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Molecular assessment of phosphorus and iron physiology in *Trichodesmium* populations from the western Central and western South Atlantic

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Abstract

Trichodesmium is capable of responding to low phosphorus (P) and low iron (Fe) concentrations through the induction of alkaline phosphatase (AP) activity (an enzyme that hydrolyzes inorganic phosphate from phosphomonoesters) and the IdiA protein (an iron deficiency protein that putatively binds iron), respectively. We monitored AP activity and IdiA expression as molecular diagnostics of P and Fe stress in *Trichodesmium* field samples on a transect from the western central to the western South Atlantic and compared the expression of these markers with physical, chemical, and N_2 fixation measurements. *Trichodesmium* AP activity was detected at many stations, whereas IdiA expression was not. Incubation additions of inorganic P at one station resulted in a loss of AP activity and an increase in N_2 fixation. Our data suggest that P, and not Fe, is the constraining factor limiting *Trichodesmium* N_2 fixation along most of the transect. These results support previous modeling efforts predicting that diazotrophs in this region are P-stressed and further validate the use of molecular diagnostics of nutritional physiology, in concert with more traditional approaches, for identifying constraints on marine N_2 fixation.

Photosynthetic fixation of carbon dioxide (CO_2) in the ocean accounts for approximately half of the total global primary production (Field et al. 1998). Cyanobacteria are prominent constituents of the marine biosphere that contribute significantly to this ''biological carbon pump'' (Waterbury et al. 1986; Partensky et al. 1999*a*; Partensky et al. 1999*b*). The factors that control the growth of cyanobacteria directly effect not only the carbon pump but also the global nitrogen cycles through the activity of the nitrogen (N_2)-fixing genera *Trichodesmium* spp. and *Crocosphaera* spp. (Waterbury and Rippka 1989; Capone et al. 1997; Zehr et al. 2001 and references therein). Observations from the tropical and subtropical North Atlantic indicate that species of *Trichodesmium* are the most significant cyanobacterial primary producers (fixing ~165 mg m^{-2} day^{-1} CO_2) (Carpenter and Romans 1991). Furthermore, numerous studies in these regions have shown that *Trichodesmium* introduces a large quantity of new nitrogen into the euphotic zone (~30 mg m^{-2} day^{-1} N), values that frequently exceed the estimated flux of nitrate across the thermocline (Carpenter and Romans 1991; Capone et al. 2005). In short, cyanobacteria are significant primary producers at the base of the marine food chain, with the genus *Trichodesmium* being particularly important in the tropical and subtropical oceans.

With the global importance of N_2-fixing organisms like *Trichodesmium*, the factors that constrain their growth, abundance, species diversity, productivity, and N_2 fixation rates are active areas of research. Previous studies have implicated a number of factors in limiting the abundance and N_2 fixation rates of *Trichodesmium* in different regions of the Atlantic Ocean. For example, in the western Central and western North Atlantic, phosphorus (P) has been shown to limit the growth and N_2 fixation rates of *Trichodesmium* by use of molecular diagnostics of P stress, P uptake rates, and P quotas as metrics of P physiology (Sanudo-Wilhelmy et al. 2001; Dyhrman et al. 2002). Other work in the eastern tropical Atlantic, a region that has been documented to receive inputs of iron (Fe) higher than those observed in the western central Atlantic, used bottle incubations to identify the potential of Fe and P to colimit N_2 fixation (Mills et al. 2004). Further, a study from the Atlantic Meridional Transect program in the U.K. has

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suggested that physical forcing, not macronutrient or trace metal availability, is the primary factor limiting *Trichodesmium* abundances and N_2 fixation rates south of the equator (Tyrrell et al. 2003). Finally, recent modeling efforts implicated P as the primary element limiting the growth of diazotrophs in the north tropical and western South Atlantic (Moore et al. 2004). Despite this progress, there remain relatively few published data on *Trichodesmium* abundance, physiology, and N_2 fixation rates from the western central and South Atlantic oceans.

The studies mentioned above emphasize the importance of Fe, P, and physical forcing in the dynamics of *Trichodesmium* populations in the Atlantic. Furthermore, they highlight the many approaches (e.g., modeling, molecular diagnostics, nutrient quotas, bottle incubations, etc.) that can be used to examine physiochemical constraints on *Trichodesmium* growth and N_2 fixation in the field. Multiple approaches are necessary because of the inherent challenges associated with characterizing the physiological state of individual genera in situ. The genus *Trichodesmium* is rare compared with other marine phytoplankton because colonies can be physically isolated from water samples and washed. This facilitates genus-specific physiological assays such as the measurement of nutrient or metal quotas in the colonies. Although *Trichodesmium* colonies can be isolated from the field, they can carry substantial populations of associated microbes (Sheridan et al. 2002), which are hard to remove. While these associated microbes may not considerably influence quota measurements, the presence of non-*Trichodesmium* biomass may cause difficulties in the implementation of some assays of physiology, such as uptake rates and traditional enzyme activities. To complement these methods we have established *Trichodesmium*-specific approaches for detecting the expression of the Fe-regulated protein IdiA (Webb et al. 2001) and the activity of the P-regulated enzyme alkaline phosphatase (AP) (Dyhrman et al. 2002), resulting in two genus-specific molecular-level diagnostics for *Trichodesmium* Fe and P physiology, respectively. When applied in the field, these molecular diagnostics can reveal increased information on the physiological status of a population, severe Fe-deficiency in the case of immunological detection of IdiA, and P stress in the case of AP activity. Here we note that a population experiencing Fe defiency or P stress indicates a response to low Fe or P, but not necessarily a limitation of growth rate or N_2 fixation because the extent to which stressed *Trichodesmium* are able to scavenge Fe or P will ultimately result in either chronic limitation or recovery from the stressed state.

Despite considerable advances in our understanding of *Trichodesmium* physiological ecology, the factors that constrain *Trichodesmium* growth and N_2 fixation in key tropical and subtropical environments remain to be determined. In this study we sought to empirically examine the influence of P, Fe, and physical forcing on *Trichodesmium* abundance and N_2 fixation rate from the western central and western South Atlantic using multiple approaches: bottle enrichments, correlations of nutrient and metal concentrations, physical parameters, and the presence of *Trichodesmium* AP activity and IdiA. To our knowledge this is the first field study to apply molecular diagnostics for both Fe and P on the same field samples. Our results show that P is the likely limiting nutrient for *Trichodesmium* at most stations sampled, whereas in some stations the primary limiting factor remains to be determined.

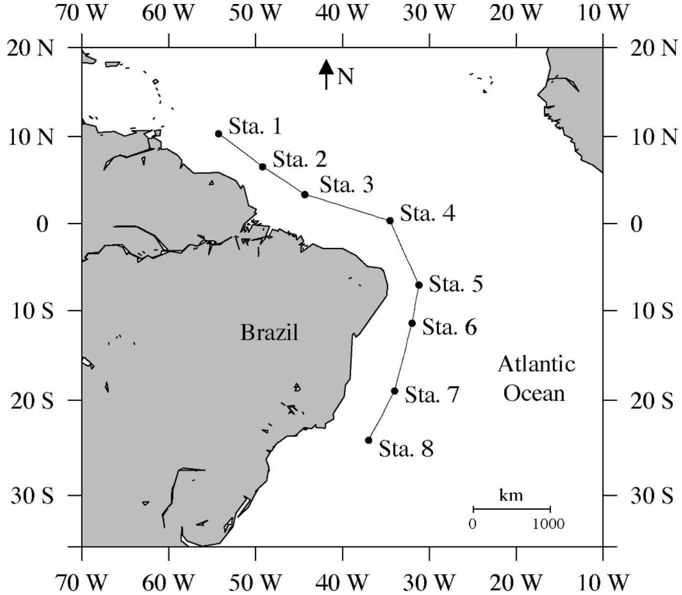

Fig. 1. EN367 cruise map sampled in March 2002.

