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FEATURE ARTICLE

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Diazotrophic bacteria respond to Saharan dust additions

Rebecca J. Langlois[1,2,*], Matthew M. Mills[1,3], Celine Ridame[1,4], Peter Croot[1,5], Julie LaRoche[1,2]

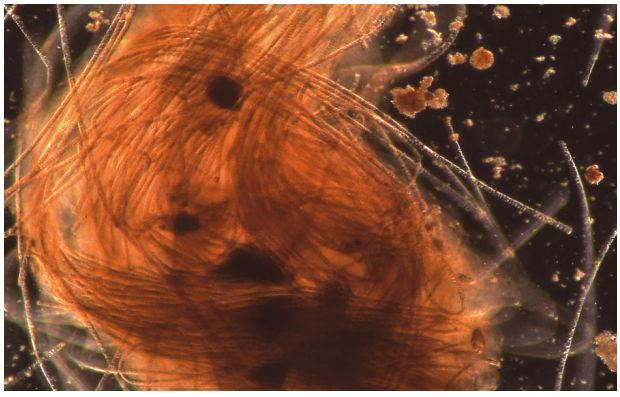
[1]Helmholtz Centre for Ocean Research Kiel|GEOMAR, 24105 Kiel, Germany

[2]*Present address:* Dalhousie University, Halifax, Nova Scotia B3H 4R2, Canada
[3]*Present address:* Environmental Earth System Science, Stanford University, Stanford, California 94305, USA
[4]*Present address:* LOCEAN-IPSL, Université Pierre et Marie Curie, 75252 Paris, France
[5]*Present address:* National University of Ireland, Galway, Ireland

ABSTRACT: Three bioassay experiments were performed to study the effects of nutrient and Saharan dust additions on natural diazotrophic communities in the tropical North Atlantic Ocean. Samples for nucleic acid analysis were collected at the beginning and end of 48 h incubations. TaqMan probes specific to 7 diazotrophic phylotypes, viz. filamentous cyanobacteria (*Trichodesmium* spp.), unicellular cyanobacterial (UCYN) Groups A, B, and C, Gamma A and P *Proteobacteria*, and Cluster III, were used to quantify *nifH* DNA abundances. N_2 fixation rates were measured in the same experiments using the $^{15}N_2$ gas bubble injection method. N_2 fixation was co-limited by P and Fe. Total *nifH* abundances increased relative to the control with additions of either Fe or P or both in combination. Additions of dissolved N, alone or in combination with phosphate, induced increases in UCYN-A and Gamma A *nifH* compared with the control. Saharan dust additions significantly stimulated fixation rates. Abundances of all cyanobacterial and Gamma A *nifH* phylotypes at least doubled after Saharan dust additions where surface water dissolved Fe concentrations were <2 nmol l^{-1}. Laboratory experiments with cultures of *T. erythraeum* demonstrated that dust addition promoted colony formation and the persistence of *T. erythraeum* biomass relative to cultures to which no Fe was added. Our results with both field and laboratory experiments indicate that Saharan dust positively affects diazotrophic phylotype abundances and changes *T. erythraeum* colony morphology.

KEY WORDS: Nutrient limitation · Nitrogen fixation · North Atlantic · Bioassay · *Trichodesmium* · UCYN-A · *nifH* · qPCR

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Saharan dust particles entrained in and around a radial colony of *Trichodesmium erythraeum* filaments.

Photo: R. J. Langlois, J. LaRoche

INTRODUCTION

Marine nitrogen inventory gain processes are biologically mediated. Fixed nitrogen enters the biogeochemical cycle through biological nitrogen (N_2) fixation, the reduction of atmospheric N_2 gas to ammonium. Only a few specialized bacteria and *Archaea*, which contain the highly conserved protein nitrogenase, are able to fix N_2. Due to the relatively low abundance of diazotrophs (N_2-fixing organisms), tracking their response to changes in environmental conditions has been problematic. Average diazotroph abundances under non-bloom conditions are estimated to be 2.5×10^4 cells l^{-1} (Langlois et al. 2008). In contrast, abundances of the common open-

*Email: rebecca.langlois@dal.ca

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ocean cyanobacterial species *Synechococcus* and *Prochlorococcus* have been reported to be 5×10^7 and 1.3×10^8 cells l^{-1}, respectively, in the Atlantic Ocean (Davey et al. 2008). Although diazotroph abundances can reach concentrations above 10^5 cells l^{-1} (Church et al. 2008, Langlois et al. 2008, Moisander et al. 2010), it is not clear what conditions or environmental factors are needed for this to occur. In addition these estimates, made using quantitative polymerase chain reaction (qPCR) of the *nifH* gene (the marker gene that encodes nitrogenase reductase) and transcripts from diverse phylotypes, have shown that the relative abundance of various diazotrophs including unicellular and filamentous diazotrophs vary widely within and between oceanic basins (Church et al. 2008, Langlois et al. 2008).

Although information on the distributions of diazotrophs and N_2 fixation in marine environments is accumulating, the environmental factors that control these distributions are still not well described (Langlois et al. 2008). Many environmental factors including availability of dissolved nutrients and atmospheric deposition of desert dust as a source of Fe and PO_4 have been hypothesized to affect diazotrophic activity in the ocean (Mills et al. 2004, Lenes et al. 2005, Ridame et al. 2011). The low concentrations of inorganic nitrogen (N) found in oceanic environments should favor high diazotroph activity and abundance, but surface waters of oligotrophic oceans are often low or depleted in both PO_4 and Fe, which may ultimately control diazotroph distributions (Moore et al. 2009). There are few sources of PO_4 in oceanic waters other than the remineralization of organic matter and atmospheric deposition (Mahowald et al. 2008), and thus the low availability of PO_4 has been considered a constraint on N_2 fixation in the North Atlantic (Wu et al. 2000, Mather et al. 2008). Diazotrophs are also hypothesized to be limited by Fe (Falkowski 1997, Karl et al. 2002) due to a relatively high requirement for the Fe-rich N_2-fixing enzyme nitrogenase (Kustka et al. 2003). Diazotrophic phototrophs are further impacted by Fe availability because of the additional high Fe demand of the photosynthetic apparatus (Shi et al. 2007).

