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Iron-Stimulated N_2 Fixation and Growth in Natural and Cultured Populations of the Planktonic Marine Cyanobacteria *Trichodesmium* spp.

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In light of recent proposals that iron (Fe) availability may play an important role in controlling oceanic primary production and nutrient flux, its regulatory impact on N_2 fixation and production dynamics was investigated in the widespread and biogeochemically important diazotrophic, planktonic cyanobacteria *Trichodesmium* spp. Fe additions, as $FeCl_3$ and EDTA-chelated $FeCl_3$, enhanced N_2 fixation (nitrogenase activity), photosynthesis (CO_2 fixation), and growth (chlorophyll *a* production) in both naturally occurring and cultured (on unenriched oligotrophic seawater) *Trichodesmium* populations. Maximum enhancement of these processes occurred under FeEDTA-amended conditions. On occasions, EDTA alone led to enhancement. No evidence for previously proposed molybdenum or phosphorus limitation was found. Our findings geographically extend support for Fe limitation of N_2 fixation and primary production to tropical and subtropical oligotrophic ocean waters often characterized by *Trichodesmium* blooms.

Iron (Fe) availability has recently been proposed as playing a major role in controlling primary production and associated nutrient flux in certain oceanic regions, including the subarctic and equatorial Pacific Ocean (1, 9, 10). In these regions, combined nitrogen (i.e., NO_3^-, NO_2^-, and NH_4^+) appears to be readily available, and, as such, primary production is dominated by non-N_2-fixing eukaryotic microalgae (e.g., diatoms and flagellates) and picoplanktonic cyanobacteria. However, in other oligotrophic open ocean and more-productive coastal regions, chronic nitrogen limitation can be a persistent feature (5, 6, 20). In these regions, the ability to fix atmospheric N_2 is an adaptive and effective means of circumventing N limitation (2, 8, 11). Nitrogen fixation, however, is also reliant on adequate Fe availability, since Fe is a critical constituent of the enzyme complex nitrogenase, which mediates this process (14, 18).

A particularly significant planktonic N_2 fixer is the filamentous, nonheterocystous, bloom-forming cyanobacterial genus *Trichodesmium*, which is widely distributed in tropical and subtropical oceans (3). *Trichodesmium* spp. are capable of contemporaneously fixing N_2 and CO_2 at relatively high rates and are considered a major contributor to oceanic primary production C and N cycling (3). It has been estimated that N_2 fixed by *Trichodesmium* spp. constitutes the largest single source of new nitrogen introduced into the north Atlantic Ocean's euphotic zone (3). Prior work has shown N_2 fixation to have a high demand for Fe in a cultured strain of *Trichodesmium* (18); it has been suggested that Fe limitation may be operative among natural populations as well (19). Clarifying the potential for Fe (and other nutrients') limitation of N_2 fixation and growth in this phytoplankter is of considerable importance in understanding oceanic production dynamics and bridging the gap between different paradigms concerning large-scale nutrient limitation of oceanic primary production.

Efforts aimed at identifying and characterizing nutrient limitation of N_2 and CO_2 fixation in naturally occurring *Trichodesmium* populations have been hampered by logistical problems, including conducting nutrient limitation bioassays at sea and the ability to maintain populations in a viable and representative state (i.e., as aggregated bundles of filaments) for the time required to detect nutrient limitation (24 to 48 h). Recently, we have been able to sample and maintain naturally occurring *Trichodesmium* populations (as aggregates) from Atlantic Ocean Gulf Stream waters off the coast of North Carolina and from a Caribbean Sea location south of Barbados for periods long enough to detect nutrient limitation. We have recently been able to culture North Carolina coastal *Trichodesmium thiebautii* populations (in aggregated forms) (16), thereby facilitating longer-term (several generations) characterization of nutrient limitation. Using both short- and long-term bioassays, we evaluated the potential for Fe as well as molybdenum (Mo) and phosphorus (P) limitation, since these nutrients have been proposed as possibly limiting marine N_2 fixation (4, 7, 8, 14). We report here on repeated evidence for Fe limitation and the absence of Mo or P limitation in freshly collected and cultured *Trichodesmium* populations. These findings reinforce the hypothesis that Fe may play a broad role in controlling marine planktonic primary production.