Material and methods

Trichodesmium cultures and field sampling—Trichodesmium erythraeum IMS101 control cultures for the molecular methods were grown in replete, P-, and Fe-omitted conditions as described previously (Webb et al. 2001).

Field samples were collected in March 2002 aboard the RV *Endeavor* on cruise EN367, which followed a cruise track from Barbados to Rio de Janeiro (Fig. 1). In the field, colonies were obtained from surface water by hand-towing a 130.0-μm plankton net (Sea-Gear) with a 25-m line. Abundance assessments were based on visual estimates of the relative amount of *Trichodesmium* colonies obtained per net tow. All net tows for *Trichodesmium* N_2 fixation rates or incubations were performed during the daylight hours when rates were predicted to be the highest (11:00–13:00 h). Colonies in the cod end were rapidly transferred to an acid-cleaned 2-liter polycarbonate beaker, shielded from direct sunlight, and taken inside for sorting and washing. Individual colony types were picked using sterile polypropylene pasteur pipettes and washed twice in 0.2-μm-filtered sterile seawater, and immediately assayed for nitrogenase activity, AP activity, or gently filtered onto 5.0-μm polycarbonate filters and stored at -20°C for subsequent IdiA immunological detection in the laboratory. Colonies for AP activity assays and IdiA immunoblotting were collected at 08:00 h local time and archived approximately 2–3 h before the collection of *Trichodesmium* samples for the N_2 fixation experiments. Samples for AP assays were collected at all stations, but because of constraints in *Trichodesmium* biomass or the timing of the station, samples for N_2 fixation could only be collected at Stas. 2, 3, 4, 7, and 8 and IdiA samples from Stas. 3, 7, and 8.

Physical and chemical parameters—The water column structure from the upper 200 m was characterized using a Sea Bird Electronics 911*plus* CTD. Samples for total dissolved Fe and nutrient analyses were collected using 10-liter Teflon-coated Go-Flo bottles (General Oceanics) on a Kevlar hydrowire. Go-Flo bottles were pressurized with filtered N_2 gas and samples transferred directly from the bottles into a laminar flow hood through acid-cleaned Teflon tubing. Samples were filtered through acid-cleaned 0.2-μm polycarbonate filters using an acid-cleaned Teflon filter rig. Nutrient samples were collected in 50-mL polypropylene screw-cap tubes (cleaned with trace-metal-grade 10% HCl before use) and immediately frozen at -20°C, while samples for total Fe were acidified using concentrated Seastar Baseline HCl (Seastar Chemical) and stored in rigorously acid-cleaned low-density polyethylene bottles until analysis. Nutrient concentrations were measured by Susan Becker and Douglas Masten at the Ocean Data Facility of the Scripps Institution of Oceanography using a standard protocol for the Skalar San Plus autoanalyzer with a detection limit of approximately 3 nmol L^{-1} for nitrate and nitrite combined and phosphate. Hereafter we refer to these parameters as dissolved inorganic nitrogen (DIN) (NO_3 + NO_2) and dissolved inorganic phosphorus (DIP). We are unable to report ammonia concentrations because the samples were stored for an extended period.

Total dissolved Fe was measured using the $Mg(OH)_2$ coprecipitation method of Wu and Boyle (1998). Sample vials were soaked in warm 1 mol L^{-1} instra-analyzed HCl (J. T. Baker) for 48 h, rinsed three times with Milli-Q water, and then filled with 5% Seastar Baseline HCl and heated for 24 h. Before use, sample vials were emptied of the 5% HCl and rinsed once with sample. Then vials were filled with 1.3 mL of sample and supplemented with an ^{57}Fe-enriched isotope supplement (Cambridge Isotope Laboratories), which was allowed to equilibrate overnight. Next, 20 μL of 10% Seastar ammonia was added to each sample. After 1.5 min, samples were centrifuged at 6611 *g* for 1 min. The supernatant was shaken off, and then the pellet was recentrifuged and any remaining supernatant again shaken off. Sample precipitates were dissolved in 2.5% Optima HCl (Fisher Scientific) and analyzed on a Finnigan ELEMENT2 inductively coupled plasma mass spectrometer (Thermo Electron Corp.). For procedural blanks, a $Mg(OH)_2$ precipitation was performed on acidified Sargasso seawater and the supernatant was saved as low-Fe seawater. The low-Fe seawater was used as a blank and prepared as samples except that a smaller volume was used (50 μL). The average blank value was 0.12 nmol L^{-1}. The detection limit, calculated as three times the standard deviation of the blank, was 0.10 nmol L^{-1}.

Nitrogenase activity—Nitrogenase activity was measured as described elsewhere (Tuit et al. 2004) using the 4 : 1 ratio for converting acetylene reduction to N_2 reduction (Capone and Montoya 2001). The following modifications to the protocol were used: 30 mL of sample were assayed in the 60-mL polycarbonate bottles, 10–15 hand-sorted *Trichodesmium* colonies were assayed, the samples were incubated at 50 μmol m^{-2} s^{-1} quanta at 28°C in a shipboard Percival incubator (Percival Scientific). After the nitrogenase activity assay was completed, the colonies were drawn onto a GF/F filter for determinations of chlorophyll *a* (Chl *a*) concentration. Briefly, the filters were extracted in 90% acetone overnight at -20°C and analyzed using a handheld Aquafluor fluorometer (Turner Designs). Chl *a* concentrations were calculated following the publicly available Bermuda Atlantic Time-Series study (BATS) protocol based on Herbland et al. (1985) and references therein. N_2 fixation results are reported as nmol N h^{-1} μg^{-1} Chl *a* or nmol N h^{-1} per colony. One-way analysis of variance (ANOVA) Tukey tests were applied to the data to determine which rates were statistically different ($p < 0.05$).

Trichodesmium incubation—Approximately 160 *Trichodesmium* puff colonies from Sta. 8 were isolated, separated, and washed as described above. These colonies were placed into 60-mL Nalgene polycarbonate bottles that were cleaned with Micro and 0.5 mol L^{-1} trace-metal-grade HCl and fitted with Teflon-coated silicone septa (I-Chem). Two replications of 20 colonies per condition were resuspended in 30 mL of 0.2-μm local-filtered seawater supplemented with the following (final concentration): (1) 30 μmol L^{-1} sodium phosphate (DIP), (2) 50 nmol L^{-1} ferric citrate (Fe), (3) both 30 μmol L^{-1} sodium phosphate and 50 nmol L^{-1} ferric citrate (Fe-DIP), or (4) no addition (cntrl). These treatments were incubated in a Percival incubator at 50 μmol m^{-2} s^{-1} quanta at 28°C for 48 h. We emphasize that these incubation conditions were chosen to mimic culture conditions known to repress *Trichodesmium* AP activity and IdiA expression, so as to assess in situ regulation of these parameters. The conditions are not representative of ambient conditions. After incubation, aliquots were removed for assays of AP activity and IdiA (described below), while N_2 fixation rates were measured in the remaining colonies as described above ($\sim$10 colonies per bottle). After completion of nitrogenase rate measurements, total Chl *a* per bottle was determined as described above. One-way ANOVA Tukey tests were used to determine which rates were statistically different ($p < 0.05$).