Aeolian inputs of desert dust supply the majority of Fe to the oligotrophic oceans (Jickells et al. 2005). The tropical North Atlantic Ocean is subjected to periodic dust storms originating in the Sahara, but indications of low Fe availability have been observed (Mills et al. 2004, Moore et al. 2006). *Trichodesmium*, the uncultured unicellular Group A cyanobacteria (UCYN-A), and other diazotrophic groups are abundant throughout dust-affected regions in the tropical and sub-tropical North Atlantic Ocean (Tyrrell et al. 2003, Capone et al. 2005), and abundances of *nifH* genes appear to be related to atmospheric dust deposition (Langlois et al. 2008).

We conducted 3 nutrient addition bioassay experiments in the sub-tropical North Atlantic Ocean to look at the effects of NO_3, PO_4, Fe, all combinations of these, and Saharan dust additions on natural diazotrophic phylotype abundances using TaqMan qPCR. Primary production in the same bioassay experiments was shown to be N-limited (Davey et al. 2008), while N_2 fixation was Fe and PO_4 co-limited (Mills et al. 2004). Abundances of 7 diazotrophic phylotypes were detected and estimated, including filamentous cyanobacteria (*Trichodesmium*, Fil), UCYN-A, Group B unicellular cyanobacteria (*Crocosphaera*, UCYN-B), Group C unicellular cyanobacteria (*Cyanothece*, UCYN-C), 2 gammaproteobacterial phylotypes (Gamma A and Gamma P), and a cluster III phylotype (CIII). To further investigate the effects of dust additions, cultures of *T. erythraeum* IMS101 were grown with or without iron and/or Saharan dust in the media, and changes in the photosynthetic efficiency, colony morphology, and *nifH* abundances were monitored.

MATERIALS AND METHODS

Bioassay experiments

Samples for molecular analysis were collected at the start and end (48 h) of 3 bioassay experiments performed in Autumn 2002 during the Meteor 55 research cruise in the sub-tropical north Atlantic Ocean (Expts A,B described by Mills et al. 2004 and Expts B,C described by Davey et al. 2008). Station locations and initial conditions are given in Fig. 1 and Table 1. Experiments were conducted using trace metal clean techniques (Graziano et al. 1996). Surface seawater was pumped on-board using a Teflon diaphragm pump (Almatec) into a trace metal clean container. Seawater was collected in 60 l carboys and then siphoned into acid-cleaned and seawater-rinsed 1 l polycarbonate Nalgene bottles. Nutrients were added alone and in combination to final concentrations of 1.0 µmol l^{-1} NH_4NO_3 (+N), 0.2 µmol l^{-1} NaH_2PO_4 (+P), and 2.0 nmol l^{-1} $FeCl_3$ (+Fe) to triplicate bottles under a laminar flow hood. Saharan dust was added alone at final concentrations of 0.5 mg l^{-1} (D1) and 2.0 mg l^{-1} (D2). Details on the chemical characterization of the dust were reported by Ridame & Guieu (2002) and Mills et al. (2004). Bottles were

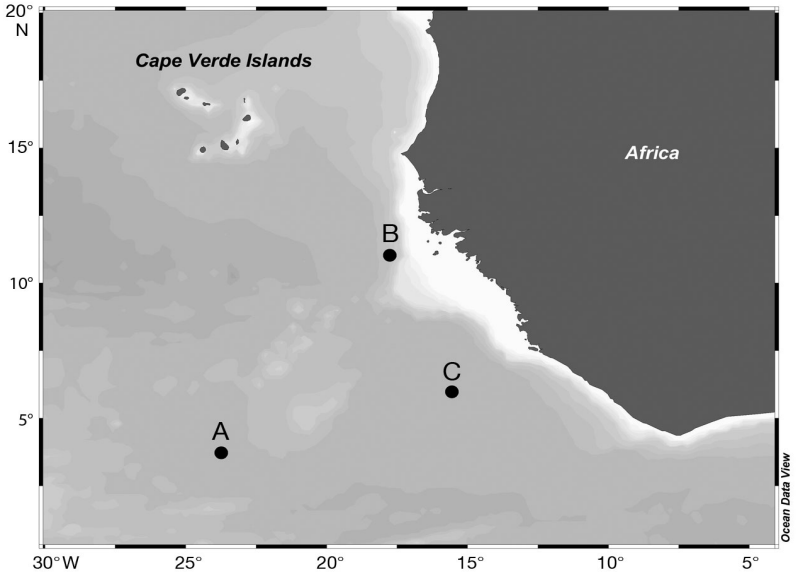


Fig. 1. North Atlantic Ocean, showing locations of the bioassay experiments (points A, B, and C) during the Meteor 55 cruise

then placed in incubators shaded to 20% of incident surface irradiance (range of 5 to 60 µmol photons m^{-1} s^{-1}) with blue filters (Lagoon Blue, Lee Filters #172). Incubator water temperature was maintained with constant flowing seawater. Results of N and CO_2 fixation rate measurements, chlorophyll *a* (chl *a*) concentrations, and changes in phytoplankton abundance and composition in response to the nutrient amendments can be found elsewhere (Mills et al. 2004, Davey et al. 2008). N fixation rates were likely underestimated (Mohr et al. 2010); however, this does not affect the conclusions drawn in this paper, as changes in rates relative to a control are being compared in bottles handled identically. Samples (1.5 l) for characterization of the initial diazotrophic community were collected during the experiment set-up and were filtered under low vacuum pressure (2 kPa) onto 25 mm diameter, 0.2 µm Durapore filters (Millipore). At the end of the 48 h incubation, the 300 to 800 ml of water remaining in the triplicate chl *a* bottles was combined to make 1 sample per treatment and filtered as described above. Thus, the average response in the triplicate bottles was sampled. Filtrations took 1.5 to 2 h, and afterwards filters were immediately frozen at −80°C until extraction of nucleic acids in the laboratory.