Trichodesmium populations, as fusiform (tuft) and spherical (puff) aggregates of filaments or trichomes (2, 13, 16), were collected by bucket or net tow in the Caribbean Sea (between Barbados and Grenada) and the Gulf Stream off the North Carolina coast. When viewed microscopically, freshly sampled natural *Trichodesmium* aggregates are assemblages of many organisms, including bacterial heterotrophs and eukaryotic grazers, which, when placed in containers, may consume *Trichodesmium* spp. or chemically alter the water so that *Trichodesmium* populations rapidly lose viability. Nutrient limitation experiments on natural populations were therefore

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executed within 48 h to minimize potential grazing and otherwise negative bottle effects. In this regard, the advantage of using cultured populations of aggregates is that we were assured of maintaining viable and uniform populations free of grazing effects. This permitted longer-term nutrient limitation assays.

Short-term bioassays (24 to 48 h) were conducted by dispensing freshly collected near-surface populations in replicated (triplicate or quadruplicate) 3.8-liter transparent polyethylene Cubitainers or 125-ml polycarbonate Erlenmeyer flasks. Containers were repeatedly and thoroughly cleaned prior to deployment, initially by two washes with 0.1 N HCl and then two washes with deionized water, followed by two rinses with ambient seawater. Western Atlantic and Caribbean Sea samples were incubated on shipboard in Cubitainers, maintained at surface water temperatures under natural irradiance. To prevent photoinhibition, two layers of neutral-density screening were placed over the Cubitainers. This reduced incident irradiance by approximately 50%, yielding 800 to 1,200 and 600 to 1,000 microeinsteins $\cdot m^{-2} \cdot sec^{-1}$ for Caribbean and North Carolina Atlantic coastal samples, respectively. A thorough description of bioassay deployment is provided elsewhere (15, 17). Samples from North Carolina coastal waters were assayed in 125-ml polycarbonate Erlenmeyer flasks in a constant-temperature (25°C) incubator illuminated by cool white-Gro Lux fluorescent ($\approx$100-microeinstein $\cdot m^{-2} \cdot s^{-1}$) lamps on a 14-h-light–10-h-dark cycle (16).

Bioassay response parameters included nitrogen fixation, estimated by the acetylene reduction assay (13, 16, 21); growth, estimated by chlorophyll *a* production (13, 16); and CO_2 fixation, estimated by $^{14}CO_2$ assimilation (15, 17). Acetylene reduction and photosynthetic $^{14}CO_2$ assimilation measurements were made under illuminated and dark conditions and reported as the values for illuminated conditions minus the values for dark conditions. Individual 2- to 4-h assays were conducted with either 20-ml subsamples withdrawn from polycarbonate flasks or 60-ml subsamples withdrawn from Cubitainers. Details of acetylene reduction and $^{14}CO_2$ fixation assays as applied to *Trichodesmium* populations were provided by Paerl and Bebout (13) and Prufert-Bebout et al. (16).

A culture of *T. thiebautii*, originally obtained from a North Carolina coastal Atlantic Ocean population, was established in November 1992 and has been maintained on oligotrophic seawater as described by Prufert-Bebout et al. (16) and outlined below. Cultured *T. thiebautii* consisted mainly of small puff and tuft aggregates. This population was utilized for longer-term (5- to 11-day) bioassays. The medium used for maintaining *Trichodesmium* spp. in the laboratory was prepared from offshore, oligotrophic seawater amended with magnesium, calcium, and trace concentrations (<1 μM) of PO_4^{3-} (as NaH_2PO_4), equimolar EDTA-chelated iron (as $FeCl_3$), and molybdenum (as Na_2MoO_4), as prescribed by Prufert-Bebout et al. (16). Cultures were transferred (after two washes in nutrient-depleted oligotrophic seawater) to unenriched seawater prior to bioassay experiments. Nutrients were then added, either individually or in combination, and nitrogenase activity and growth were determined.

During the course of this study, *Trichodesmium* spp. proved to be a frequent inhabitant of North Carolina coastal waters, being transported shoreward by the oligotrophic Gulf Stream meanders. From 1992 through mid-1993, we observed at least four nearshore *Trichodesmium* blooms, all actively fixing N_2 (Table 1).

