have supported the contention that N_2 fixers have greater iron requirements than non-diazotrophs. This becomes particularly clear when considering that diatoms in the open Atlantic Ocean (presumably with less available iron than coastal populations) can sustain nitrate supported growth and do not appear to experience iron limitation [32].

4. Phylogenetic and metabolic diversity

Twenty seven phylogenetically distinct diazotrophs were recently identified from nanoplankton ($< 10 \mu m$) collections at the well studied Hawaii Ocean Time series (HOT) station [35]. Based on the RT-PCR of *nifH* (the gene encod-

ing for the Fe protein of nitrogenase) mRNA extracts, this study provides conclusive evidence of the phylogenetic and metabolic potential of organisms other than the more extensively studied *Trichodesmium* to act as net sources of fixed nitrogen to pelagic marine ecosystems. Nineteen of the clones sequenced were inferred to be of cyanobacterial origin, while the remainder appear to derive from heterotrophic bacteria. Some of the cyanobacterial isolates exhibited maximal *nifH* gene expression during the day and others at night, suggesting different diel rhythms of N_2 fixation within the assemblage of diazotrophs. This new phylogenetic evidence may indicate that iron's potential regulatory role on pelagic diazotrophy is not simply related to the iron costs of cyanobacterial diazotrophs which fix N_2 during the day. Nighttime N_2 -fixing phototrophs could enjoy a more efficient use of their cellular iron due to the potential for “iron recycling” associated with the diel cycle of N_2 ase synthesis and degradation [17]. In addition, cyanobacteria may have the ability to share certain electron transport chains among metabolic steps in photosynthetic, respiratory and nitrogen fixation pathways [30]. Theoretically, temporally decoupled processes (night-time N_2 fixation and daytime C fixation) could minimize the redundancy of certain iron containing catalysts and allow for an increase in the iron use efficiency.

The identification of heterotrophic pelagic diazotrophs at HOT [35] suggests these bacteria may be significant N_2 fixers in the marine environment. The constraints of the high iron demand for diazotrophy and low iron bioavailability in marine waters may be less significant for heterotrophic diazotrophs. First, heterotrophs should require less iron than diazotrophic phototrophs for net growth, due to the absence of photosynthetic electron transport chains. Second, due to considerations of the surface area dependence of iron uptake rates in cultures [31], these heterotrophs may have carbon-specific iron uptake rates as high as those of similar sized cyanobacteria, and higher than those of colonial diazotrophs such as *Trichodesmium*.

Since nothing is yet known of the iron status of these recently discovered heterotrophic diazotrophs, we review the potential roles of iron on non-diazotrophic heterotrophic bacterial growth in the marine environment, and attempt to frame these results in the context of the combined metabolic (Fe) costs for heterotrophy and diazotrophy. A full review of factors limiting heterotrophic growth is well beyond our scope and is not the intent here. Most studies of iron limitation in heterotrophs or phototrophs have focused on HNLC regions. In these regions, some studies suggest Fe limitation of heterotrophic growth, but others suggest Fe limitation only after addition of C or N [4]. Presumably, such additional iron would only be necessary to fuel growth processes at an increased rate after relief from C or N limitation. These observations suggest the biosynthetic machinery contain adequate Fe for the achieved, C or N limited, growth rates. Kirchner et al. [16] suggested that

subarctic Pacific bacteria could be limited instead by the quantity and quality of DOM.