AP activity—In this study we used the modified enzyme-labeled fluorescence (ELF) assay described previously (Dyhrman et al. 2002) to assess *Trichodesmium*-specific AP activity. This procedure can distinguish between the AP activity of *Trichodesmium* and associated microbes on the basis of the presence or absence of a fluorescent green precipitate. The ELF assay can be used to identify the presence or absence of AP activity, but the assay does not generate specific hydrolytic rates for the enzyme. In this regard, it differs from more commonly applied assays of AP activity in *Trichodesmium* (Sohm and Capone 2006), which provide hydrolysis rates, but are not species specific, as AP activity can come from multiple sources. For each sample, two washed colonies were placed into 100 μL of sterile seawater combined with 5 μL of the ELF-97 reagent (Molecular Probes). This sample was allowed to react for 45 min in the dark at room temperature. After this incubation the colonies were drawn onto a 25-mm 5.0-μm polycarbonate filter and washed twice with 1.0 mL of sterile seawater. The biomass and the filter were placed in 1.0 mL of histology-grade 10% neutral buffered formalin (Surgipath) and incubated for 45 min to 1 h in the dark at room temperature. To aid in the microscopic determination of ELF-positive cells, the samples were shaken vigorously for 10 s to remove any existing free trichomes from the filter and break up the colonies into free trichomes. The resulting suspension was drawn onto a second 25-mm 5-μm polycarbonate filter, and the filter was mounted onto glass slides with the mounting media provided in the ELF Phosphatase Detection Kit (Molecular Probes) and covered with a 25-mm coverslip. Using the above protocol the samples are stable for several months when kept in a damp container at 4°C in the dark. The slides were imaged using a Zeiss Axioplan2 equipped for phase contrast, epifluorescence microscopy, and digital photomicroscopy in the laboratory, and a Zeiss Axioscop equipped for epifluorescence microscopy at sea. To avoid biases in the scoring, slides were coded and initially scored by two investigators at sea and then again using a double-blind approach back in the laboratory. Three classifications were obtained: positive (+) when all filaments were labeled with the green fluorescent ELF precipitate, weak positive (±) when heterogeneous labeling was observed, or negative (−) when there was no observable labeling.

IdiA expression—IdiA protein expression was assayed using polyclonal antisera raised against freshwater *Synechococcus* PCC6301 IdiA (generously provided by E. Pistorius) as described previously (Webb et al. 2001) with minor exceptions. In brief, protein extracts were generated by resuspending filtered trichomes and colonies in 100–300 μL of Bug-Buster (Novagen) in 1.5-mL microfuge tubes at 4°C and sonicating with a Branson 545 sonifier with a high-intensity microcup horn attachment (Branson Ultrasonics). The samples were sonicated for 1 min with alternating 0.5-s on–off pulses, returned to the ice for cooling, and repeated for a total sonication time of 3 min. To keep the samples from overheating while in the sonicator, ice water was pumped through the microcup horn using a peristaltic pump. Cellular lysis was verified visually using a Zeiss Axioplan2 microscope. Protein in each extract was quantified using the BioRad DC protein assay per the manufacturer's instructions. For Western blot analyses, 7 μg of total extract from severely Fe-deficient control cultures of *Trichodesmium erythraeum* IMS101 and from sorted field samples (*Trichodesmium* puffs and tufts) were loaded in each lane. Under these assay conditions, and with constraints in the cross-reactivity of the primary antibody, the *Trichodesmium* IdiA protein can be detected under acute Fe depletion, but not at the early stages of Fe depletion, hereafter referred to as Fe stress. With the incubation samples we also probed for *Trichodesmium* NifH using polyclonal antisera (1 : 8000 dilution) raised against the *Azotobacter vinelandii* NifH protein (generously provided by Dr. Paul Ludden) using the Immun-Blot Amplified Alkaline Phosphatase Kit (BioRad).

Results

Transect hydrography and apparent Trichodesmium *abundances*—Surface samples of *Trichodesmium* were obtained using plankton net tows on cruise EN367 transecting from Barbados to Rio de Janeiro in March 2002 (Fig. 1). Multiple colony morphologies were present and identifiable at every station, though qualitative abundances of *Trichodesmium* changed along the cruise track (Table 1). *Trichodesmium* abundance was highest at the two most southern stations (Stas. 7 and 8) and lowest at 7–11°S (Stas. 5 and 6). Two morphotypes, puffs and tufts (tentatively identified as *Trichodesmium thiebautii* and *T. erythraeum*, respectively), were frequently much more abundant at each station than other species and therefore were the primary foci for subsequent physiological characterization.

During EN367, there was a general shoaling of the mixed layer along the transect with a concomitant trend of increasing salinity (Fig. 2), as has been previously observed in this area (Memery et al. 2000). There was no consistent trend between mixed layer depth and *Trichodesmium* abundance. Although *Trichodesmium* was most abundant at the station with the shallowest mixed layer (40 m, Sta. 8), other stations with intermediate mixed layer depths (Sta. 5, 75 m; Sta. 6, 50 m) had very low apparent surface *Trichodesmium* abundances (Fig. 2 and Table 1). Furthermore, Stas. 2 and 3 had relatively deep (~100 m) mixed layer depths with intermediate *Trichodesmium* abundances. Salinity values were lowest (~35–36) north of the Equator and increased (≥37) to the south (Fig. 2 and Table 1). The southernmost stations (Stas. 7 and 8) were two of the saltiest in character, and were associated with the highest relative *Trichodesmium* abundances (Fig. 2 and Table 1).

Surface nutrients and Fe concentrations—Nutrient concentrations along the entire transect were consistent with oligotrophic conditions (<5 μmol L^{-1}), with DIN concentrations ranging from 0.01 μmol L^{-1} at Sta. 1 to 0.19 μmol L^{-1} at Sta. 5 (Table 1). At Stas. 5 and 6 the DIN concentrations were the highest of the transect, with values of 0.19 and 0.12 μmol L^{-1}, respectively (Table 1). DIP concentrations were variable along the transect, with the highest concentrations (0.12 μmol L^{-1}) found at Sta. 2 and the lowest values obtained at Sta. 8 (0.01 μmol L^{-1}) (Table 1). As with DIN, the DIP concentrations from Stas. 5 and 6 were some of the highest values that were measured during the cruise (~0.10 μmol L^{-1}) (Table 1). This macronutrient elevation was associated with decreased *Trichodesmium* abundances (Table 1) and may be attributed to the effect of the equatorial upwelling combined with the nutrient-enriched waters being drawn south via the Southern Equatorial Current in this part of the transect.

The total dissolved Fe concentrations in the surface mixed layer ranged between 0.14 and 0.93 nmol L^{-1} (Table 1). Total Fe concentrations followed the predicted trend of higher levels in the north and lower levels in the south (Table 1), data that are in agreement with the

Table 1. Station location, physiochemical conditions, and *Trichodesmium* abundance and physiology at stations on transect EN367.

