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Nitrogen-fixation strategies and Fe requirements in cyanobacteria

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Abstract

Diazotrophic (nitrogen-fixing) cyanobacteria are important contributors of new nitrogen to oligotrophic environments and greatly influence oceanic productivity. We investigated how iron availability influences the physiology of cyanobacterial diazotrophs with different strategies for segregating nitrogen fixation and photosynthesis. We examined growth, photosynthesis, nitrogen fixation, and Fe requirements of the filamentous nonheterocystous *Trichodesmium*, the filamentous heterocystous *Anabaena*, and the unicellular *Cyanothece* under a range of Fe concentrations. Under similar Fe concentrations the three species differed in N_2-fixation rates, photosynthetic activity, the relative abundance of the photosynthetic units PSI : PSII, elemental stoichiometry, and Fe use efficiency. Complex colonial forms such as *Trichodesmium* and *Anabaena* are more likely to be Fe limited in their natural environments and are more efficient at utilizing Fe than unicellular diazotrophs such as *Cyanothece*. The varied physiological responses to Fe availability of the three cyanobacteria reflect their nitrogen-fixation strategies, cell size, unicellular or colonial organization, and may explain, at least in part, the ecological distribution of these photosynthetic bacteria.

Biological fixation of atmospheric nitrogen in the oceans (primarily by diazotrophic cyanobacteria) is an important source of ''new'' nitrogen into the surface oceans that fuels primary production, phytoplankton blooms, and influences global carbon cycling (Dugdale and Goering 1967; Falkowski 1997; Karl et al. 1997). Several factors control aquatic nitrogen fixation. The atmospheric concentration of oxygen and the corresponding dissolved oxygen in aquatic systems exert a fundamental control, as nitrogenase, the enzyme responsible for N_2 fixation, is irreversibly damaged by molecular oxygen in vitro (Postgate 1998) and operates at a fraction of its potential activity in vivo (Bergman 2001; Gallon 2001; Berman-Frank et al. 2003). Additional regulation of diazotrophic cyanobacterial populations may be via other factors such as temperature (Staal et al. 2003*b*), phosphorus limitation (Wu et al. 2000; Sanudo-Wilhelmy et al. 2001), iron availability (Paerl et al. 1987; Reuter et al. 1992; Berman-Frank et al. 2001*a*), or in some areas colimitation by Fe and P (Mills et al. 2004).

Cyanobacteria are the only organisms that actively evolve oxygen as a by-product of oxygenic photosynthesis within the same cell or colony of cells where N_2 fixation occurs. The presence of oxygen triggered biochemical and morphological adaptations in diazotrophic phototrophs aimed at limiting the inhibitory effects of oxygen on nitrogenase (Gallon 1992; Bergman et al. 1997; Berman-Frank et al. 2003). Cyanobacterial diazotrophs can temporally segregate photosynthesis and N_2 fixation (fixing C during the day and N_2 at night); they can spatially separate photosynthesis and N_2 fixation; or they can use a combination of spatial and temporal separation (Adams 2000; Gallon 2001; Berman-Frank et al. 2003) (Fig. 1).

In many unicellular diazotrophs (e.g., *Cyanothece, Croccosphaera*) nitrogen is fixed during the night when

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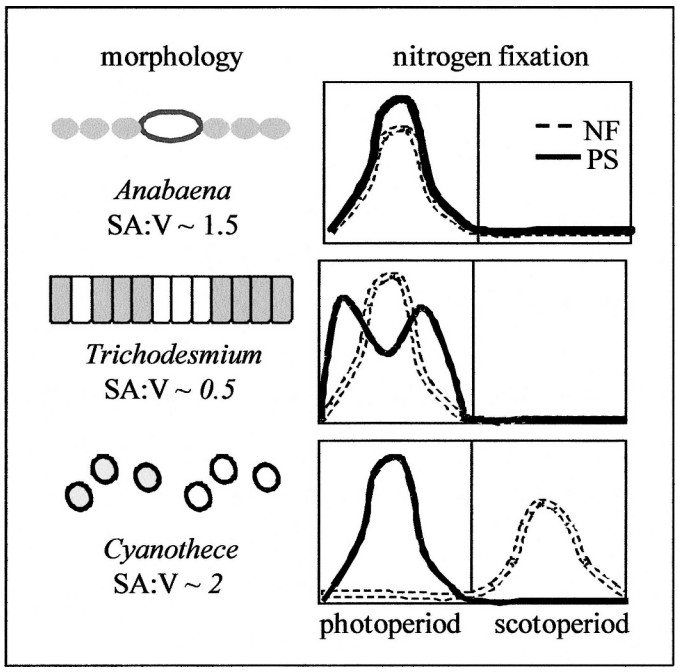

Fig. 1. Schematic representation of the different diazotrophic morphologies, strategies of separation of nitrogen fixation and photosynthesis, and typical surface area : volume (SA : V) ratios of the three model organisms used in this study: the filamentous heterocystous *Anabaena*, the filamentous nonheterocystous *Trichodesmium*, and the unicellular *Cyanothece*. White coloring indicates the localization of nitrogenase within the cells. Dark gray coloring indicates actively photosynthesizing vegetative cells. The graphs in the panels illustrate the relative timing of photosynthesis (solid black line) and nitrogen fixation (dashed line) during the diel cycle. Figure is modified from Berman-Frank et al. (2003).

grown under a light : dark (LD) cycle, or under the subjective scotophase when grown under continuous light (Bergman et al. 1997). High nitrogenase activity coincides with high respiration rates; with a phase difference of up to 12 h from the peak of photosynthetic activity (Fig. 1). Energy and reductants are provided via respiration and use of photosynthetically fixed carbon while cellular pools such as Fe and Mo are recycled between day and nighttime cellular requirements (Tuit et al. 2004).

In heterocystous filamentous cyanobacteria such as *Anabaena*, nitrogenase is confined to an anaerobic cell, the heterocyst, that differentiates completely and irreversibly 12–20 h after combined nitrogen sources are removed from the medium (Adams 2000). Heterocysts are characterized by a thick membrane that slows the diffusion of O_2, high photosystem I (PSI) activity, the absence of photosystem II (PSII), and loss of division capability. Heterocystous organisms cannot obtain reductant directly from noncyclic photosynthetic electron flow and rely on the supply of fixed carbon from adjacent vegetative cells for reducing equivalents and on cyclic electron flow around PSI to supply adenosine triphosphate (ATP) (Adams 2000).