We compared the measured Fe:C contents of heterotrophs to the expected Fe demand for heterotrophic growth. Phytoplankton at an open ocean subarctic Pacific station have a measured Fe:C of 3.7 $\mu\text{mol}:\text{mol}$ (± 2.3 $\mu\text{mol}:\text{mol}$, s.d.) [33], while the Fe:C contents of heterotrophic bacteria are 6.05 $\mu\text{mol}:\text{mol}$ (± 2.5 $\mu\text{mol}:\text{mol}$, s.d.). Growth rates of these bacterial populations have been reported as $\mu = 0.06$ d^{-1} [33, and references therein]. From a synthesis of published values, we have calculated the minimum iron requirements to support a growth rate of 0.06 d^{-1} for heterotrophic growth without the additional costs of diazotrophy (as diazotrophy would not be a suitable strategy for sub-Arctic populations) and extend this calculation to include diazotrophy. Consideration of the heterotrophic growth efficiency [33], and the iron costs for respiration (for C assimilation) [25] yields a calculated minimum required Fe:C for non-diazotrophic growth of 0.3 $\mu\text{mol}:\text{mol}$ (Table 1). The additional costs for N_2 fixation [17], and the extra respiration necessary to fuel diazotrophy [17], suggests that a diazotrophic heterotroph would require a minimum of 2.3–5.7 $\mu\text{mol}:\text{mol}$ Fe:C to grow at 0.06 d^{-1} . This suggests that diazotrophic heterotrophs require about an order of magnitude more Fe than heterotrophs growing on other sources of N. These estimates do not consider basal metabolic iron costs, or any additional iron costs associated with diazotrophy (such as a possible increased demand for Fe-superoxide dismutase). In addition, bacterial growth efficiency depends on the quality of available DOM and is much greater with the incorporation of preformed amino acids as compared to inorganic nutrients [23]. Nonetheless, these calculations suggest that the iron cost for heterotrophic growth on N_2 at $\mu = 0.06$ d^{-1} (2.5–5.7 $\mu\text{mol}:\text{mol}$ Fe:C) may be low enough to preclude direct iron limitation. This would depend on the growth and iron uptake rates of these diazotrophs. It is unclear whether the greater mean Fe quotas of heterotrophic bacteria in the sub-Arctic Pacific, relative to the phytoplankton assemblages (6.1 vs. 3.7 $\mu\text{mol}:\text{mol}$ Fe:C), simply reflect a combination of increased carbon specific iron uptake rates and depressed growth rates (due to C limitation) or whether these heterotrophs truly require more iron than their phototroph counterparts.

In general, marine bacterial productivity may be limited by the quantity and quality of organic carbon [16]. Within an oligotrophic microbial assemblage, relief of iron limitation in phototrophs may increase labile DOC production, which could then serve as energy and growth substrate for the diazotrophic heterotrophs. Therefore, heterotrophic diazotrophy may be indirectly limited by iron. These calculations do not support a high Fe demand for heterotrophic growth. Clearly, the role of and constraints on heterotrophic diazotrophy in the marine environment deserve more attention.

5. Future research considerations

The three approaches used to address iron limitation in *Trichodesmium* (namely estimating biochemical iron demands, evaluating growth – Fe quota relationships and measuring field Fe quotas and growth responses due to added iron) do not seem to present consistent evidence of iron satiation or limitation. Relying solely on nutrient quotas to diagnose growth limitation in the field is compromised by the capacity of phytoplankton for luxury uptake of iron. Furthermore, co-limitation by other nutrients or light cannot be ignored. While culture work helps put field observations in perspective, an integrated field approach including measurements of molecular indicators of iron stress (or iron storage) would aid in determining the iron status of field populations.

A promising probe for iron limitation has been developed and tested in cultures of three open ocean cyanobacteria, including *Trichodesmium* [34]. 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 *Crocospaera* spp., *Trichodesmium*, and *Synechococcus* WH7803. In addition, within three days 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 depending on the minimum time required for repression of idiA synthesis, perhaps in field iron addition experiments as well. Coupling this diagnostic indicator with measurements of cellular iron quota and growth processes should help refine our quantitative understanding of the role of Fe on C specific rates of nitrogen fixation in various oceanographic regimes.

The relative ease of culturing, rapid growth rates and high achievable biomass for heterotrophic diazotrophs, such as *Azotobacter vinelandii* and *Klebsiella pasteurianum*, have allowed these organisms to be models for biochemical studies on nitrogen fixation. This is apparent in literature searches, as very few biochemical studies of nitrogenase proteins have been conducted in cyanobacteria! Fortunately, advances in molecular techniques are obviating the need for purification of gram-quantities of proteins required for traditional experimentation.