Sta.	Date	Latitude	Longitude	Sample depth	M.L. depth	Total Fe (nmol L^{-1})	DIN (μmol L^{-1})	DIP (μmol L^{-1})	T* (°C)	RTA†	ELF‡	IdiA§
1	02 Mar	10.36°N	54.23°W	15 m	85 m	0.93±0.06	0.01±0.00	0.11±0.00	27.2	Med	+/+	n.a./n.a.
2	04 Mar	06.58°N	49.19°W	15 m	100 m	0.60±0.02	0.07±0.01	0.12±0.00	27.2	Med	−/+	n.a./n.a.
3	06 Mar	03.29°N	44.30°W	15 m	96 m	0.46 (n=1)	0.04±0.00	0.05±0.01	27.5	Med	−/+	n.a./−
4	10 Mar	00.33°S	34.54°W	15 m	70 m	0.31±0.02	0.06±0.00	0.04±0.01	28.1	Med	−/−	n.a./n.a.
5	12 Mar	07.10°S	31.20°W	15 m	75 m	n.a.	0.19±0.00	0.11±0.00	28.2	Low	−‖	n.a./n.a.
6	13 Mar	11.42°S	32.00°W	40 m	50 m	0.35±0.09	0.12±0.01	0.10±0.00	27.6	Low	−/−	n.a./n.a.
7	16 Mar	19.02°S	34.02°W	20 m	55 m	0.14±0.01	0.07±0.01	0.04±0.01	28.1	High	±/−	−/−
8	18 Mar	24.3°S	37.01°W	20 m	40 m	0.27±0.01	0.09±0.00	0.010±0.00	27.3	High	−/+	−/−

* Sea temperature at 9 m.
† Relative surface *Trichodesmium* abundances.
‡ ELF results in the puff/tuft morphologies are expressed as + indicating highly labeled, ± indicating intermediate labeling, and − indicating little or no labeling.
§ IdiA in the puff/tuft morphologies; − indicates no target protein was detected, n.a. indicates not assayed.
‖ Puff and tuft morphologies were pooled.

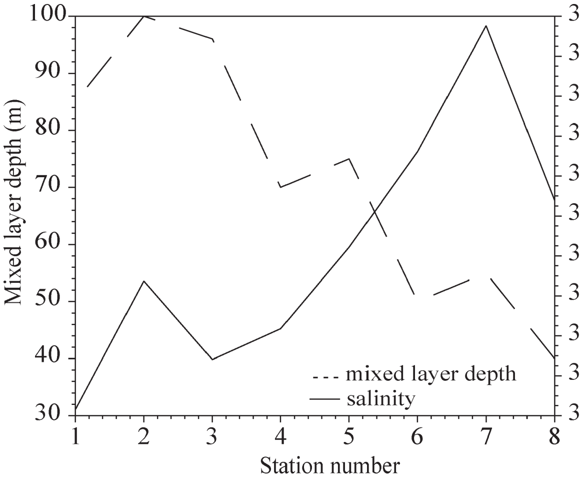

Fig. 2. Hydrological characterization of the stations on EN367. Mixed layer depth as determined from σ_T (dashed line) and salinity at 9 m (solid line) are plotted from all stations sampled along the transect.

documented high flux of aeolian dust (that can deposit Fe) from Africa into the Caribbean and equatorial Atlantic (Husar et al. 1997). The Fe concentrations are also consistent with 0.4-μm filtered Fe values measured by other researchers on the same cruise (Bergquist and Boyle 2006; Cullen et al. 2006). Interestingly, Stas. 7 and 8 had the lowest measured Fe concentrations for the cruise track, fairly low nutrient concentrations, but the highest *Trichodesmium* abundances of the transect (Table 1).

Nitrogen fixation rates—At discrete stations along the transect in situ N_2 fixation rates were determined for both puff and tuft morphotypes (Fig. 3). The N_2 fixation rates were calculated in both nmol N h^{-1} μg^{-1} Chl *a* and nmol N h^{-1} per colony and are expressed as the average number value obtained from 11:00 h to13:00 h with standard error. Chl *a* normalized N_2 fixation rates ranged from 1.36 to 15.9 nmol N h^{-1} μg^{-1} Chl *a*. The average Chl *a*-normalized N_2 fixation rate was higher in the tuft morphology than in the puff morphology at every station (Fig. 3A). Colony-normalized N_2 fixation rates ranged from 0.004 to 0.095 nmol N h^{-1} per colony. There was not as pronounced a difference in N_2 fixation rates between morphotypes when rates were normalized to colony rather than Chl *a* (Fig. 3A,B). The overall spatial trend in N_2 fixation rates was similar using either normalization scheme, with the highest N_2 fixation rates observed at Sta. 8 in both morphotypes, whereas the lowest N_2 fixation rates were seen at Sta. 4 (Fig. 3A,B). For the Chl *a*-normalized puff samples, Sta. 8 was shown to be significantly higher than Stas. 4 and 7, but not higher than Stas. 1 and 2 (Fig. 3A). Conversely, for the Chl *a*-normalized tuft samples, Stas. 2 and 3 were shown to be statistically different from Stas. 4 and 8, but not from Sta. 7 or from each other (Fig. 3A). In the case of the colony-normalized N_2 fixation rate, the tuft-specific N_2 fixation rates obtained from Sta. 8 were statistically higher than every other station while all of the other rates were not statistically different (Fig. 3B). The

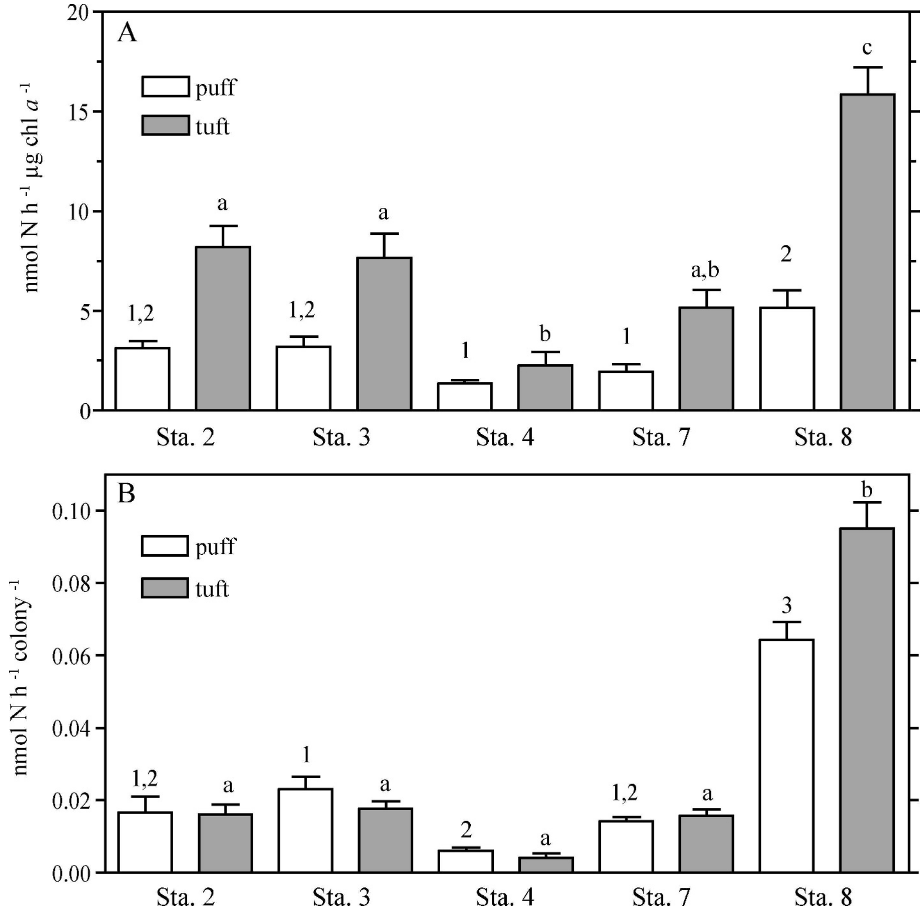

Fig. 3. In situ N_2 fixation rates were determined for both *Trichodesmium* morphotypes (tufts and puffs) and normalized to (A) micrograms of Chl *a* and (B) colony number. Standard error between replicates is displayed. Stations that have statistically different N_2 fixation rates on the basis of one-way ANOVA Tukey tests are indicated by different numbers (puffs) or letters (tufts) above the bar.

colony-normalized puffs showed a different pattern, where Sta. 3 was different from Stas. 4 and 8 but not different from Stas. 2 and 7 (Fig. 3B).