Culture experiments

Non-axenic cultures of *Trichodesmium erythraeum* ISM101 were grown in YBCII media (Chen et al. 1996). Cultures were inoculated in sterile plastic tissue flasks under a laminar flow hood to limit bacterial and Fe contamination and grown under a 12:12 h light:dark cycle with an irradiance of 150 µmol photons m^{-2} s^{-1}. Cultures were gently shaken to re-suspend settled filaments and visually inspected daily. At the start of the experiment, 4 ml of 1 stock culture in late exponential growth phase was used to inoculate 45 bottles (9 replicates for 5 treatments) of 40 ml YBCII media. Fe concentrations were modified to 0 (0 µmol EDTA l^{-1}), 0.41 (2 µmol EDTA l^{-1}), or 0.82 µmol l^{-1} (4 µmol EDTA l^{-1}). Additionally, a Saharan dust treatment (+Dust) was conducted in which 9 mg of an uncharacterized ≤90 µm sifted soil fraction, collected 20 km north-west of Agadir in Morocco (courtesy of Dr. A. Baker), was added to 18 bottles of YBCII media containing either 0 or 0.41 µmol l^{-1} Fe (9 bottles each) for a final dust concentration of 0.23 g l^{-1}. Saharan dust samples from 9 sites in a remote section of Morocco where the dust used in the culture experiments was collected were characterized by Guieu et al. (2002). This area is a known dust source region, and the soil collected here is typical of aerosols entrained under high winds and blown out to sea (Guieu et al. 2002). There is no single dust source, and even aerosols collected at sea show variation (Shi et al. 2011). Three bottles from each treatment were used for biomass analysis, 3 for quantifying *nifH* abundance after 60 h, and 3 for quantifying *nifH* abundance after 132 h. The entire culture volume (44 ml) was used for estimating gene abundances. Starting inoculation amounts (4 ml of stock culture) were filtered in triplicate for initial (0 h) measurements of *nifH* abundances. The experiment was repeated 3 times and showed replicable outcomes in visual growth and changes in colony morphology. Samples for nucleic acid extraction and qPCR analysis were collected for only one of the experiments.

DNA extraction, cDNA synthesis, *nifH* amplification, and cloning

DNA from the bioassay experiments was extracted using the Qiagen DNeasy Plant extraction kit. Filters were broken up by holding the cryo-tube containing the filter in liquid nitrogen for 30 s and then using a sterile pipette tip as a pestle. Lysis buffer was applied directly to the filter pieces, and extraction followed

Table 1. Summary of the 3 bioassay experiments (Expts A, B, and C). Primary production and chlorophyll *a* were N-limited. Nutrient(s) limiting nitrogen fixation (Nutr. added) are shown, as are initial phosphate and dissolved iron concentrations, temperature, and nitrogen and carbon fixation rates. NO_3 was not detected initially in any of the experiments. Detection limits were 0.3 µmol l^{-1}, 0.03 µmol l^{-1}, and 80 pmol l^{-1} for NO_3, PO_4, and Fe, respectively. nm: not measured. Initial abundances of *Synechococcus* (*Syn.*), *Prochlorococcus* (*Pro.*), and picoeukaryotes (Picoeuk.) are given. *nifH* enrichment was calculated by normalizing the *nifH* concentration in the D2 treatment to that of the control. See 'Materials and methods' for detailed descriptions of treatments

Expt	Date (2002)	Site	Nutr. added	PO_4 (µmol l^{-1})	Fe (nmol l^{-1})	Temp. (°C)	Fixation rate (l^{-1} d^{-1}) N_2 (nmol)	CO_2 (µmol)	*Syn.* ($\times 10^6$ cells l^{-1})	*Pro.* ($\times 10^8$ cells l^{-1})	Picoeuk. ($\times 10^5$ cells l^{-1})	*nifH* dust enrichment[c]
A[a]	31 Oct	4° N, 24° W	+PFe	≤0.03	1.3	28	4.09	0.76	6.1	1.0	3.8	6.8
B[a,b]	6 Nov	11° N, 18° W	+PFe	≤0.03	1.1	30	6.48	1.66	39	1.3	4.3	54.0
C[b,c]	10 Nov	6° N, 16° W	+P, +PFe[c]	nm	7[d]	29	1.85	1.14	20	1.2	3.4	0.4

[a]From Mills et al. (2004); [b]from Davey et al. (2008); [c]present study; [d]Croot et al. (2004)

Table 2. TaqMan primers and probes and SYBR-Green primers. UCYN: unicellular cyanobacterial group

Taxon	Forward	Reverse	Probe
Filamentous[a]	TGGCCGTGGTATTATTACTGCTATC	GCAAATCCACCGCAAACAAC	AAGGAGCTTATACAGATCTA
UCYN-A[a]	TAGCTGCAGAAAGAGGAACTGTAGAAG	TCAGGACCACCGGACTCAAC	TAATTCCTGGCTATAACAAC
UCYN-B[a]	TGCTGAAATGGGTTCTGTTGAA	TCAGGACCACCAGATTCTACACACT	CGAAGACGTAATGCTC
UCYN-C[a]	TCTACCCGTTTGATGCTACACACTAA	GGTATCCTTCAAGTAGTACTTCGTCTAGCT	AAACTACCATTCTTCACTTAGCAG
Gamma A[a]	TTATGATGTTCTAGGTGATGTG	AACAATGTAGATTTCCTGAGCCTTATTC	TTGCAATGCCTATTCG
Gamma P[a]	TTGTGCAGGTCGTGGTGTAATC	CATCGCGAAACCACCACATAC	CCTATGACGAAGACCTAGAC
Cluster III[a]	ACCTCGATCAACATGCTCGAA	GCAGACCACGTCACCCAGTAC	CCTGGACTACGCGTTC
Trichodesmium erythraeum nifH	GGTCCTGAGCCTGGTGTAGG	GATAGCAGTAATAATACCACGGCCA	–

[a]From Langlois et al. (2008)

according to the manufacturer's instructions. RNA and DNA were simultaneously extracted from the culture experiment samples using the Qiagen All-Prep DNA/RNA extraction kit and the same sample preparation steps described above. DNA and RNA were eluted into 50 µl PCR grade water. Amplification and cloning of *nifH* was performed as described by Langlois et al. (2005), using the nested PCR protocol and *nifH* primers described by Zani et al. (2000). Bands of the correct size (354 bp) from the control, +PFe, and dust treatments were inserted into the Topo TA vector and into Top 10 chemically competent *Escherichia coli* cells according to the manufacturer's instructions (Invitrogen). Clones were screened for inserts using T3 and T7 primers, and screened inserts of the correct size were sequenced. *nifH* sequences from the initial microbial populations have already been published (Langlois et al. 2005).