A mixed temporal and spatial separation strategy characterizes the marine nonheterocystous filamentous cyanobacteria *Trichodesmium* and *Katagnymene* (*Katagnymene* has been reaffiliated to *Trichodesmium* [Lundgren et al. 2001]). Unlike all other nonheterocystous species of cyanobacteria, these species fix nitrogen during the day in a fraction of the cells that are often arranged consecutively along the trichome (Fig. 1). Active photosynthetic components (such as PSI and PSII complexes, Rubisco, carboxysomes) are found in all cells, even those harboring nitrogenase (Janson et al. 1994; Fredriksson et al. 1998; Berman-Frank et al. 2001*a*). Hence, spatial aggregation of nitrogenase into certain zones (or cells) is not sufficient to protect against oxygen. Protection against oxygen in *Trichodesmium* is a complex interaction between spatial and temporal segregation of the photosynthetic, respiratory, and nitrogen-fixation processes (Chen et al. 1999; Berman-Frank et al. 2001*a*) with down-regulation of photosynthetic oxygen evolution and high oxygen consumption through processes such as the Mehler reaction occurring during the peak of nitrogen fixation (Berman-Frank et al. 2001*b*; Küpper et al. 2004; Milligan et al. in press) (Fig. 1).

As photosynthesis and N_2 fixation both require Fe, it is likely that the varying morphological and biochemical strategies used to separate N_2 fixation from photosynthesis will affect the Fe requirements for the different types of diazotrophs. Availability of iron influences N_2 fixation in cyanobacteria by its direct effect on Fe-rich protein synthesis of nitrogenase, and by effects on photosynthesis, growth, and global productivity (Paerl et al. 1987; Falkowski 1997). Higher intracellular iron quotas were calculated and found in diazotrophic cyanobacteria in comparison with nondiazotrophic phytoplankton (Kustka et al. 2003; Tuit et al. 2004). Several studies, mostly focused on the nonheterocystous filamentous *Trichodesmium* as the model organism, have shown a positive correlation between Fe availability and nitrogen fixation and growth (Reuter et al. 1992; Paerl et al. 1994; Berman-Frank et al. 2001*a*). However, differences in Fe requirements and use by diazotrophs with different morphological and biochemical adaptations for segregation of N_2 fixation and photosynethesis have not yet been determined.

In this study, we focused on how iron availability influences cyanobacterial diazotrophs with different strategies for nitrogen fixation: the filamentous nonheterocystous *Trichodesmium*, the filamentous heterocystous *Anabaena*, and the unicellular *Cyanothece* (Fig. 1). We tested whether the biochemical and morphological differences (such as cell and colony size) characterizing the spatial or temporal (or both) segregation of N_2 fixation and photosynthesis affect the utilization of Fe as reflected in rates of nitrogen fixation, photosynthesis, and growth over a range of Fe concentrations.

Materials and methods

Culture growth conditions—Cultures of *Trichodesmium* IMS101, *Cyanothece* sp. (WH8904), and *Anabaena flos aqua* (UTEX 2557) were grown at 26°C under a 12 : 12 LD cycle at 85 μmol m^{-2} s^{-1} quanta supplied by VHO fluorescent tubes. *Trichodesmium* data were obtained from Berman-Frank et al. (2001*a*) and additional unpublished

data from associated experiments. *Trichodesmium* was grown in YBCII media (Chen et al. 1996) amended as below for Fe and ethylenediaminetetra-acetic acid (EDTA). *Cyanothece sp.* cultures were grown in a modified Aquil seawater media prepared as described in Ho et al. (2003). *Anabaena* was grown in an artificial freshwater media, Fraquil, prepared as described by Morel et al. (1975) except that the trace-metal stock, nutrients (N and P), and vitamins for the modified Aquil recipe were used rather than those described for Fraquil. For cultures of all three species, Fe ($FeCl_3$) was added at five concentrations between 4 nmol L^{-1} and 4 μmol L^{-1}, and complexed with 20 μmol L^{-1} EDTA (see Berman-Frank et al. 2001*a* for details). The media was microwave-sterilized at least 24 h before use. *Cyanothece* and *Anabaena* were grown in each Fe treatment for a minimum of five generations before the commencement of experiments to fully acclimate cells to their respective Fe treatments. Cultures were kept optically thin throughout the experiment to prevent physiological changes due to decreased irradiance rather than Fe effects. Unless noted otherwise, cells were harvested for experiments during the exponential phase of growth. Although cultures were not axenic, extremely low bacterial counts were observed during the exponential phase. Growth rates were determined from linear regression analysis of the increase of logarithmic transformed C and N concentrations versus time. CHN was assayed on precombusted Gelman AE filters using a Carlo-Erba CHN analyzer. Cultures were filtered under low vacuum (<13 kPa) and stored frozen until CHN analysis could be performed.

Chlorophyll a—Samples for chlorophyll *a* (Chl *a*) analysis were filtered gently onto 13-mm 0.45-μm Durapore (Millipore) filters and frozen at -20°C. Filters were defrosted slowly on ice in dim light. Methanol (90%) was added and the samples were sonicated with a Branson sonicator while on ice. Samples were returned to the freezer overnight and centrifuged the next day to clarify. Chl *a* concentrations were assayed at 664 and 750 nm using a HP 8451A diode array spectrophotometer. At least two samples were taken for each of the three cultures of *Cyanothece* and *Anabaena* grown in each Fe treatment and replicate results were averaged.

Metal quotas—Inductively coupled mass spectrometry (ICPMS) measurements were made essentially as described previously (Berman-Frank et al. 2001*a* ; Quigg et al. 2003) with the following modifications for *Anabaena* and *Cyanothece*. Samples were collected onto 25-mm Poretics filters that had been pretreated as described in Cullen and Sherrell (1999) on a filter apparatus detailed in Ho et al. 2003 under gentle filteration (<13 kPa). All filtering was done in a laminar flow hood to reduce contamination of samples by airborne particles. Additional filters were washed with Aquil or Fraquil and 0.4 mol L^{-1} NaCl to assay background contamination. *Trichodesmium* was examined as described in Berman-Frank et al. (2001*a*).