Normalizing rates to the number of colonies is one of the most common techniques for measuring *Trichodesmium* N_2 fixation in the field (Capone et al. 2005), but to directly compare and calibrate our field numbers with rates calculated from laboratory cultures of *T. erythraeum* IMS101 (which does not form colonies) we also determined the Chl *a*-normalized rates. With IMS101 cultures grown in replete conditions, we commonly measure N_2 fixation rates as high as 10–15 nmol N h^{-1} μg^{-1} Chl *a* (data not shown); similar values have been reported by others (Mulholland and Bernhardt 2005). N_2 fixation in the tuft morphology from Sta. 8 falls near this range, but the rates are substantially lower at all the other stations regardless of colony morphology.

Differences in Chl *a* content between morphotypes was observed in samples from EN367, as has been previously described in *Trichodesmium* samples from the Red Sea (Post et al. 2002). Chl *a* analyses of 330 colonies from each colony type obtained during EN367 showed that although there was great variation, the puff forms contained more Chl *a* on average than tufts (7.4 ± 4.8 and 3.0 ± 1.7 ng for puffs and tufts, respectively). The variation in Chl *a* concentration per colony affected the N_2 fixation values calculated from some stations. For example, when N_2 fixation rates of Stas. 2 and 3 are normalized to Chl *a,* the tufts have higher rates, whereas conversely, when the rates are normalized to colony, the values are equivalent between tufts and puffs. Because of the uncertainty in these variations, the N_2 fixation rates are most valuable for intraspecific comparisons between the same colony type.

AP activity and IdiA expression—To assess the in situ P and Fe physiology of the *Trichodesmium* populations along the transect, we collected samples for AP activity and IdiA immunoblotting. AP activity was determined at all stations using the cell-specific ELF assay, and therefore should be considered *Trichodesmium* specific. AP activity was present in one or more morphotypes at most stations along the transect (Stas. 1, 2, 3, 7, and 8, Fig. 4A). There was no detectable AP activity in either morphotype at Stas. 4, 5, and 6. There was substantial heterogeneity in the presence of AP activity between the tufts and puffs obtained from the same station (Fig. 4A).

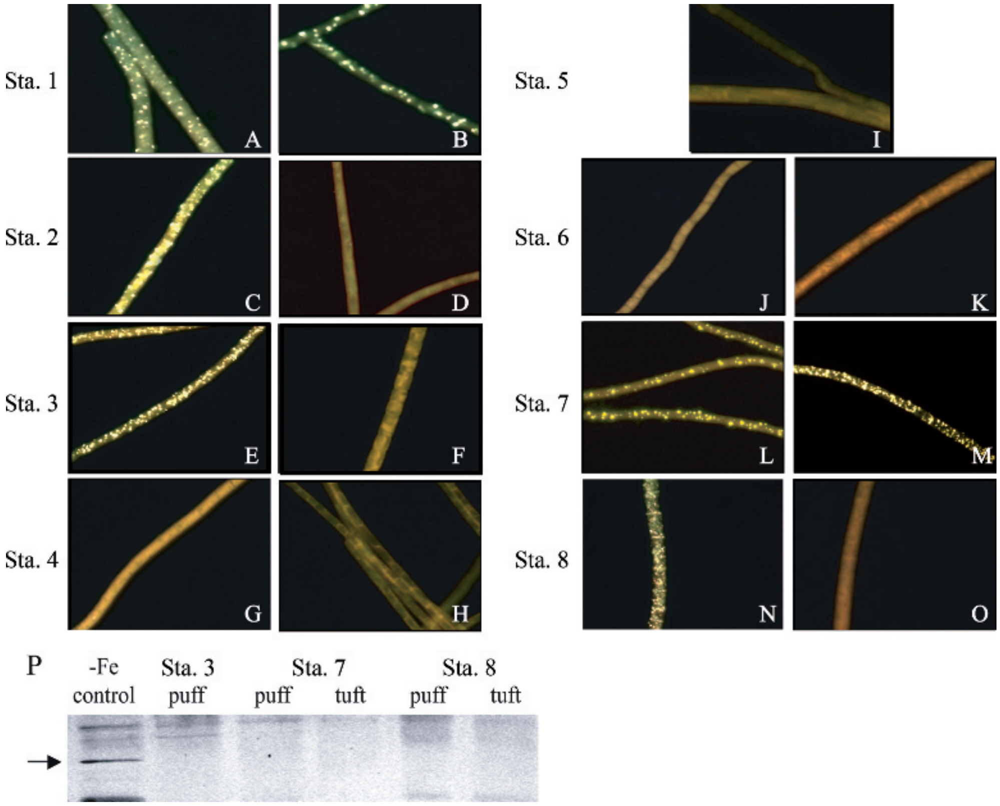

Fig. 4. In situ molecular diagnostics for P and Fe. (A–N) *Trichodesmium*-specific AP activity (P stress diagnostic) as determined by the generation of a green precipitate on normally orange autofluorescing trichomes. For each station, data are expressed as the reaction detected with sorted puffs (panels A, C, E, G, J, L, N) and tufts (panels B, D, F, H, K, M, O), except in panel I where the morphotypes were pooled. (P) Immunoblot of IdiA expression (severe Fe deficiency diagnostic) at selected stations, normalized to total protein. The arrow denotes the IdiA protein detected in extracts from laboratory-grown axenic cultures of severely Fe deficient *Trichodesmium.*

While the puff morphotypes had AP activity at almost every station (Stas. 1, 2, 3, 7, and 8), the tufts only had AP activity at Stas. 1 and 7 (Fig. 4A). Chl *a*-normalized N_2 fixation rates are consistent with this differential response, in that the tuft morphology typically had a higher N_2 fixation rate relative to the puff morphology (Fig. 3A). Colony-normalized N_2 fixation rates were not as consistent with the differential AP activity in the mophotypes when compared with the Chl *a*-normalized N_2 fixation rates (Fig. 3B). The presence of AP activity and DIP concentrations did not show a clear inverse relation in situ. For example, AP activity was present in both morphotypes at Sta. 1 where DIP was relatively high (0.12 μmol L^{-1}), yet at Sta. 8, where the DIP was 10-fold lower (0.01 μmol L^{-1}), AP activity was only present in the puff morphotype. Although AP activity was present at two stations (Stas. 1 and 2) with DIP $>$ 0.110 μmol L^{-1}, *Trichodesmium* AP activity has been frequently detected at similar DIP concentrations (or above) in other systems (Mulholland et al. 2002). Although there was not a specific inverse relation between the presence of AP activity and DIP concentrations in our study system, the presence of AP activity is clearly responsive to DIP addition (see below).