DNA and RNA concentrations were measured using the Picogreen DNA and Ribogreen RNA quantitation kits (Molecular Probes), respectively. RNA samples from the culture experiment were transcribed to cDNA using the Quanti-Teq cDNA synthesis kit and DNase digest step (Qiagen) according to the manufacturer's instructions. Abundances of filamentous (*Trichodesmium* spp., Fil), Group A unicellular (UCYN-A), Group B unicellular (UCYN-B), Group C unicellular (UCYN-C), Gamma A CIII and Gamma P proteobacterial, and CIII *nifH* DNA were estimated in the bioassay samples using the specific TaqMan probe and primer sets described by Langlois et al. (2008; Table 2). Plasmid standards for each primer/probe set (Langlois et al. 2008) were used, and all samples were run with the same set of standards. Primer efficiencies ($E = 10^{-1/slope} - 1$) were 92% for Fil (R^2 = 0.996), 92% for UCYN-A (R^2 = 0.994), 94% for UCYN-B (R^2 = 0.993), 79% for UCYN-C (R^2 = 0.999), 96% for Gamma A (R^2 = 0.991), 86% for Gamma P (R^2 = 0.999), and 91% for CIII (R^2 =

0.997). TaqMan master mix (Applied Biosystems) was used and samples were run in an ABI Prism 7000 Real-Time PCR cycler, with the default program set to 45 cycles. Every experimental sample was run in triplicate, while standards and no-template controls (NTCs) were run in duplicate. No amplification was observed in NTCs.

Abundances of *Trichodesmium erythraeum nifH* in culture experiment samples were estimated using the primer set *T. erythraeum nifH* (Table 2). All DNA, cDNA, and RNA samples from culture experiments were run in triplicate using SYBR-Green Master Mix (Invitrogen), and the default thermocycler program was modified as follows: the activation time was reduced to 2 min and the annealing time to 30 s. The Fil standard was used to quantify the number of copies in these reactions (E = 97%, R^2 = 0.998). No amplification was observed in NTC and RNA control reactions, indicating that all reagents were clean and that there was no contaminating DNA in cDNA reactions. Quantities of *nifH* phylotypes in the qPCR reactions were calculated using the ABI Sequence Detection Software (v. 1.2.3) with RQ application.

qPCR detection limits and statistical analyses

The average cycle threshold (Ct) deviation for all samples was 0.63 Ct with a mean coefficient of variation of 1.6% (range 0.02 to 4.63%, median 1.7%). These values were nearly identical for all primer/probe sets. The detection limit of qPCR analysis is 1 gene copy per reaction, which corresponds to a Ct value of 39 for primer/probe sets UCYN-B, Gamma A, and Gamma P or a Ct value of 40 for the remaining sets. As different sample volumes were filtered for the field data set, the actual detection limit for the bioassay sample set varied from 33 copies l^{-1} (1.5 l filtered) to 170 copies l^{-1} (0.3 l filtered); however, the majority (75%) of the samples had a detection limit of 100 copies l^{-1}. The detection limit for the culture experiment samples was higher (1250 copies l^{-1}) due to the smaller sample volume filtered. Samples which amplified at Ct values of >38 or >39 (1 cycle less than the Ct for 1 copy) in at least duplicate reactions were called 'detectable, but not quantifiable' and are noted in the text. Values of $p < 0.05$ were considered significant. Due to limited experimental water resources, it was not possible to collect replicate samples for nucleic acid analysis. Instead, multiple experiments were conducted. Analysis of triplicate cultures showed small variations in abundances, indicating that the extraction and analysis techniques are highly reproducible. Relative change was calculated as $(\text{abundance}_{\text{treatment}} \times \text{abundance}_{\text{control}}^{-1}) - 1$. Statistical analyses were performed using Statistica 8.0. The nitrogen fixation rates and laboratory experiment qPCR data were analyzed using a 1-way analysis of variance (ANOVA) with a Fisher LSD post hoc test.

RESULTS

Diazotrophic community composition in bioassay experiments

All samples from bioassays experiments conducted at 3 sites in the tropical North Atlantic Ocean (Table 1, Fig. 1) were tested for the presence and abundance of 7 diazotrophic phylotypes. Fil (which detects both *Trichodesmium* and *Katagnymene*), UCYN-A, UCYN-B, UCYN-C, and Gamma A phylotypes were quantifiable in at least 1 bioassay experiment. The CIII phylotype was not detected in any samples, and the Gamma P phylotype was detected in only the D2 treatment of all experiments, but was not quantifiable. Analysis of clone libraries from the various treatments showed that no new phylotypes were stimulated by the nutrient/dust additions and that the qPCR probes targeted all dominant diazotrophic phylotypes (data not shown). The diversity of the diazotroph community decreased from west to east as seen by the number of phylotypes detected by qPCR; 5 were quantifiable in Expt A, while only 2 were quantifiable in Expt C. The Fil and UCYN-A phylotypes were detected in all experiments and were most abundant.