Nitrogenase activity—Nitrogen-fixation rates for all three species were measured using the acetylene reduction method according to Capone (1993). Ten to 30 mL of cultures (depending on biomass) were sealed in serum bottles, injected with acetylene (20% of headspace volume), and incubated for 2 h at ambient light and temperature. Ethylene production was measured on a SRI 310 gas chromatograph with a flame ionization detector and quantified relative to an ethylene standard. Results were normalized to carbon and nitrogen.

Variable fluorescence and photosynthetic parameters—We used a fast repetition rate fluorometer, which measures fluorescence transients induced by a series of subsaturating excitation pulses from a bank of blue-green light-emitting diodes to derive photosynthetic parameters (Kolber et al. 1998). The photochemical quantum yield ($F_v : F_m$) was determined from the initial, dark-adapted fluorescence (F_o) and the maximal fluorescence (F_m) when all PSII reaction centers are photochemically reduced ($F_v : F_m = (F_m - F_o)/F_m$). Blanks of sterile media were subtracted from each measurement. The functional absorption cross-section of photosystem II (σ_{PSII}) was calculated according to Kolber et al. (1998).

Relative abundance of PSI and PSII—The ratio between PSI and PSII was estimated from 77K fluorescence emission spectra (excitation wavelength 435 nm) performed on cells that were flash-frozen in liquid nitrogen. The spectra were analyzed on an AB2 Aminco spectrofluorometer equipped with a liquid nitrogen Dewar, which allowed measurements at 77K using a 2-nm bandpass. Spectra were resolved, machine baseline subtracted, and the peak ratio was determined using PeakFit™ Software (SPSS). Spectra were normalized to emission at 685 nm.

Surface area and volume parameters—Biovolume and surface area were calculated using Biovol2.1 software (courtesy David Kirschtel) assuming cylinder shapes for *Trichodesmium* (filament lengths 100 to 1000 μm, cellular radius 4 μm) and *Anabaena* (filament lengths 100 to 1000 μm, cellular radius 1.3 μm) and a sphere (average radius 1.4 μm) for *Cyanothece*.

Results

The effects of iron on growth, photosynthesis, the relative ratios of PS I and II, cellular iron quotas, and nitrogen-fixation rates were measured in exponential-phase cultures of *Anabaena* and *Cyanothece*. We include some new data and also findings from earlier work (Berman-Frank et al. 2001*a*) on *Trichodesmium* for reference since it was grown under identical conditions to the marine diazotroph, *Cyanothece*.

Growth rates—All cyanobacteria were acclimated and grown under varying iron regimes (range 4 nmol L^{-1} to 4 μmol L^{-1}) for a minimum of five generations. Of the three species tested, carbon-specific growth rates were highest in the unicellular *Cyanothece* at all Fe concentrations (Table 1, Fig. 2). An increase in iron concentrations in the growth media of *Cyanothece* cultures resulted in

Table 1. The influence of Fe availability on growth rates and elemental ratios of Fe to C and P in Trichodesmium, Cyanothece, and Anabaena. Values are averages of n=3 for Anabaena and Cyanothece, n=4 for Trichodesmium at all Fe concentrations except Fe=4 μmol L^{-1} where n=12. Numbers in parentheses represent standard errors.

	External Fe concentration (μmol L^{-1})				
	0.004	0.04	0.1	0.4	4.0
Carbon-based growth, μ (d^{-1})					
Anabaena	0.14 (0.003)	0.15 (0.009)	0.23 (0.03)	0.26 (0.02)	0.26 (0.02)
Trichodesmium	0.05 (0.004)	0.06 (0.007)	0.09 (0.008)	0.1 (0.009)	0.12 (0.01)
Cyanothece	0.23 (0.02)	0.26 (0.005)	0.29 (0.018)	0.31 (0.05)	0.28 (0.04)
Fe : P (mmol : mol)					
Anabaena	4.2 (3)	5.6 (1)	2.4 (1)	5.4 (2)	30 (7)
Trichodesmium	0.8 (0.006)	1.6 (0.8)	2 (0.1)	3 (0.5)	22 (7)
Cyanothece	6 (2)	7.3 (1)	26 (3)	40 (2)	271 (22)
FPUE (mmol Fe mol P^{-1} h^{-1})					
Anabaena	0.025	0.04	0.023	0.06	0.32
Trichodesmium	0.002	0.004	0.007	0.01	0.11
Cyanothece	0.06	0.09	0.31	0.52	3.16
Fe : C (μmol : mol)					
Anabaena	0.076 (0.05)	0.32 (0.012)	0.16 (0.02)	0.2 (0.018)	2.4 (0.3)
Trichodesmium	13 (9.9)	30 (2.7)	33 (1.8)	48 (2.2)	168 (23)
Cyanothece	0.076 (0.014)	0.074 (0.014)	0.37 (0.08)	0.58 (0.07)	9.38 (2.4)
FUE (μmol Fe mol C^{-1} h^{-1}) $\times$ 10^{-3}					
Anabaena	0.4	2	1.5	2.2	26
Trichodesmium	27	75	124	194	840
Cyanothece	0.73	0.8	4.5	7.5	109

a modest increase of up to 36% for the C-specific growth rates (Fig. 2). Both *Anabaena* and *Trichodesmium* responded to increases in external Fe with a sigmoidal-type growth curve. The lowest growth rates occurred at 4 nmol L^{-1} to 0.04 μmol L^{-1}, and increased linearly until Fe concentrations reached 0.4 μmol L^{-1}. Fe concentrations greater than ~1 μmol L^{-1} did not result in higher growth rates. Both *Cyanothece* and *Anabaena* grew significantly faster than *Trichodesmium* at the same Fe concentrations; with division rates at 0.25, 0.27, and 0.12 d^{-1} respectively at 4 μmol L^{-1} Fe (Fig. 2).