IdiA immunoblots were performed on samples with particularly low Fe concentrations (Stas. 3, 7, and 8) using *Synechococcus* PCC6301 IdiA polyclonal antisera (Fig. 4P). With this antisera, IdiA was identified in extracts from a severely Fe-deficient control culture (−Fe), but the protein was not detectable in samples from stations with total Fe concentrations down to 0.14 nmol L^{-1} (Sta. 7). It is critical to note that the –Fe control is a culture with high IdiA expression. Because of sensitivity issues with the antisera, it is not possible to completely eliminate the possibility of low to moderate Fe stress in the field samples, even though IdiA was not detected.

Incubation—A shipboard incubation was performed at Sta. 8 using the puff morphology to examine the sensitivity of AP activity, IdiA expression, and N_2 fixation to inputs of DIP and Fe. Puffs were resuspended in 0.2-μm filter-sterilized local seawater with the following supplements: no addition (cntrl), Fe, DIP, or Fe-DIP, and incubated for 48 h. After the incubation, the N_2 fixation rates in all treatments were consistently lower than the rates obtained with in situ puffs, a finding that was likely due to viability reductions in *Trichodesmium* once it is placed in a bottle (Burns et al. 2006). N_2 fixation rates are presented as

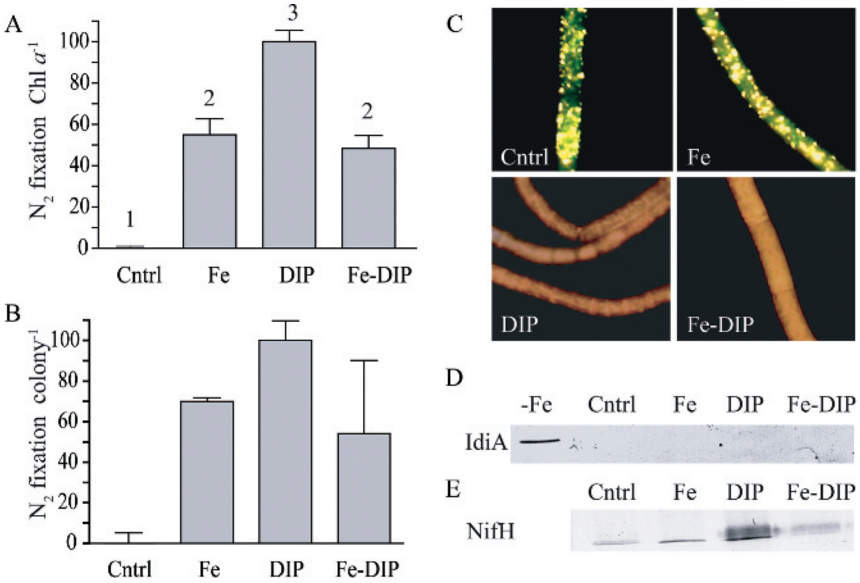

Fig. 5. Incubation results from *Trichodesmium* puff colonies collected at Sta. 8. Treatment designations are as follows: Cntrl, no-addition control; Fe, Fe addition; DIP, DIP addition; and Fe-DIP, combined addition of Fe and DIP. N_2 fixation rates are expressed as the percentage maximum rate obtained per (A) Chl *a* or (B) colony with the standard error. Stations that have statistically different N_2 fixation rates on the basis of one-way ANOVA Tukey tests are indicated by different numbers. (C) *Trichodesmium*-specific AP activity (green) detected in the different treatments. Orange color indicates pigment autofluorescence. Immunoblot detection of (D) *Trichodesmium* IdiA and (E) NifH normalized to total protein. Lanes are designated with the incubation treatment (Cntrl, Fe, DIP, Fe-DIP) or the severely Fe-deficient *Trichodesmium* culture control (−Fe).

percentages of the maximum N_2 fixation rate obtained in the DIP treatment, corrected for Chl *a* content or colony number (Fig. 5A,B). The trends between treatments are consistent (excluding the Fe-DIP treatment, which showed variation) regardless of whether or not the data are normalized to Chl *a* or colony (Fig. 5A,B). Relative to the no-addition control, there was an increase in N_2 fixation in all the treatments, with the highest being in the DIP treatment (Fig. 5A,B). There was also an increase in the N_2 fixation rates upon incubation with Fe, although not as large as the increase seen with DIP (Fig. 5A,B). The results for the Fe-DIP addition were similar to those of the Fe addition alone in the Chl *a*-normalized data (Fig. 5A); however, there was substantial variation in this treatment, which can be visualized in the colony-normalized data (Fig. 5B). When incubation N_2 fixation rates were normalized to Chl *a*, the Fe and Fe-DIP treatments were significantly higher than the control, and the DIP treatment was significantly higher than the Fe and Fe-DIP treatments. However, when the rates were normalized to colony, the variation in the Fe-DIP treatment replicates eliminated any statistical differences between the treatments.

AP activity was observed in the time-zero puff samples for Sta. 8 (Fig. 4A), the no-addition control (Fig. 5C), and the Fe addition (Fig. 5C). In contrast, no AP activity was observed in the two treatments that received DIP additions (Fig. 5C). Although there was an increase in the N_2 fixation rates in the treatments supplemented with Fe, the IdiA protein was not detectable in any of the treatments (similar to the field results), whereas the positive control blots with the same protein samples did detect the nitrogenase Fe protein, NifH (Fig. 5D,E).

Discussion

The factors that constrain the growth and N_2 fixation of marine diazotrophs such as *Trichodesmium* are varied and difficult to predict, yet identifying these factors is critical to modeling marine N_2 fixation and its ramifications. In this study, we examined the importance of Fe and P in constraining *Trichodemsium* N_2 fixation and abundance in the western Central and western South Atlantic. A combination of molecular assays, an incubation experiment, and water column chemical and hydrological analyses were performed to establish linkages between *Trichodesmium*-specific N_2 fixation rates and these physiochemical limitation factors.

Trichodesmium distribution—The preferred habitat for *Trichodesmium* is thought to be warm, oligotrophic, and salty (e.g., warm, salty, anticyclonic eddies [Davis and McGillicuddy 2006]). Elevated *Trichodesmium* abundances have also been inversely related to mixed layer depth in the South Atlantic (Tyrell et al. 2003). Although present at every station, the abundances of *Trichodesmium* varied dramatically along the EN367 cruise track, with moderate relative abundances found in the four most northern stations (Stas. 1–4), low abundances found at Stas. 5 and

6, and high abundances observed at the two most southern stations (Stas. 7 and 8). It is noteworthy that the highest *Trichodesmium* abundances were observed at relatively salty stations with shallow mixed layer depths (Stas. 7 and 8). Despite this observation, no relation between relative *Trichodesmium* abundances and mixed layer depth could be established across the whole transect.