Trends in both total *nifH* abundances and N_2 fixation rates were observed in the bioassay experiments (Fig. 2). N_2 fixation rates were co-limited by P and Fe in all 3 experiments ($p < 0.05$), as previously determined by Mills et al. (2004). Despite this clear association in N_2 fixation rates, total *nifH* abundances (the sum of all phylotypes detected) were higher in +PFe than the control in Expt C only. Dust additions nearly doubled and tripled N_2 fixation rates relative to the control in Expts A and B, respectively (Mills et al. 2004). In contrast, Saharan dust additions increased total *nifH* concentrations by over an order of magnitude in Expts A and B with D2, the higher dust concentration, causing an even larger increase in abundances than D1 in Expt A.

A different response was elicited in Expt C, where N_2 fixation rates were the lowest. In this experiment, additions of P and PFe increased N_2 fixation rates to statistically similar values, while N_2 fixation rates in

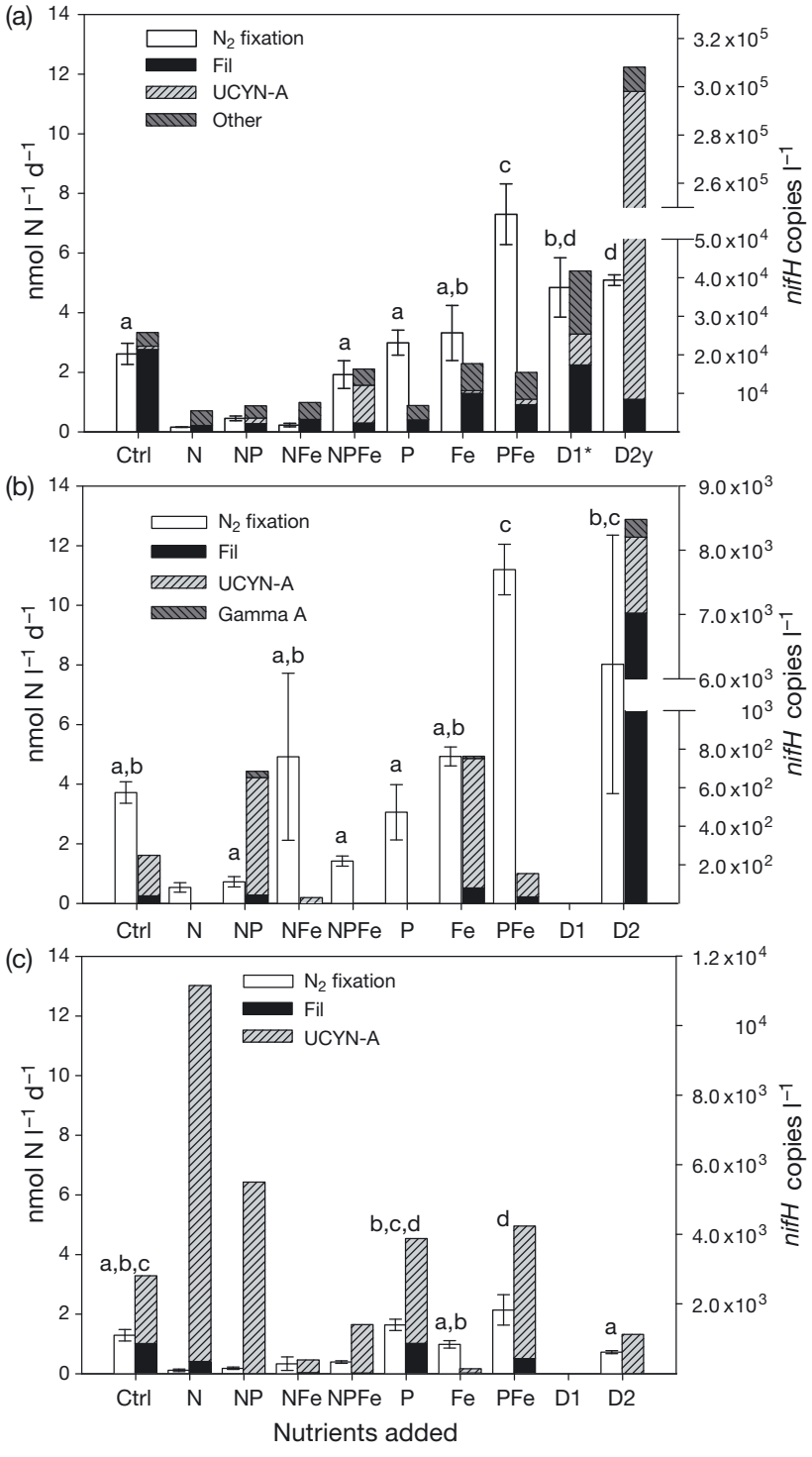


Fig. 2. Average (±SE) N_2 fixation rates (nmol N l^{-1} d^{-1}) and abundances of *nifH* phylotypes (*nifH* copies l^{-1}) detected in bioassay: (a) Expt A, (b) Expt B, (c) Expt C. Fixation rates for Expts A and B have been previously published (Mills et al. 2004) and have been re-plotted here. Letters identify mean N_2 fixation rates that are higher than or equal to the control (Ctrl) rate, and same letters denote means which are not statistically different from one another. Note that not all phylotypes were detectable in each experiment. *Only Expt A had the D1 (Dust) treatment. See 'Materials and methods' for detailed descriptions of treatments

D2 were statistically similar to rates measured in the control, +Fe, and +P treatments. Saharan dust additions did not impact diazotroph phylotype abundances in this experiment either. The initial Fe concentrations measured in this experiment were much higher (20 nmol l^{-1}) than in Expts A and B (<2 nmol l^{-1}), which may have been due to contamination during sampling for dissolved Fe. Water samples collected from an independent group showed concentrations of 7 nmol l^{-1} (Croot et al. 2004), still much higher than in Expts A and B. Surface waters had low salinity at this site due to heavy rainfall from the Intertropical Convergence Zone which also contained Fe and N (Baker et al. 2007), indicating that the diazotrophic community may not have been Fe-stressed at this site. Surprisingly additions of N alone, and in combination with P (+NP), resulted in increases of UCYN-A abundances to 1.1 × 10^4 and 5.5 × 10^3 *nifH* copies l^{-1}, respectively, from 2.0 × 10^3 *nifH* copies l^{-1} in the control. In fact, UCYN-A abundances appeared to increase in the +NP treatment in all 3 experiments, although dust additions resulted in the largest increases in UCYN-A *nifH* DNA.