There are special experimental challenges that must be considered in the case of rearing fastidious marine phytoplankton under trace metal controlled conditions. For *Trichodesmium*, suitable growth conditions have entailed using relatively low concentrations of metal chelators such as EDTA (i.e., ≤ 20 μM). These chelators are employed to ensure a constant concentration of “available” iron, defined here as the sum of all iron species not bound to EDTA (denoted Fe’), over the course of experimentation. The constancy of the concentration of Fe’ is a result of the apparent stability constants of Fe-EDTA complexes which depend on the total Fe and EDTA added, and specific conditions of irradiance, pH, and ionic strength [11], as

well as the rate of Fe' uptake. To ensure that the buffering capacity of relatively low ligand concentrations remains effective at maintaining steady state concentrations of Fe', only a fraction of the maximum achievable biomass can be obtained under batch culture conditions. Low biomass is also a requirement in order to strictly control the nitrogenous nutrition of *Trichodesmium* cultures. Batch cultures under apparent diazotrophic growth conditions exhibit biomass-specific N₂ fixation rates which progressively decline as biomass reaches excessive concentrations (i.e., $\geq 100 \mu\text{g L}^{-1}$ chl *a*), thus becoming decoupled from C fixation [20]. At these levels of biomass, up to 70% of nitrogen nutrition is supplied by regenerated nitrogenous compounds in media where no combined N was initially added [20]. Excluding such high rates of N uptake from regenerated sources under nominally 'diazotrophic' conditions may be critical in comparative proteonomic studies and is essential during quantitative determinations of energy and iron requirements for diazotrophy, as both costs are significantly elevated relative to ammonium-supported growth in *Trichodesmium* (Fig. 2b).

6. Summary

Quantitative data on factors limiting marine nitrogen fixation will be invaluable for modeling the influences of marine diazotrophy on global climate as well as the impact of global climate change on marine diazotrophy [9]. From a biochemical perspective, *Trichodesmium* and other marine diazotrophs are poorly studied and exist in an environment drastically different from that of model diazotrophs. The extremely nutrient deplete conditions of the oligotrophic ocean suggest that biochemical and physiological adaptations may exist which are not found in model diazotrophs. Coupling molecular tools to physiological and geochemical measurements should improve our understanding of marine diazotrophs and the factors that regulate their rates of N₂ fixation.

Acknowledgements

We thank K. Orcutt for providing *Trichodesmium* C and N data complementing data in Ref. [21], and I. Berman-Frank for providing raw data in Ref. [1]. D. Capone provided N₂ fixation data from the Australia 1999 work from [1] cited in Fig. 4.