Photosynthetic performance—The photochemical quantum yield of PS II photochemistry, $F_v : F_m$, provides an early diagnostic for iron limitation (Kolber et al. 1994; Behrenfeld and Kolber 1999). Photochemical quantum yields were positively correlated with carbon-specific growth rates for the three species with a saturation response of $F_v : F_m$ observed at high Fe concentrations (>0.1 μmol L^{-1}) for the three species examined (Fig. 3). The unicellular *Cyanothece* outperformed the two filamentous species and maintained high photosynthetic efficiency as reflected in the maximum quantum yields of PSII, which increased from 0.5 to 0.78 over a range from 4 nmol L^{-1} to 4 μmol L^{-1} Fe (Fig. 3). *Anabaena* responded to enhanced Fe availability by a linear increase in $F_v : F_m$ from values of ~0.35 at 4 nmol L^{-1} Fe to values ~0.6 as Fe availability increased to 0.1 μmol L^{-1} (Fig. 3). The slight decline in $F_v : F_m$ observed for *Anabaena* at 4 μmol L^{-1} Fe is not significant and falls within the range of saturated values . *Trichodesmium* showed a response similar to the other two cyanobacteria examined but maintained consistently lower photosynthetic efficiencies. Photochemical yields, $F_v : F_m$, determined at the peak of nitrogenase activity (i.e., about 5 h from light induction) were 50% lower ($F_v : F_m$ = 0.25) for iron-depleted cultures at 4 nmol L^{-1} Fe compared with iron-replete cultures ($F_v : F_m$ = 0.54 at 4 μmol L^{-1} Fe) (Fig. 3).

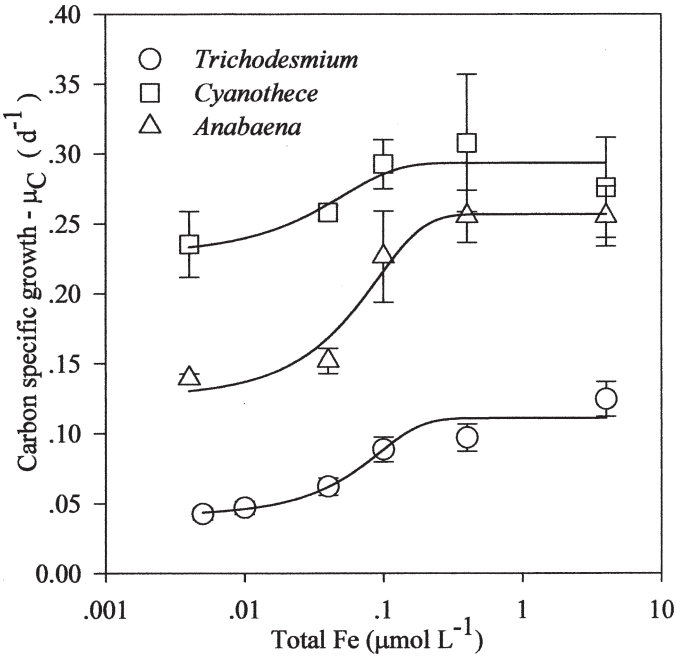

Fig. 2. (A) Effect of Fe availability on carbon-specific growth rates μ_c (d^{-1}) in *Trichodesmium*, *Cyanothece*, and *Anabaena*.

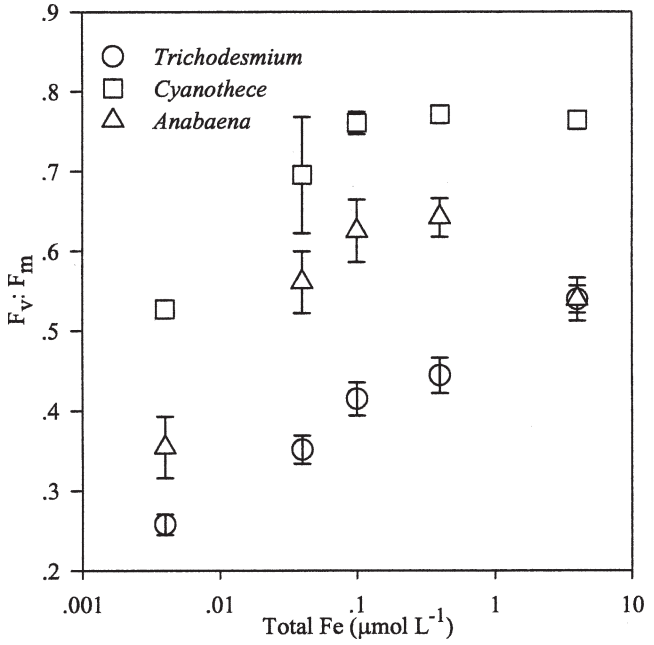

Fig. 3. Influence of Fe availability on photochemical quantum yields of PSII ($F_v : F_m$) in exponentially growing cultures of *Trichodesmium*, *Cyanothece*, and *Anabaena*.

Stoichiometry of photosynthetic units—The relative abundances of PSI and PSII vary in diazotrophs because of the additional roles that the two photosystems play in the nitrogen-fixation process, and because of their high atomic requirement. For all three species, emission spectra at 77K showed distinct changes in the spectral signature as Fe availability changed (Fig. 4A–C). In general, Fe-depleted cultures displayed spectra with a more dominant PSII component compared with PSI (Fig. 4 A–C), sometimes resulting in both the 685-nm and 695-nm peaks representing the core antennae complexes of PSII, CP43 and CP47, respectively. Both *Trichodesmium* and *Anabaena* exhibited much more prominent PSI signatures at high Fe (Fig. 4A,B) than *Cyanothece* (Fig. 4C), in which PSII was always dominant.

In *Trichodesmium* IMS101, Fe availability influenced the relative abundance of PSI and PSII. Peak area ratios of PSI : PSII decreased threefold, from 1.3 ± 0.3 to 0.25 ± 0.06 as Fe was reduced from 4 μmol L^{-1} to 4 nmol L^{-1} (Figs. 4A, 5A). The changes in PSI : PSII in *Trichodesmium* were correlated with Fe availability (Fig. 5A) and with nitrogen-fixation rates ($r^2 = 0.98$) (Fig. 5B). In *Cyanothece* PSI : PSII ratios increased with Fe availability (Fig. 5A), but no correlation was observed between PSI : PSII ratios and nitrogen-fixation rates for this unicellular organism (Fig. 5B). In the heterocystous *Anabaena*, PSI : PSII ratios were consistently higher at all Fe concentrations (Fig. 5A) with two levels of ratios measured: PSI : PSII ~ 1.5 at the two lowest Fe concentrations, then significantly higher ratios (at Fe > 0.1 μmol L^{-1}) ranging from 4.5 to 6.5. (Fig. 5A). In *Anabaena*, no change in the PSI : PSII was observed for all but the lowest nitrogen-fixation rates (when Fe is limited) (Fig. 5B).