A closer look at Expt A, where diazotrophic phylotypes were most abundant, revealed complexity among the individual phylotype responses to nutrient amendments (Fig. 3). Although the relative change in total abundances to the control was negative in all treatments except for dust amendments (Fig. 3a), increases in abundances were observed when each group was studied individually. The relative change of Fil to the control was negative in Expt A (Fig. 3b); however, abundances in the +Fe, +PFe, D1 and D2 treatments were at least double those measured in treatments where N was added (>7 × 10^3 versus <3.2 × 10^3 *nifH* copies l^{-1}, Fig. 2a). The same trend in abundances measured in the +Fe, +PFe, D1 and D2 treatments compared to +N treatments was observed in the UCYN-C group (Fig. 3d), and the relative change to the

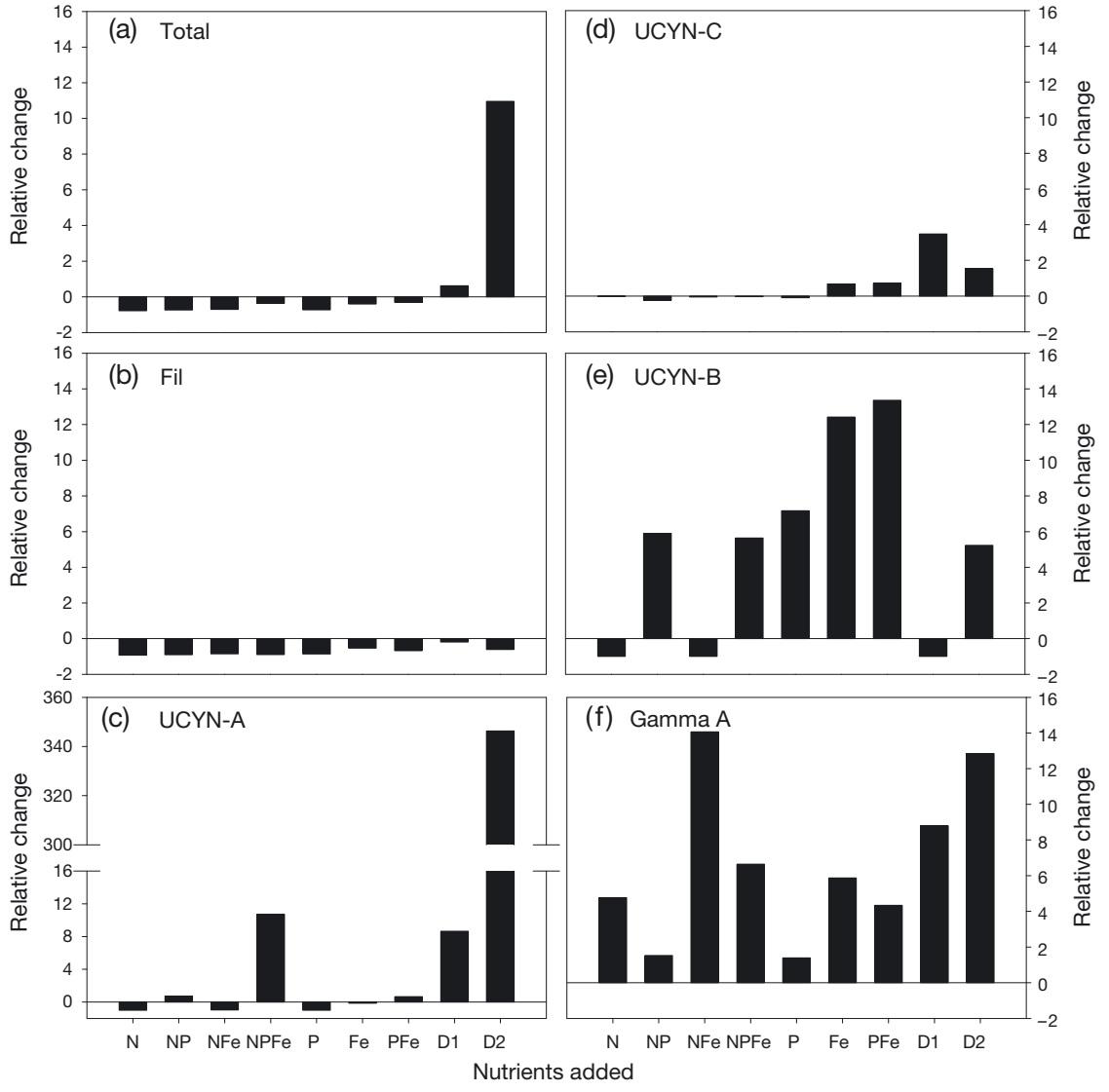


Fig. 3. Relative changes in phylotype concentrations with respect to the control in Expt A. Individual graphs are lettered in order of decreasing abundances. (a) The total is the sum of all phylotypes detected in a treatment. (b) Filamentous (Fil) was the dominant phylotype detected in nearly all treatments. (c) Unicellular cyanobacterial group A (UCYN-A) was dominant in +NPFe and D2, and (d) UCYN-C was the next most abundant phylotype. (e) UCYN-B and (f) Gamma A had roughly the same concentrations. Note the different *y*-axis scale in (c). See 'Materials and methods' for detailed descriptions of treatments

control was positive for this group. In contrast, UCYN-B abundances increased with additions of P and Fe alone or combined and sometimes with N (Fig. 3e). Interestingly, UCYN-A (Fig. 3c) and Gamma A (Fig. 3f) abundances were positively influenced by additions of N either alone or in combination with other dissolved nutrients. Combined, these results indicate that diverse diazotrophic phylotypes respond differently to changes in nutrient concentrations.