The Amazon River plume and equatorial upwelling are two regional factors that may influence *Trichodesmium* abundance over portions of the western central and South Atlantic transected in this study. The Amazon River plume can extend for many months and thousands of kilometers, and has been shown to cause large variations in N_2 fixation rates and *Trichodesmium* abundances (Carpenter et al. 1999; Capone et al. 2005). Although the highest Amazon flow rates are in June through September, the NO_3 concentrations in the plume are typically the highest in March when the river flow rate is increasing and the rising water resuspends deposited nutrients from the previous season (Demaster and Pope 1996). Despite the fact that NO_3 concentrations in the Amazon are the highest in March, the lower flow rate and seasonal currents generally restrict the effect of the Amazon Plume to the South American coastal zone, thereby reducing the likelihood that it was influencing *Trichodesmium* in the offshore waters sampled during our March EN367 cruise.

Equatorial upwelling, on the other hand, may have affected *Trichodesmium* abundances and N_2 fixation rates at Stas. 5 and 6 during EN367. The westernmost WOCE (World Ocean Circulation Experiment) leg A17 followed a very similar cruise track to EN367 (Memery et al. 2000; Wienders et al. 2000). In WOCE-A17, high-resolution hydrographic data was collected from January to March 1994 that allowed for characterization and definition of the water masses along the western boundary of the South and equatorial Atlantic. Although limited in resolution, the physiochemical data from EN367 supports the hypothesis that equatorial upwelling was influencing *Trichodesmium* at Stas. 5 and 6. For example, although still submicromolar, the surface nutrient concentrations at Stas. 5 and 6 were the highest DIN values, and close to the highest DIP values (both >0.1 μmol L^{-1}), observed on this cruise. It is possible that in these upwelling-influenced waters, *Trichodesmium* was outcompeted by other nondiazotrophic phytoplankton species that could have taken advantage of the higher DIN conditions at Stas. 5 and 6.

The factor(s) causing the observed moderate abundances and N_2 fixation of *Trichodesmium* at Sta. 4 are not easy to define. This station is located where it could be influenced by equatorial upwelling; however, the DIN or DIP concentrations measured were not consistent with this hypothesis. Furthermore, it is important to note that the DIN values obtained from Stas. 4, 5, and 6 are not high enough to cause reductions in N_2 fixation rates in cultured *Trichodesmium* (Holl and Montoya 2005); therefore it is likely that many, possibly related, factors contribute to the low abundances observed at these stations, including increased competition by other phytoplankton species, nutrient-driven niche change, P supply (see below), or zooplankton and viral predation.

The most northern (Stas. 1–3) and southern (Stas. 7–8) stations were out of the range of influence by equatorial upwelling. *Trichodesmium* abundance was depressed at Stas. 1–3 relative to Stas. 7 and 8. Although nutrient concentrations were favorable for *Trichodesmium* growth at Stas. 1–3 (low DIN, moderate-high DIP), the hydrographic parameters were less conducive to *Trichodesmium* growth (deeper mixed layer depths, lower salinities) than in the southern part of the transect (Stas. 7 and 8). These data likely reflect the importance of physical parameters (shallower mixed layer depth and higher salinities) on *Trichodesmium* abundance.

P physiology—The presence of AP activity is often used as a P stress indicator because the enzyme activity is typically regulated in response to P supply. In the case of *Trichodesmium*, the presence of ELF-assayed AP activity has previously been shown to be sensitive to P supply in culture and field populations in the western North Atlantic (Dyhrman et al. 2002). Here we underscore that this diagnostic captures the P physiology of *Trichodesmium*, which we term P stress. A population experiencing P stress indicates a response to low P, but not necessarily a limitation of growth rate or N_2 fixation because the extent to which stressed *Trichodesmium* are able to scavenge P will ultimately result in either chronic limitation or recovery from the stressed state.

AP activity was examined at all stations along the transect using this ELF assay, which can distinguish the presence of AP activity in *Trichodesmium* from adhered microbes. AP activity was present in one or more morphotypes at all but three stations along the transect (Stas. 1, 2, 3, 7, and 8). However, there was no detectable AP activity in either morphotype at Stas. 4, 5, and 6. To cross-validate the sensitivity of AP activity as a P stress indicator for *Trichodesmium* in the western Central and western South Atlantic, an incubation experiment was performed at Sta. 8 where the in situ population had AP activity. In the 48-h incubation, AP activity (positive ELF signal) was lost in treatments that received DIP, but not in the Fe-addition or the no-addition control treatment. When treatments were released from P stress with DIP addition, there was also an increase in the N_2 fixation rate relative to the P-stressed control. This increase was not as pronounced in the Fe-DIP addition, although there was higher variability between replicates for this treatment. The N_2 fixation rates were lower than the values obtained with fresh samples, and this can be attributed to both the acclimation to growth in a mechanical incubator and the documented die-off of *Trichodesmium* that can occur in bottle enrichments (Burns et al. 2006). Despite these caveats, the loss of AP activity in the DIP additions emphasizes the utility of ELF-assayed AP activity as a P stress indicator and suggests that the presence of AP activity in *Trichodesmium* is indicative of P stress-induced reductions in maximal N_2 fixation.

The presence of AP activity in the different morphotypes and the regulation data from the incubations suggest that P is an important factor constraining N_2 fixation along the transect. The physiological data indicate that P may be particularly important at Sta. 1 and Sta. 7 where both morphotypes were P-stressed and in the case of Sta. 7 where N_2 fixation rates were low (relative to Sta. 8). P stress does not appear to be a constraining factor on *Trichodesmium* abundance and N_2 fixation at Stas. 4, 5, and 6, where there was no detectable AP activity. Despite the apparent importance of P stress at several stations in this system, there was no clear inverse relation between DIP concentration and *Trichodesmium* AP activity. Some stations (e.g., Sta. 4) had relatively low DIP but no AP activity. Conversely, there were some stations (e.g., Sta. 1) that had AP activity and relatively high (0.10 μmol L^{-1}) DIP. Although AP activity in *Trichodesmium* colonies has been detected at DIP > 0.10 μmol L^{-1} in other systems (Mulholland et al. 2002), these observations underscore the challenges in using DIP concentrations as an indicator of P physiology. There are several possible explanations as to why *Trichodesmium* AP activity may not inversely track with DIP concentrations. First, the standing stock concentration of a nutrient does not indicate cycling rate and both can affect the physiological status of a cell. For example, the lack of AP activity at Sta. 4, with a relatively low DIP concentration (0.35 μmol L^{-1}), may be the result of a particularly fast cycling rate at this station. Another explanation is that there may be microscale variability in the DIP field, where DIP is depleted in the near vicinity of the colony, a feature that is not resolvable with bulk water assays of DIP. Microscale features in physiology and biogeochemistry have been discussed elsewhere (Azam 1998; Azam and Worden 2004; Ruttenberg and Dyhrman 2005). Additionally, the AP activity reflects the nutritional history of the colony (e.g., a roughly 48-h window of P stress [Dyhrman et al. 2002 and the incubation result described herein]), not necessarily its instantaneous nutrient environment.

Comparing the Chl *a*-normalized N_2 fixation rates with the AP activity suggests that P supply may have a differential effect on the two colony morphologies. While the puff morphotypes had AP activity and thus appear to be P-stressed, at five of the eight stations, the tufts were mostly nonstressed (positive for AP activity at only two of the eight stations). Chl *a*-normalized N_2 fixation rates are consistent with this differential P stress response in that the tuft morphology typically had a higher N_2 fixation rate relative to the puff morphology. These results underscore the potential of AP activity to be an indicator of P-related reductions in maximal N_2 fixation for this genus. Additionally, the data suggest that each *Trichodesmium* species may respond differently to changes in the environment such as P depletion.