Short-term bioassays of a freshly collected North Carolina natural population in 1992 indicated that Fe additions (as equimolar EDTA-chelated $FeCl_3$ or FeEDTA) led to distinct and significant (one-way analysis of variance; $P < 0.05$) stimulation of *T. thiebautii* N_2 fixation, reported as nitrogenase activity (Fig. 1a). Analogously, Caribbean populations exhibited Fe-stimulated growth, determined as photosynthetic CO_2 fixation (Fig. 1b). In contrast, neither Mo (as Na_2MoO_4, 1 to 10 μM [results for 1 μM are shown]) nor P (as NaH_2PO_4, 10 to 50 μM [results for 10 μM are shown]) enrichment enhanced nitrogenase activity or growth relative to that of untreated controls. No significant enhancement (above Fe stimulation) of N_2 fixation or growth was observed when Mo or P was provided in addition to Fe.

TABLE 1. Chlorophyll *a*-specific rates of N_2 fixation (as acetylene reduction) determined on four individual *T. thiebautii* blooms sampled in North Carolina Atlantic coastal waters from 1992 to mid-1993[a]

Date sampled	Chlorophyll *a*-specific N_2 fixation rate (nmol of C_2H_4/μg of chlorophyll *a*/h)	
	Light	Dark
5 July 1992	3.45 (±1.60)	0.21 (±0.11)
3 September 1992	1.10 (±0.55)	0.14 (±0.05)
29 October 1992	4.33 (±1.98)	0.16 (±0.08)
18 June 1993	3.79 (±1.24)	0.11 (±0.04)

[a] All bloom populations were collected within 15 km of the shoreline, near Morehead City, N.C. Values for standard deviations among triplicates are shown in parentheses.

During 1993, we examined individual effects of FeEDTA, EDTA, and unchelated $FeCl_3$. In longer-term (11-day; 2.3-generation) bioassays utilizing cultured North Carolina *T. thiebautii*, populations were maintained on oligotrophic Gulf Stream water which was initially supplemented with a trace (<0.5 μM) of P (to avoid P exhaustion during the course of the 11-day bioassay). Among five bioassays (results for only one are shown here), iron enrichment (added as FeEDTA) consistently and significantly (one-way analysis of variance; $P < 0.05$) enhanced nitrogenase activity (Fig. 2a), suggesting limitation by Fe in these oligotrophic waters. Fe limitation was further confirmed in a 5-day growth experiment in which the addition of either $FeCl_3$ or FeEDTA yielded significantly higher (one-way analysis of variance; $P < 0.05$) biomass (as chlorophyll *a*) relative to that of the control (Fig. 2b). We noted that the addition of EDTA alone also enhanced *T. thiebautii* growth. A similar stimulatory EDTA effect has been reported for a *Trichodesmium* population collected near Barbados (18). Copper (Cu) addition of up to 10 nM did not negatively impact the growth of *T. thiebautii*, suggesting that the observed EDTA effect was not necessarily related to the relief of trace metal toxicity resulting from complexation by EDTA. The pH values for *Trichodesmium* cultures and Caribbean and North Carolina Atlantic coastal seawater ranged between 8.1 and 8.3. Under this pH condition, a synergistic (stimulatory) FeEDTA effect was evident. We speculate that the addition of EDTA might have enhanced Fe availability, either by increasing the total soluble Fe or by chemically or photochemically reducing Fe(III) to Fe(II). In general, FeEDTA addition yielded additional stimulation above that with the addition of EDTA alone, demonstrating an Fe enhancement effect.

It has previously been proposed that N_2 fixation in this and other oxygenic diazotrophs might be Mo limited because of competitive inhibition by high ambient concentrations ($\approx$28 mM) of SO_4^{-2}, a structural analog of MoO_4^{-2}, the biologically available form of Mo in oxic seawater (7). Our short- and long-term Mo-enrichment bioassays (total of 10) collectively