Comparisons of changes in picophytoplankton (*Synechococcus*, *Prochlorococcus*, and picoeukaryotes) relative abundances in the dust treatments were made to investigate the possibility that the Saharan dust additions may have been toxic to the phytoplankton community and showed variability between experiments (Fig. 4a). Dust additions had an increasingly negative effect on *Synechococcus* and *Prochlorococcus* abundances, but an increasingly positive effect on picoeukaryotes moving eastward. Interestingly, the positive effect of Saharan dust on UCYN-A abundances decreased from Expts A to C (Fig. 4d). The effect of dust on picophytoplankton abundances was clearly variable and probably due to an environmental variable not constrained in this study, as no other treatment produced such a pattern in relative abundances of this group.

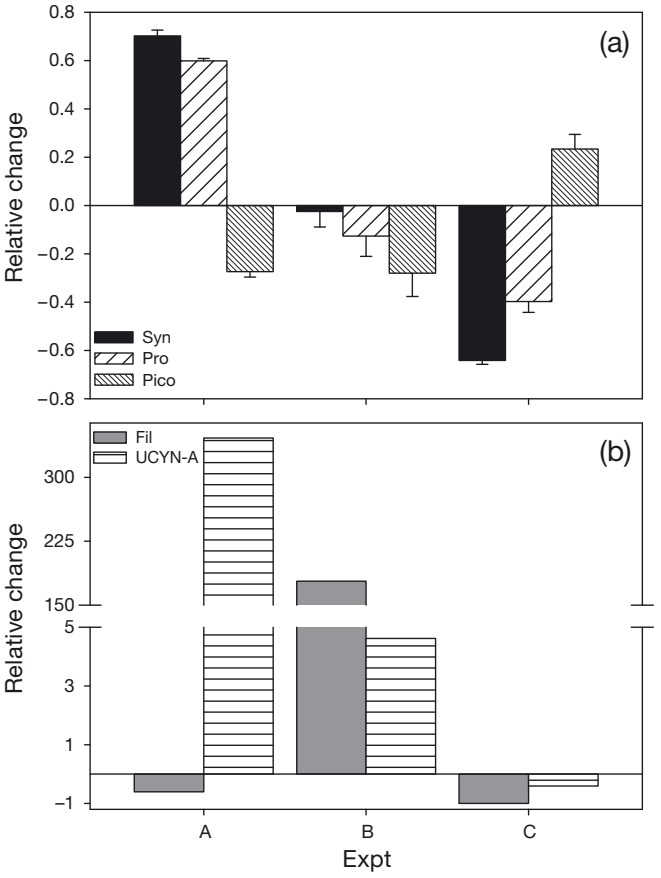


Fig. 4. Relative changes in abundance of (a) picophytoplankton cells and (b) dominant phylotypes in the D2 treatment compared to the respective control. Mean abundances of *Synechococcus* (Syn), *Prochlorococcus* (Pro), and picoeukaryotes (Pico) as measured by analytical flow cytometry are shown. Error bars in (a): SE (n = 3) Abundances of filamentous (Fil) and UCYN-A *nifH* are shown in (b). See 'Materials and methods' for detailed descriptions of treatments

Effects of Saharan dust on *Trichodesmium* cultures

Culture experiments where *T. erythraeum* was grown in media without Fe (0.0 µmol Fe l^{-1}), Fe concentrations of normal YBC II media (0.41 µmol Fe l^{-1}), Saharan dust (0.0 µmol Fe l^{-1} +Dust and 0.41 µmol Fe l^{-1} +Dust), and twice the Fe concentration of YBCII media (0.82 µmol Fe l^{-1}) demonstrated that the effect of dust on *nifH* abundances observed in field samples can be partially replicated in laboratory cultures. *T. erythraeum* colony morphology was different in the various treatments (Fig. 5). No colonies were observed in 0.0 µmol Fe l^{-1} bottles after 60 h, and within 48 h of the transfer into Fe-free medium, filaments became very short, thick, and dark red. In contrast, some colonies formed and persisted until the end of the experiment (132 h) in the dust-only bottles. Large radial colonies (puffs) were observed in all dust-amended bottles after 24 h, while only filaments were observed in bottles with just Fe added.

Changes in biomass were followed by estimating *nifH* DNA and cDNA at 60 and 132 h using TaqMan qPCR (Fig. 6). At 60 h, the highest *nifH* abundances were observed in 0.82 µmol Fe l^{-1} bottles, with statistically similar abundances for 0.41 µmol Fe l^{-1} +Dust bottles. Abundances in 0.41 µmol Fe l^{-1} bottles were lower and statistically different from 0.82 µmol Fe l^{-1} bottles but not from the 0.41 µmol Fe l^{-1} +Dust bottles. By 132 h, these 3 treatments contained similar amounts of *nifH* DNA. A small decrease in the amount of *nifH* over the 7 d in the 0.0 µmol Fe l^{-1} +Dust treatment was observed, while *nifH* decreased by 2 orders of magnitude in 0.0 µmol Fe l^{-1} media.

The abundances of *nifH* cDNA over time were more variable. The quantity of *nifH* cDNA decreased in all treatments after 60 h. The largest decrease occurred in the 0.0 µmol Fe l^{-1} treatment where *nifH* abundances decreased by over 2 orders of magnitude; in fact, *nifH* abundances decreased to undetectable levels in 2 of the triplicate bottles by the end of the experiment. *nifH* cDNA abundances in the 0.0 µmol Fe l^{-1} +Dust treatment also declined, although they were still significantly higher ($p < 0.05$) than in the 0.0 µmol Fe l^{-1} treatment. These results suggest that *Trichodesmium erythraeum* was able to access and utilize iron from Saharan dust for maintaining its biomass.