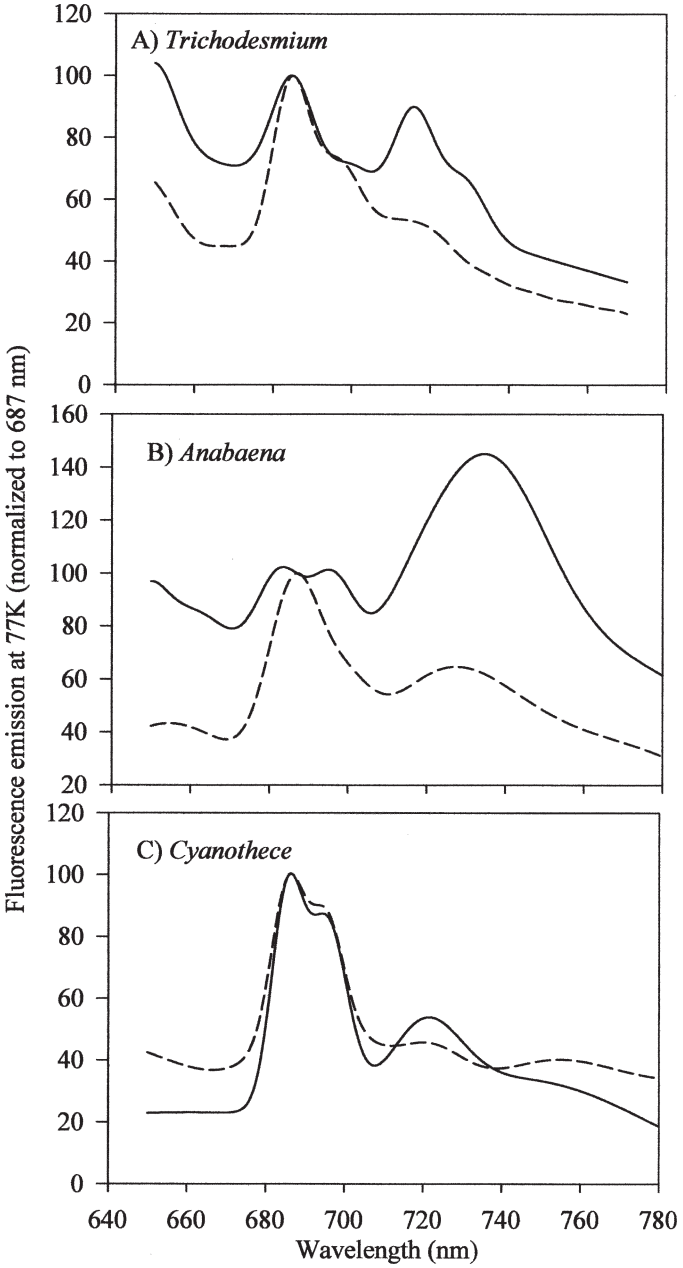

Fig. 4. The influence of Fe availability on the 77K fluorescence emission spectra and PSI : PSII for whole cells of (A) *Trichodesmium*, (B) *Anabaena*, and (C) *Cyanothece*. Spectra are representative of the highest Fe concentration (4 μmol L^{-1}, solid lines) and the lowest concentrations (4 nmol L^{-1}, dashed lines). Excitation wavelength is 435 nm. Spectra were normalized to peaks at 687 nm (PSII).

Nitrogen fixation—Nitrogen-fixation rates in *Trichodesmium* were significantly affected by iron availability with a sigmoidal pattern observed over the range of Fe concentrations in this study (Fig. 6). When Fe was reduced from 4 to 0.1 μmol L^{-1}, rates of ethylene produced decreased by 50% and by more than 85% when Fe availability was reduced to 4 nmol L^{-1} , with nitrogen-fixation rates declining sevenfold from 0.5 to 0.07 mmol N mol C^{-1} h^{-1} (Fig. 6). *Anabaena* and *Cyanothece* did not

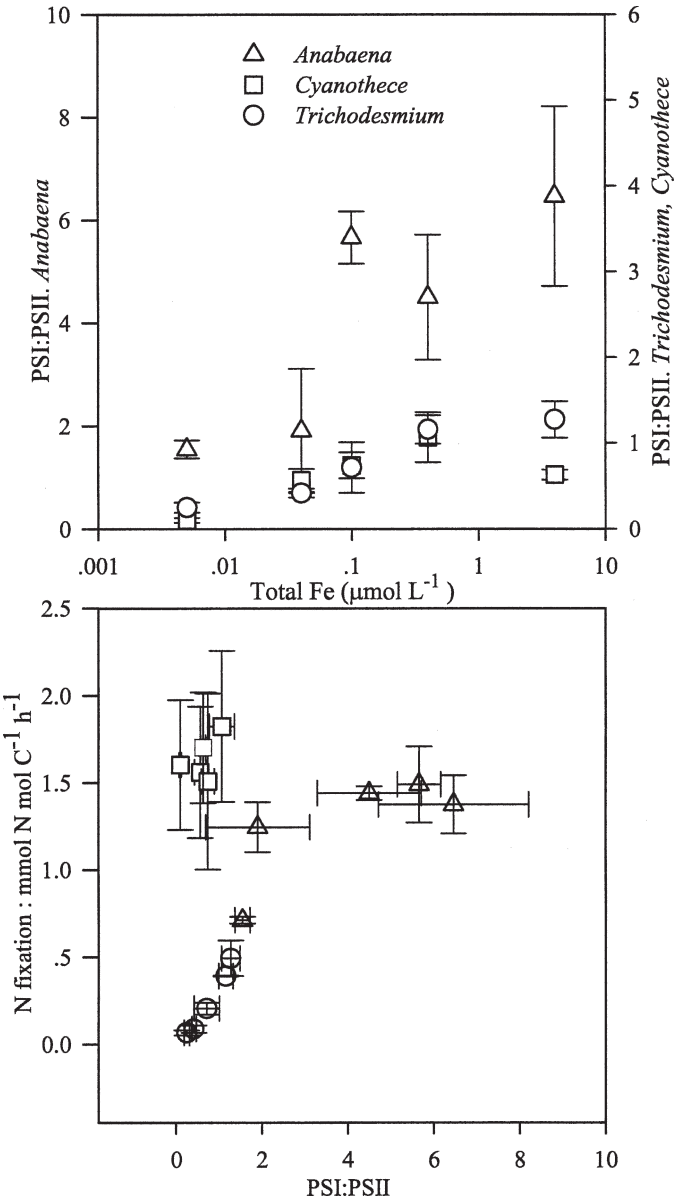

Fig. 5. (A) Influence of Fe availability on PSI : PSII ratios, and (B) relation between PSI : PSII ratios and nitrogen-fixation rates in *Trichodesmium*, *Cyanothece*, and *Anabaena*.