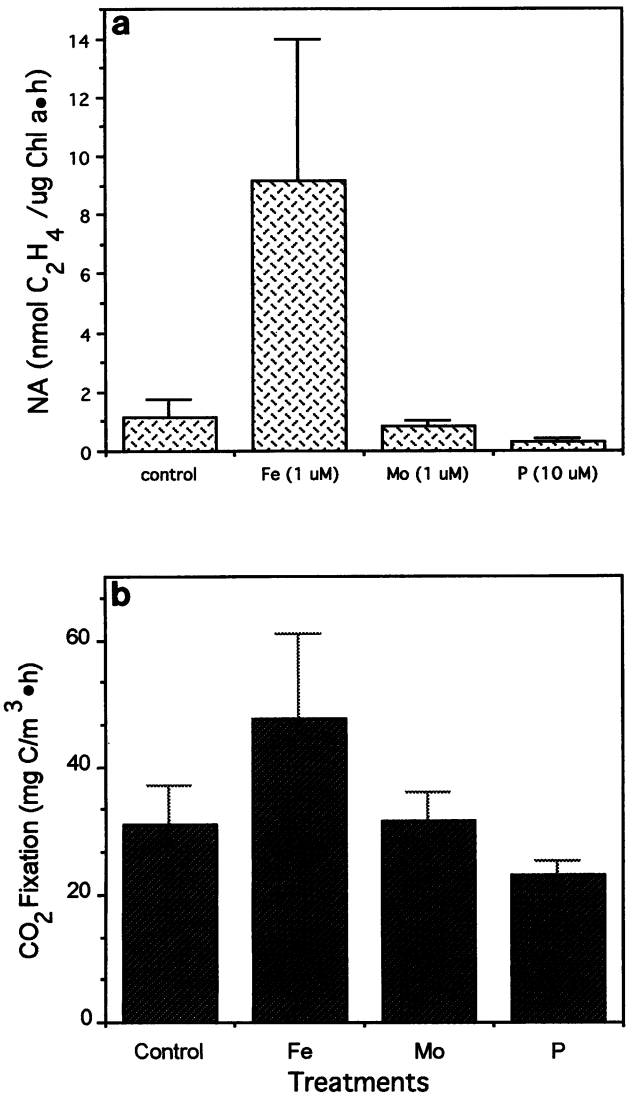

FIG. 1. Short-term bioassays evaluating impacts of Fe (1 μM), Mo (1 μM), and P (10 μM) on nitrogenase activity (NA) of *T. thiebautii* from the Atlantic Ocean off the North Carolina coast (24-h incubation) (a) and photosynthetic CO_2 fixation of a Caribbean *Trichodesmium* population (48-h incubation) (b). Chl a, chlorophyll *a*.