References

- [1] I. Berman-Frank, J.T. Cullen, Y. Shaked, R.M. Sherrell, P.G. Falkowski, Iron availability, cellular iron quotas, and nitrogen fixation in *Trichodesmium*, *Limnol. Oceanogr.* 46 (2001) 1249–1260.
- [2] L.E. Brand, Minimum iron requirements of marine phytoplankton and the implications for the biogeochemical control of new production, *Limnol. Oceanogr.* 36 (1991) 1756–1771.
- [3] D.G. Capone, J.P. Zehr, H.W. Paerl, B. Bergman, E.J. Carpenter, *Trichodesmium*, a globally significant marine cyanobacterium, *Science* 276 (1997) 1221–1229.
- [4] M.J. Church, D.A. Hutchins, H.W. Ducklow, Limitation of bacterial growth by dissolved organic matter and iron in the Southern Ocean, *Appl. Environ. Microbiol.* 66 (2000) 455–466.
- [5] H. de Baar, von Liebig law of the minimum and plankton ecology (1899–1991), *Prog. Oceanogr.* 33 (1994) 347–386.
- [6] M.R. Droop, Vitamin B₁₂ and marine ecology. IV. Kinetics of uptake growth and inhibition in *Monochrysis lutheri*, *J. Mar. Biol. Assoc. UK* 48 (1968) 689–733.
- [7] R.C. Dugdale, D.W. Menzel, J.H. Ryther, Nitrogen fixation in the Sargasso Sea, *Deep Sea Res.* 7 (1961) 297–300.
- [8] R.R. Eady, B.E. Smith, in: R.W.E. Hardy, F. Bottomley, R.C. Burns (Eds.), *A Treatise on Dinitrogen Fixation*, Wiley-Interscience, New York, 1979, pp. 399–490.
- [9] P.G. Falkowski, Evolution of the nitrogen cycle and its influence on the biological sequestration of CO₂ in the ocean, *Nature* 387 (1997) 272–275.
- [10] N. Gruber, J.L. Sarmiento, Global patterns of marine nitrogen fixation and denitrification, *Global Biogeochem. Cycles* 11 (1997) 235–266.
- [11] R.J.M. Hudson, D.M. Covault, F.M.M. Morel, Investigations of iron coordination and redox reactions in seawater using Fe-59 radiometry and ion-pair solvent-extraction of amphiphilic iron complexes, *Mar. Chem.* 38 (1992) 209–235.
- [12] D.A. Hutchins, Iron and the marine phytoplankton community, *Prog. Phycol. Res.* 11 (1995) 1–49.
- [13] G.B. Jones, C. Burdon-Jones, F.G. Thomas, Influence of *Trichodesmium* red tides on trace metal cycling at a coastal station in the Great Barrier Reef lagoon, *Oceanol.* 1 (1982) 319–326.
- [14] D.M. Karl, A. Michaels, B. Bergman, D.G. Capone, E.J. Carpenter, R. Letelier, F. Lipschultz, H. Paerl, D. Sigman, L. Stal, Dinitrogen fixation in the world's oceans, *Biogeochemistry*, in press.
- [15] A. Kerry, D.A. Laudenbach, C.G. Trick, Influence of iron limitation and nitrogen source on growth and siderophore production by cyanobacteria, *J. Phycol.* 24 (1988) 566–571.
- [16] D.L. Kirchman, Limitation of bacterial growth by dissolved organic matter in the subarctic Pacific, *Mar. Ecol. Prog. Ser.* 62 (1990) 47–54.
- [17] A. Kustka, S.A. Sañudo-Wilhelmy, D.G. Capone, E.J. Carpenter, J.A. Raven, A revised estimate of the iron cost of nitrogen fixation, with special emphasis on the marine cyanobacterium, *Trichodesmium* spp. (Cyanophyta), *J. Phycol.* (2002) in review.
- [18] A. Kustka, S.A. Sañudo-Wilhelmy, E.J. Carpenter, W.G. Sunda, Growth and Fe quotas of N₂ and NH₄⁺ supported cultures of *Trichodesmium* (IMS 101): comparison with nitrogen fixation rates, iron quotas and incubation experiments from field populations, *Mar. Ecol. Prog. Ser.* (2002) in prep.
- [19] J.M. Lenes, B.P. Darrow, C. Cattrall, C.A. Heil, M. Callahan, G.A. Vargo, R.H. Byrne, J.M. Prospero, D.E. Bates, K.A. Fanning, J.J. Walsh, Iron fertilization and the *Trichodesmium* response on the West Florida shelf, *Limnol. Oceanogr.* 46 (2001) 1261–1277.
- [20] M.R. Mulholland, D.G. Capone, Stoichiometry of nitrogen and carbon utilization in cultured populations of *Trichodesmium* IMS 101: implications for growth, *Limnol. Oceanogr.* 46 (2001) 436–443.
- [21] K.M. Orcutt, F. Lipschultz, K. Gundersen, R. Arimoto, A.F. Michaels, A.H. Knap, J. Gallon, A seasonal study of the significance of N₂ fixation by *Trichodesmium* spp. at the Bermuda Atlantic Time-series Study (BATS) site, *Deep Sea Res. II* 48 (2001) 1583–1608.
- [22] H.W. Paerl, L.E. Prufert-Bebout, C. Guo, Iron stimulated N₂ fixation and growth in natural and cultured populations of the planktonic marine cyanobacteria *Trichodesmium* spp., *Appl. Environ. Microbiol.* 60 (1994) 1044–1047.
- [23] W.J. Payne, W.J. Wiebe, in: M.P. Starr, J.L. Ingraham, S. Raffel (Eds.), *Ann. Rev. Microbiol. Annual Reviews, Inc.*, Palo Alto, 1978, pp. 155–183.
- [24] A. Puppo, J. Rigaud, Superoxide dismutase: an essential role in the protection of the nitrogen fixation process?, *FEBS Lett.* 201 (1986) 187–189.