show the same dependence on Fe availability over the same concentration range as *Trichodesmium* (Fig. 6). In *Anabaena*, only the lowest Fe concentration (4 nmol L^{-1}) showed any significant reduction (about 50% from maximal values) in nitrogen-fixation rates. At all other Fe concentrations nitrogen-fixation rates (normalized to carbon) were saturated and not significantly different from one another. In *Cyanothece* similar rates of nitrogen fixation (1.6–1.8 mmol N mol C^{-1} h^{-1}) were observed across the three-orders-of-magnitude changes in Fe concentrations and were only 3.5-fold higher than measured rates of nitrogen fixation in Fe-replete *Trichodesmium* (Fig. 6).

Fe utilization—The effects of cellular size and resource utilization were reflected in the Fe : C and Fe : P ratios,

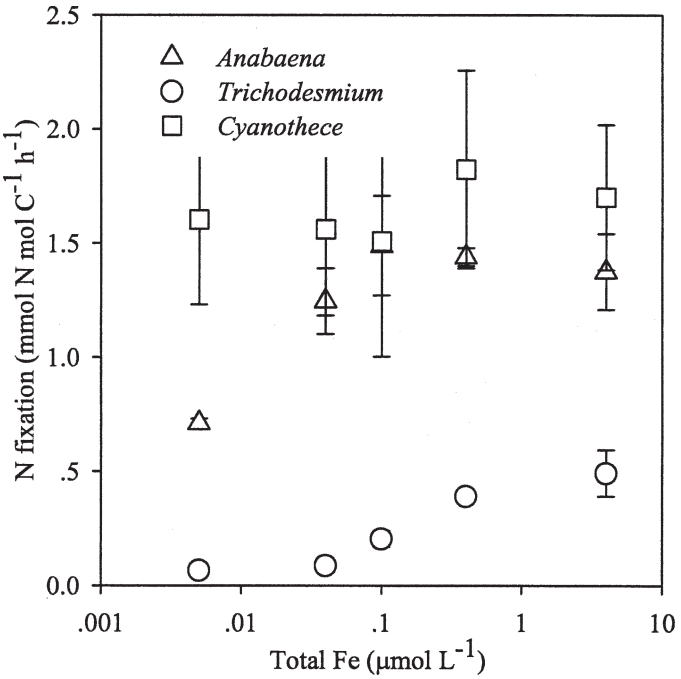

Fig. 6. Influence of Fe availability on nitrogen fixation rates in exponentially growing cultures of *Trichodesmium*, *Cyanothece*, and *Anabaena*.

which also varied with Fe availability for all three species (Table 1). *Trichodesmium* with the lowest surface area-to-volume ratio (SA : V) and largest-size filaments displayed much higher intracellular quotas than either *Anabaena* or the unicellular small *Cyanothece* with the largest SA : V. (Fe : C of *Trichodesmium* ranged from 13 to 168; for *Anabaena* 0.08 to 2.4; or the small unicellular *Cyanothece* 0.08 to 9.4 from 0.004 to 4 μmol L^{-1} Fe respectively) (Table 1). These differences in Fe : C quotas were also reflected in the iron use efficiency (FUE, Fe [C h] $^{-1}$), which quantifies the amount of Fe utilized per carbon relative to time. To avoid the potential bias of extracellular accumulation of Fe, the FUE is best determined under low Fe. *Trichodesmium* with the largest quotas of Fe : C also had FUE ~27–75, higher than both *Anabaena* and *Cyanothece* (Table 1). In contrast, the Fe : P ratios were the lowest in *Trichodesmium*, ranging from 0.8 to 22 compared with *Anabaena* (4.2 to 22) and *Cyanothece* (6 to 271) at 0.004 to 4 μmol L^{-1} Fe respectively, with highest values possibly due to extracellular Fe accumulation. The low Fe : P of *Trichodesmium* corresponded to a very inefficient utilization of Fe per P relative to time (FPUE, Fe [P h]$^{-1}$) in comparison with both *Anabaena* and *Cyanothece*. The smallest *Cyanothece* with the largest SA : V also had a 30-fold higher FPUE, Fe (P h)$^{-1}$ than *Trichodesmium*. These results may reflect issues of luxury consumption of P and extracellular P absorption (see Discussion).

Discussion

Anabaena, *Cyanothece*, and *Trichodesmium* use different strategies to balance demands of photosynthesis and

nitrogen fixation that can be characterized as spatial, temporal, or a combination of spatial and temporal, along with use of specialized cells (Fig. 1). In phototrophic diazotrophs, nitrogen fixation, photosynthesis, and respiration are intricately coupled, with both photosynthesis and respiration providing ATP and reductant for the energy-expensive process of nitrogen fixation. Respiration provides the substrates required for the assimilation of the fixed nitrogen and for consuming the oxygen derived from photosynthesis that irreversibly inhibits nitrogenase (Postgate 1998). The evolutionary selection of strategy by *Anabaena*, *Cyanothece*, and *Trichodesmium* to separate N_2 fixation from oxygenic photosynthesis and related morphological and biochemical adaptations have consequences for nutrient demand, metabolic performance, and preferred habitat. Growth rates for *Cyanothece* showed only a very modest decrease for the two lower Fe concentrations and overall do not differ significantly across the range of Fe assayed. In contrast, *Trichodesmium* and *Anabaena* exhibited a sigmoidal response as a function of Fe availability, indicating that these species were exposed to a range of conditions from Fe-limiting to Fe-replete in our experiments. Although the differential responses in PSI : PSII, N_2 fixation, and growth rates may be due to differences in metabolic strategies, covariations in cell size and colonialism indicate there are likely trade-offs related to Fe or other nutrient requirements, maximum growth rates, and the effectiveness of protecting nitrogenase from oxygen.