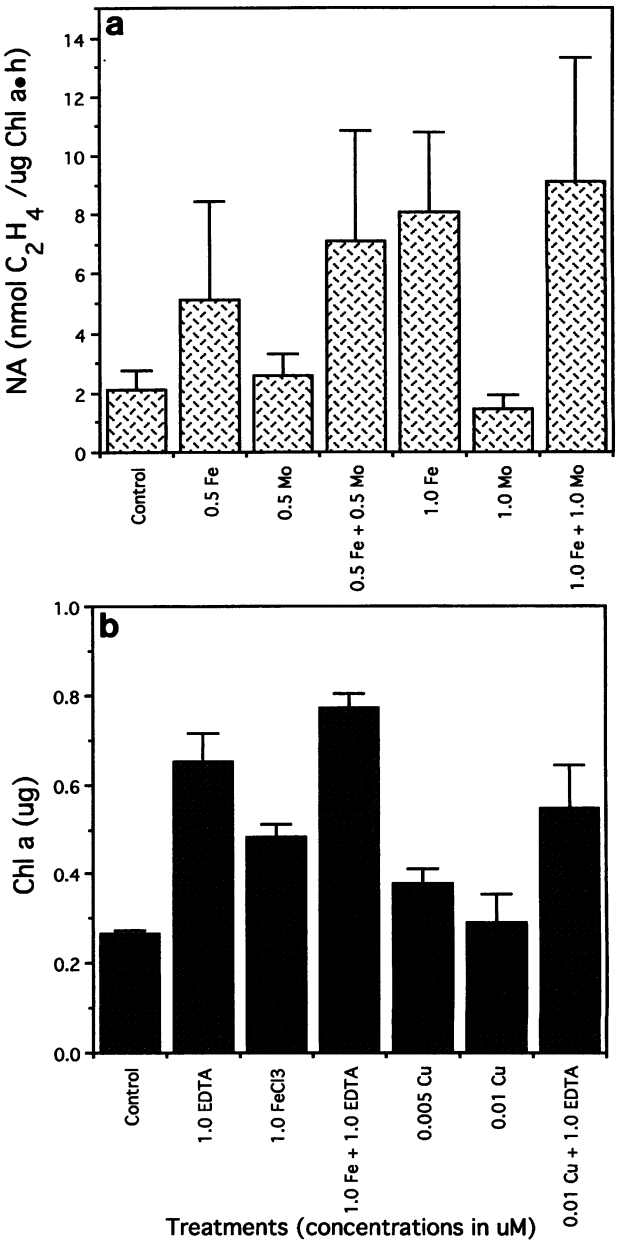

FIG. 2. Longer-term bioassays of *T. thiebautii* cultures originally isolated from a November 1992 bloom in North Carolina coastal waters. (a) Effects of Fe (as FeEDTA) and Mo addition on nitrogenase activity (NA) measured after 11 days of incubation (~2.3 generations); (b) effects of Fe (as $FeCl_3$ or FeEDTA), Cu, and EDTA on growth (chlorophyll *a* [Chl a] production) measured after 5 days of incubation (~1.2 generations).

indicated that Mo concentrations in natural seawaters were not imiting (Fig. 1 and 2; Table 2). Furthermore, no negative impact on nitrogenase activity or chlorophyll *a* production could be found by increasing ambient SO_4^{-2} concentrations, giving an SO_4^{2-}/MoO_4^{2-} ratio of 3.7×10^5, in large excess of the natural seawater ratio of 2.6×10^5 (Table 2). In fact, 20 mM SO_4^{2-} enrichment led to a slight (though statistically insignificant) enhancement of chlorophyll *a*-specific N_2 fixation, relative to that of untreated controls. The nature of such SO_4^{2-} is unclear, but it has been observed in North Carolina coastal waters on previous occasions (14).

Both short- and long-term bioassays provide a direct demonstration that Fe can be a limiting nutrient for natural populations of *Trichodesmium* spp. occurring in tropical and subtropical ocean waters. Our measurements of N_2 fixation (nitrogenase activity), photosynthesis (CO_2 fixation), and growth (chlorophyll *a* production) consistently point to the importance of Fe availability in the regulation of N_2 fixation and growth in this diazotroph. Iron limitation of primary production has been proposed for certain oceanic regions characterized by high (excess) nitrate and low chlorophyll *a* concentrations (1, 9, 10). Our results broaden the case for Fe limitation of primary production to include N-depleted ocean regions where the N_2 fixers *Trichodesmium* spp. account for a significant fraction of the new C and N inputs. Interestingly, N_2

TABLE 2. Impacts of 1 μM Fe (as $FeCl_3$), 1 μM Mo, and 20 mM SO_4^{2-} additions on chlorophyll *a*-specific rates of N_2 fixation and chlorophyll *a* concentrations in cultured *T. thiebautii*[a]

Treatment	Chlorophyll *a* concn (μg/liter)	Chlorophyll *a*-specific N_2 fixation rate (nmol of C_2H_4/μg of chlorophyll *a*/h)	SO_4^{2-} or MoO_4^{2-} concn
None (control)	10.2 (±2.8)	20.1 (±4.8)	2.6×10^5
1 μM Fe	15.7 (±3.1)	37.2 (±8.7)	2.6×10^5
1 μM Mo	8.9 (±2.3)	19.3 (±2.3)	0.2×10^5
20 mM SO_4^{2-}	9.7 (±1.9)	29.1 (±12.6)	3.7×10^5

[a] Samples were incubated for 6 days following nutrient additions. Significant ($P < 0.05$; analysis of variance) stimulation of both N_2 fixation and chlorophyll *a* production by Fe was observed, while neither Mo nor SO_4^{2-} additions had any significant impacts. Means and standard deviations among triplicates are indicated.

fixation in marine surficial and benthic microbial (cyanobacterial and bacterial) communities generally reveals a lack of Fe limitation (11, 12, 14). Rather, in these communities nitrogenase activity is more closely controlled by other factors, including organic matter inputs (11, 14). We suggest that this disparity may be related to Fe sequestering by sedimentation and effective cycling in benthic and surficial communities, in stark contrast to the planktonic environment in which Fe loss due to sedimentation presents both an acute and chronic problem. In these environments, new Fe inputs from the atmosphere, terrestrial discharge, and upwelling may represent key limitations to maintaining N_2 fixation and production potentials.

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