The differences in AP activity detected in tuft and puff colony morphotypes suggest that some species of *Trichodesmium* might be better adapted to coping with low DIP than others. Additionally, although there was considerable variation, the puffs had a higher average Chl *a* content in all of the samples from EN367. These two observations suggest that the puff forms may be adapted to live at depth where the light levels are lower (requiring them to have more Chl *a* to compensate for light attenuation) and where the DIP concentrations are higher (allowing them to evolve a higher P quota). Two recent studies lend further support to this hypothesis. Post et al. (2002) found that puffs contained more Chl *a* and were more numerous at depth than tufts. Furthermore, a high-resolution subtropical transatlantic study came to similar conclusions on the basis of observations made using a video plankton recorder to image and enumerate *Trichodesmium* down to 130 m (Davis and McGillicuddy 2006). Davis and McGillicuddy (2006) found that the tuft forms were most abundant in the surface with the highest numbers associated with warm salty anticyclonic eddies. Although the puffs were also associated with anticylconic eddies, they were more enriched at depth. These findings, and those from this study, lend further support for the presence and the ecological importance of niche differentiation within the genus *Trichodesmium*, and argue for continued analyses of *Trichodesmium* species and their differences, both in the laboratory and in the field.

Fe physiology—The total Fe concentrations measured during EN367 were low (all <1 nmol L^{-1}) and are consistent with previous studies in this region (Bergquist and Boyle 2006; Cullen et al. 2006). Dissolved Fe concentrations were highest at the stations north of the Equator (Stas. 1–3), where there is higher potential for aeolian Fe inputs associated with the Saharan dust plume (Husar et al. 1997). DIN and DIP concentrations increased by a factor of 2 or more between Sta. 4 and Sta. 6; however, there was no significant increase in Fe concentration (Sta. 4, 0.31 ± 0.02; Sta. 6, 0.35 ± 0.09). The lack of increase in Fe concentration is consistent with the interpretation that the increased nutrient concentrations were due to equatorial upwelling rather than the Amazon plume, since upwelling is not expected to be a large source of Fe.

Trichodesmium was screened for IdiA expression at Stas. 3, 7, and 8 where biomass was high enough for the assay and Fe concentrations were <0.5 nmol L^{-1}. As described above, IdiA expression detected via western blotting is indicative of severe Fe deficiency. Despite the very low Fe concentrations, no evidence of acute Fe deficiency could be detected in these samples using the IdiA western blotting approach. Measurements of the partitioning of dissolved and colloidal Fe on EN367 cruise reveal that most of the Fe was truly dissolved (i.e., in the 0.2-μm filterable fraction [Cullen et al. 2006]). Previous workers have argued that this fraction is more biologically available than the colloidal fraction (Wu et al. 2001), which might contribute to the lack of acute Fe deficiency we observed.

In the incubation done at Sta. 8 (Fe = 0.27 nmol L^{-1}), there was also no molecular evidence of severe Fe deficiency. Although IdiA expression was undetectable in protein extracts from any of the incubation conditions tested, the samples showed an intermediate increase in N_2 fixation in the Fe addition treatments relative to the control. Therefore, the combination of the lack of IdiA detected in the western blots (indicating no severe Fe deficiency) and the moderate increase in the observed N_2 fixation rates in the Fe-amended incubations (indicating possible Fe stress) suggests that, although Fe was not the proximal limiting nutrient, by the end of the incubation the *Trichodesmium* internal Fe pool was not at optimal concentrations. In short, the in situ population along the transect could have been experiencing some degree of Fe stress, but not the more severe Fe deficiency conditions to which the assay is tuned. Although the PCC6301 anti-IdiA antisera used herein can readily detect *Trichodesmium* IdiA (Webb et al. 2001), its sensitivity is limited to severe deficiency conditions and is not the optimal method of probing more subtle *Trichodesmium* Fe stress. We have recently developed a new technique based on transcription (RNA expression) of the stress-induced genes that has been successfully applied to P-stressed *Trichodesmium* samples from the Sargasso Sea (Dyhrman et al. 2006). We have modified the same technique to allow monitoring of the expression of the *idiA* gene as a biomarker for Fe stress in *Trichodesmium* cultures and have detected its expression in samples from the Sargasso Sea (unpubl. data). These more recent results validate the use of *idiA* as a marker of Fe stress in *Trichodesmium* field samples. Unfortunately, samples for RNA expression were not collected during EN367; hence, we are unable to retest the samples with the more sensitive RNA approach.

Model predictions imply that robust quantitative relations exist between Fe concentrations and N_2 fixation patterns (Moore et al. 2004). A comparison of the predicted global distributions of Fe and N_2 fixation by the Moore et al. model indicate that there is effectively an Fe limitation threshold of $\sim$0.5 nmol L^{-1}. Clearly, for any model designed to predict the status of Fe limitation, determining such a threshold is very important. At the present time, model inputs for Fe limitation are derived entirely from laboratory culture studies (Kustka et al. 2002, 2003). However, such studies can underestimate overall N_2 fixation fluxes relative to values determined from field measurements for many regions. Moreover, our results from the South Atlantic showed no evidence of severe Fe deficiency (e.g., no expression of the Fe-responsive protein IdiA) at Fe concentrations as low as 0.14 nmol L^{-1} (Fig. 4B). A lower Fe limitation threshold would result in a smaller region where N_2 fixation is predicted to be Fe limited, especially in the Pacific Ocean. However, we do not propose a universal Fe limitation value from our data, as the threshold for Fe limitation may vary because of other physical and chemical factors. For example, under P-limited conditions, *Trichodesmium* growth rates may be significantly slower than optimal, so Fe uptake rates may be sufficient to meet cellular needs, even at lower exogenous Fe concentrations. Therefore, in a P-replete environment, we may well see IdiA expression at Fe concentrations in the 0.14 nmol L^{-1} range. Further empirical investigation of the relation between Fe limitation in *Trichodesmium* using a more sensitive marker of Fe stress (such as the RNA approach discussed above) coupled with detailed measurements of Fe distribution may reveal an Fe limitation scenario that is significantly different from what is predicted by current models.

Despite the above considerations, the finding that *Trichodesmium* colonies were frequently P-stressed during the EN367 cruise track is in good agreement with recent modeling efforts (Moore et al. 2004). However, this is partly because the models predict higher-than-observed Fe concentrations of Fe in surface waters in our study region. Assumptions made in these models about sources of and removal processes for Fe are critical in N_2 fixation estimates, and we anticipate such assumptions will be refined as more extensive surface Fe data become available in the future.

Identifying the factors that constrain *Trichodesmium* growth and N_2 fixation in different systems is challenging, yet critical, to modeling marine N_2 fixation and its ramifications. The finding that the *Trichodesmium* puff morphology frequently had AP activity (an indicator of P-stress) in the western central and South Atlantic, and that N_2 fixation increased with P addition, is in good agreement with recent modeling efforts (Moore et al. 2004), which predict the potential for P limitation of N_2 fixation in this region. The differential expression of AP detected in the two *Trichodesmium* morphologies (puffs generally expressing AP and tufts not) further emphasizes the physiological heterogeneity of this genus, and the value of approaches that can discern this heterogeneity in situ. Although P is clearly an important factor in influencing N_2 fixation in this region, the low N_2 fixation rates and decreased *Trichodesmium* abundance at some stations that did not have AP activity also underscore the importance of additional biogeochemical or physical parameters.

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