Temporal segregation of N_2 fixation and photosynthesis into dark and light phases of the day (as in *Cyanothece* sp.) is a common mechanism used by unicellular diazotrophs to separate oxygen evolution of PSII from the O_2-sensitive nitrogenase complex. In this way energy and substrates are generated during the light hours to fuel the energetically expensive process of nitrogen fixation, thus reducing the costs involved in protection of nitrogenase against photosynthetically produced oxygen. Moreover, elements such as Fe and Mo can be recycled between different cellular processes such as photosynthesis and nitrogen fixation and the cells can maintain low cellular quotas. Diel cycling of Fe : C has been documented in unicellular *Croccosphaera*, similar in size ($\sim$2 μm) and N_2-fixation strategy to *Cyanothece* (Tuit et al. 2004). In *Croccosphaera* higher Fe : C was measured at night (N_2-fixing period) and low Fe : C ratios during the photoperiod. Ratios of Fe : C during the nitrogen-fixing period ranged from $\sim$7 to 27 μmol mol^{-1} (Tuit et al. 2004), similar to the values we measured in Fe-replete cultures of *Cyanothece*. These investigators calculated that an average internal concentration of 19–1,000 μmol L^{-1} Fe in *Croccosphaera* at abundances of 10^6 cells L^{-1} would require only 2.4 pmol L^{-1} Fe compared with 0.1 nmol L^{-1} available in seawater, indicating that Fe limitation in this organism in the ocean was unlikely (Tuit et al. 2004). The same appears to be true for *Cyanothece* under our experimental conditions. *Cyanothece* had a high quantum yield for photosynthesis ($F_v : F_m$) at all external levels of Fe, with only a modest decrease in photosynthetic efficiency at the lowest Fe concentration (Fig. 3). This translated into growth rates and nitrogen-fixation rates in *Cyanothece* that were almost independent of Fe over the three orders of magnitude tested here, contrasting with the Fe dependence exhibited by the two other diazotrophs (Fig. 6). Under Fe-replete conditions, *Cyanothece* exhibited an increase in Fe : P and Fe : C that may reflect a combination of higher efficiency for P uptake (Table 1) and decreased energy costs associated with replete Fe conditions. The apparent insensitivity of nitrogen fixation in *Cyanothece* to low Fe concentrations (Fig. 6), combined with its low iron use efficiency, further supports the suggestion that small diazotrophs such as *Cyanothece* are unlikely to be strongly growth limited by Fe even at nanomolar concentrations.

The effect of cell size on cellular Fe to C noted by Sunda and Huntsman (1997) was confirmed in our results (Table 1, Fig. 1): *Trichodesmium* (SA : V $\sim$ 0.5) displayed the highest Fe : C and both *Anabaena* (SA : V $\sim$ 1.5) and *Cyanothece* (SA : V $\sim$2) had extremely low Fe : C (Table 1). These characteristics may allow *Cyanothece* and other small unicellular diazotrophs to flourish in traditional Fe-limited areas of the oceans (Falcon et al. 2004; Langlois et al. 2005) as long as all other environmental parameters are favorable. Although the small size and large SA : V of *Cyanothece* provides an advantage for Fe and other nutrient uptake, the potential of the cells for luxury uptake, storage of nutrients, or spatial division of cellular processes is restricted.

Spatial segregation of N_2 fixation is advantageous because the photosynthate required for the energetically costly N_2 fixation need not be stored until the dark phase but can be generated and used in the light. *Anabaena*, known for its high growth rates (Denobel et al. 1997), generally maintained growth rates that were $\sim$two- to fourfold greater than those measured for *Trichodesmium* over a 1,000-fold range in ambient Fe concentration (Fig. 2).

Although both *Trichodesmium* and *Anabaena* fix nitrogen during the day, in *Anabaena* the process occurs in the specialized heterocysts that allow nitrogen fixation to proceed throughout the photoperiod. Thus N-fixation rates of *Anabaena* were higher at all Fe concentrations than those of *Trichodesmium* (Fig. 6). This strategy requires colonial organization and implies intercellular transportation costs (e.g., transportation of fixed N to vegetative cells) that reduce overall growth rates relative to smaller cells such as *Cyanothece*. Many metabolic processes in colonial organisms are still regulated at the cell level, so the "effective organism size" varies from that of a single cell to the whole colony, depending on the process.

The major disadvantage of spatially segregating oxygenic photosynthesis and nitrogen fixation arises under Fe-limiting conditions. In this case, Fe demand is much higher than in strategies of temporal segregation because of the need for concomitant N_2 fixation and photosynthesis and the higher PSI : PSII ratios (Fig. 4). It has generally been assumed that the primary role of PSI in cyanobacteria is to provide ATP via cyclic electron flow around the reaction center (Wolk et al. 1994). However, the photocatalyzed reduction of O_2 via PSI (Mehler reaction), which also generates ATP, is potentially a major sink for both photosynthetically generated and ambient O_2 (Berman-Frank et al. 2001*b*; Milligan et al. in press). Heterocysts lack the water-splitting, oxygen-evolving PSII and contain

only PSI complexes that supply ATP and reduce O_2. The two photosystems vary in their Fe demand as PSII is associated with only three iron atoms (two in cytochrome b559 and one nonheme iron) and coordinates the two core reaction center proteins, whereas PSI contains at least 12 iron atoms and is a major sink for iron in all oxygenic photoautotrophs (Falkowski and Raven 1997).

The importance of PSI for heterocystous diazotrophs was expressed as a large increase in PSI : PSII in *Anabaena* relative to *Trichodesmium* or *Cyanothece* at all iron concentrations, even under very low Fe concentrations (PSI : PSII $>$ 1 in *Anabaena*, Fig. 4B). Additionally, PSI : PSII ratios correlated positively with Fe availability. When Fe was limiting, PSI declined in relative abundance and a spectral shift to PSII was observed (Fig. 4A–C). This was probably due to the induction (or enhancement) of the *isiA* gene, which encodes the chlorophyll binding protein CP43′ and may work as an auxiliary PSI antenna (Xu et al. 2003) as reflected in the 77K emission spectra (Fig. 4).