- [25] J.A. Raven, The iron and molybdenum use efficiencies of plant growth with different energy, carbon and nitrogen sources, *New Phytologist* 109 (1988) 279–287.
- [26] J.P.H. Reade, L.J. Dougherty, L.J. Rogers, J.R. Gallon, Synthesis and proteolytic degradation of nitrogenase in cultures of the unicellular cyanobacterium *Gloeothece* strain ATCC 27152, *Microbiol.* 145 (1999) 1749–1758.
- [27] J.G. Rueter, D.A. Hutchins, R.W. Smith, N.L. Unsworth, in: E.J. Carpenter, D.G. Capone, J.G. Rueter (Eds.), *Marine Pelagic Cyanobacterium: Trichodesmium and other diazotrophs*, Kluwer Academic, Dordrecht, 1992, pp. 289–306.
- [28] J.G. Rueter, Iron stimulation of photosynthesis and nitrogen fixation in *Anabaena* 7120 and *Trichodesmium* (Cyanophyceae), *J. Phycol.* 24 (1988) 249–254.
- [29] S.A. Sañudo-Wilhelmy, A.B. Kustka, C.J. Gobler, D.A. Hutchins, M. Yang, K. Lwiza, J. Burns, D.G. Capone, J.A. Raven, E.J. Carpenter, Phosphorus limitation of nitrogen fixation by *Trichodesmium* in the central Atlantic Ocean, *Nature* 411 (2001) 66–69.
- [30] S. Scherer, H. Almon, P. Boger, Interaction of photosynthesis, respiration and nitrogen fixation in cyanobacteria, *Photosynth. Res.* 15 (1988) 95–114.
- [31] W.G. Sunda, S.A. Huntsman, Iron uptake and growth limitation in oceanic and coastal phytoplankton, *Mar. Chem.* 50 (1995) 189–206.
- [32] K.R. Timmermans, M. Gledhill, R.F. Nolting, M.J.W. Veldhuis, H.J.W. de Baar, C.M.G. van den Berg, Response of marine phytoplankton in iron enrichment experiments in the northern North Sea and northeast Atlantic Ocean, *Mar. Chem.* 61 (1988) 229–242.
- [33] P.D. Tortell, M.T. Maldonado, J. Granger, N.M. Price, Marine bacteria and biogeochemical cycling of iron in the oceans, *FEMS Microbiol. Ecol.* 29 (1999) 1–11.
- [34] E.A. Webb, J.W. Moffett, J.B. Waterbury, Iron stress in open-ocean cyanobacteria (*Synechococcus*, *Trichodesmium*, and *Crocospaera* spp.): Identification of the IdiA protein, *Appl. Environ. Microbiol.* 67 (2001) 5444–5452.
- [35] J.P. Zehr, J.B. Waterbury, P.J. Turner, J.P. Montoya, E. Omoregie, G.F. Steward, A. Hansen, D.M. Karl, Unicellular cyanobacteria fix N₂ in the subtropical North Pacific Ocean, *Nature* 412 (2001) 635–638.