Heterocystous cyanobacteria are predominantly terrestrial, freshwater, and coastal species, inhabiting eutrophic or brackish environments with a few epiphytic and symbiotic representatives in the marine environment (Paerl and Zehr 2000). Very few heterocystous free-living species are found in the pelagic oceans (Carpenter and Janson 2001). The heterocyst glycolipid envelope appears to confer a competitive advantage in low-temperature brackish and freshwater by protecting against the increased O_2 flux compared with that in seawater (Staal et al. 2003*a*). At higher temperatures, typical of tropical waters, the glycolipid envelope of the heterocyst does not provide additional protection against oxygen, which may explain the dearth of heterocystous free-living cyanobacteria in such environments (Staal et al. 2003*a*). *Anabaena* grows mostly in freshwater and brackish environments (Baltic Sea) where Fe concentrations are at least an order of magnitude higher than in the oligotrophic oceans. The higher availability of Fe allows for increased PSI in the heterocysts and thus high PSI : PSII at all but the very lowest Fe concentrations (Fig. 5A). This enables concurrent high rates of nitrogen fixation and photosynthesis yielding high growth rates that more than compensate for the nonphotosynthesizing heterocyst biomass (Figs. 2, 3, 6). When Fe is limiting, which may occur at times in the Baltic Sea (Sikorowicz et al. 2005), the lower efficiency in utilizing Fe results in declining nitrogen fixation (Fig. 6) and growth rates in *Anabaena* sp. (Fig. 2).

The combined spatial and temporal segregation strategy of *Trichodesmium* is intermediate between the strategies exhibited by *Cyanothece* and *Anabaena*. The lack of heterocysts requires fine-tuning of photosynthesis and nitrogen fixation during the photoperiod, limiting the hours of nitrogen fixation and the amount of energy and substrates produced by photosynthesis. Photosynthesis and N_2 fixation both occur in the light, but are necessarily spatially separated. Limited spatial separation occurs with nitrogen fixation seemingly concentrated in ''diazocytes'' (Lin et al. 1998; Berman-Frank et al. 2001*b*). Incomplete spatial segregation necessitates temporal separation during hours of peak nitrogen fixation with the down-regulation of PSII, up-regulation of the Mehler reaction, and other oxygen consumption pathways (Berman-Frank et al. 2001*b*). The co-occurring demands for both photosynthesis and nitrogen fixation in *Trichodesmium* dictate a high degree of efficiency and regulation of Fe uptake at all external Fe concentrations. *Trichodesmium,* which inhabits some oceanic areas that are extremely poor in Fe or P (or both), has a much higher iron use efficiency than either *Anabaena* or *Cyanothece* (Table 1). When ambient Fe is not limiting, *Trichodesmium* can increase Fe uptake and store the excess Fe (Tuit et al. 2004). *Trichodesmium* may also utilize natural colloidal Fe although the actual bioavailability of Fe is dependent on the colloidal chemical characteristics (Wang and Dei 2003). The finding by Tuit et al. (2004) that, in the absence of a source of fixed N, the nitrogenase complex in *Trichodesmium* is more metal (Fe) efficient, is confirmed by our observation of high iron use efficiency under low iron concentrations.

Another characteristic providing enhanced plasticity for *Trichodesmium* (despite its inherent disadvantage of small SA : V and low growth rates) is luxury uptake and storage of P (Krauk et al. 2006; White et al. 2006). Additionally, *Trichodesmium*'s ability to utilize phosphonates from the dissolved organic phosphorus pool (Dyhrman et al. 2006) and surface-absorbed phosphate (Sanudo-Wilhelmy et al. 2004) may account for the relatively low Fe : P and FPUE we calculated for this cyanobacterium (Table 1). The plasticity in P utilization may thus enable higher rates of N fixation when Fe is replete. Thus at high Fe concentrations N-fixation rates of *Trichodesmium* were only 3–3.5-fold lower than those of the other two diazotrophs tested here (Fig. 6).

Ancestral nitrogen fixers developed a variety of strategies for coping with their ''oxygen problem.'' These included a range of temporal and spatial structuring of metabolic processes within unicellular and colonial organizations. The morphological and biochemical adaptations of diazotrophs to oxygen impose a suite of associated physiological constraints that lead to specific trade-offs under different aquatic environments. The varied physiological responses to Fe availability of the three diazotrophs in this study reflect a combination of their nitrogen-fixation strategies, cell size, and unicellular or colonial organization, and may explain, at least in part, the ecological distribution of these organisms.

Unicellular cyanobacterial diazotrophs were traditionally thought to be insignificant in global N fixation, but their contribution may be greatly underestimated (Zehr et al. 2001; Falcon et al. 2004; Langlois et al. 2005). Their small size and rapid intracellular Fe recycling capacity makes unicellular diazotrophs relatively resistant to low concentrations of Fe and P, giving them an advantage in stable, low-nutrient waters. Colonial organization in diazotrophic cyanobacteria (e.g., *Trichodesmium*) permits a temporal protection of nitrogenase from photosynthetically evolved oxygen and the simultaneous fixation of nitrogen and carbon during the day but leads to an increase in cell size and decrease in SA : V, resulting in a decrease in metabolic rates such as growth.

Heterocystous cyanobacteria require a high investment of both energy and nutrients for cellular differentiation and may not be able to grow successfully in high-temperature and low-Fe and -P environments that characterize large oceanic regions. In such circumstances either the small unicellular forms such as *Cyanothece* or large partially spatially structured colonial forms such as *Trichodesmium* are better adapted. Current estimates suggest that *Trichodesmium* may be two- to threefold more abundant than previously reported and may account for the missing sink of ~90 Tg of N required to support observed oceanic new production (Davis and McGillicuddy 2006; Kolber 2006). The relative success of *Trichodesmium* in oceanic environments may be due to a complex combination of spatial and temporal separation of nitrogen and carbon fixation, high iron use efficiency, gas vacuoles, and plasticity in Fe and P storage that allow this organism to successfully exploit pulses of nutrients from dust events (Lenes et al. 2001) or from depth. Low grazing pressures (O'Neil 1998) or even the presence of an autocatalytic programmed cell death pathway in *Trichodesmium* that could select for more resistant cells (Berman-Frank et al. 2004) may act to further enhance survival of these larger (colonial bloom-forming) forms although their growth and nutrient-uptake rates are lower compared with small unicellular forms such as *Cyanothece*.

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