DISCUSSION

Comparison of experimental sites

Although the distribution of diazotrophs, especially that of *Trichodesmium*, has often been observed to be correlated with atmospheric mineral dust deposition (Tyrrell et al. 2003, Mahaffey et al. 2005, Moore et al. 2009), until now no studies have looked at how diazotrophs are directly affected by dust at the molecular level. The bioassay experiments presented here demonstrate that the abundance of diazotrophic organisms increases, as estimated by *nifH* gene copy numbers, with variable nutrient additions, while N_2 fixation is P and Fe co-limited. Saharan dust additions caused large increases in *nifH* concentrations in the 2 experiments that also showed stimulation of N_2 fixation rates in response to the dust amendments.

While dust additions increased the abundance of diazotrophic phylotypes in bioassay Expts A and B,

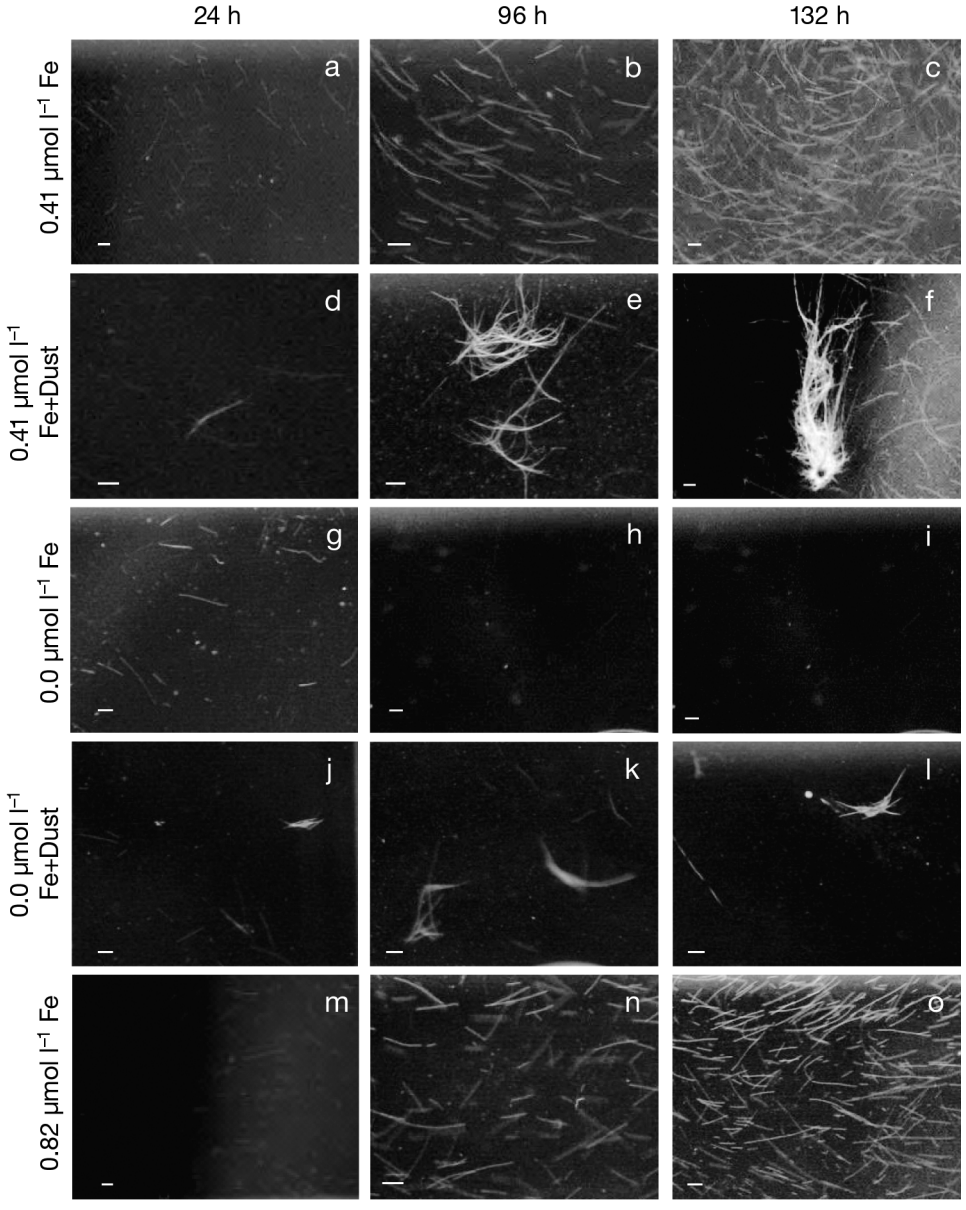


Fig. 5. *Trichodesmium erythraeum.* Changes in morphology during growth on dissolved iron and/or Saharan dust. *T. erythraeum* was grown in media with (a–c) 0.41 µmol Fe l^{-1}, (d–f) 0.41 µmol Fe l^{-1} + Dust, (g–i) 0.0 µmol Fe l^{-1}, (j–l) 0.0 µmol Fe l^{-1} + Dust, and (m–o) 0.82 µmol Fe l^{-1}. Pictures of culture flasks were taken 24 h (column 1), 96 h (column 2), and 132 h (column 3) after the start of the experiment. No colonies or filaments were observed in the 0.0 µmol Fe treatment (h and i) after 24 h. Scale bars = 90 µm

the +P, +PFe, +N, and +NP additions resulted in high *nifH* concentration increases in Expt C, where dust amendments had no effect. Like Expts A and B, primary productivity was N-limited in Expt C (Davey et al. 2008), and N_2 fixation was P and Fe co-limited. In contrast to Expts A and B, the initial Fe concentration of Expt C was much higher (Table 1). Fe concentrations measured during the same cruise increased eastwardly across the Atlantic Ocean (Croot et al. 2004). Also unlike Expts A and B, Fe additions did not result in any increases in phylotype abundance above control abundances at site C (see Fig. 1). Instead, P and N/NP additions resulted in higher Fil and UCYN-A concentrations, respectively. P additions resulted in statistically similar N_2 fixation rates to +PFe. This indicates that diazotrophs may have been more P-stressed at Site C as opposed to Fe-stressed at the other 2 stations.

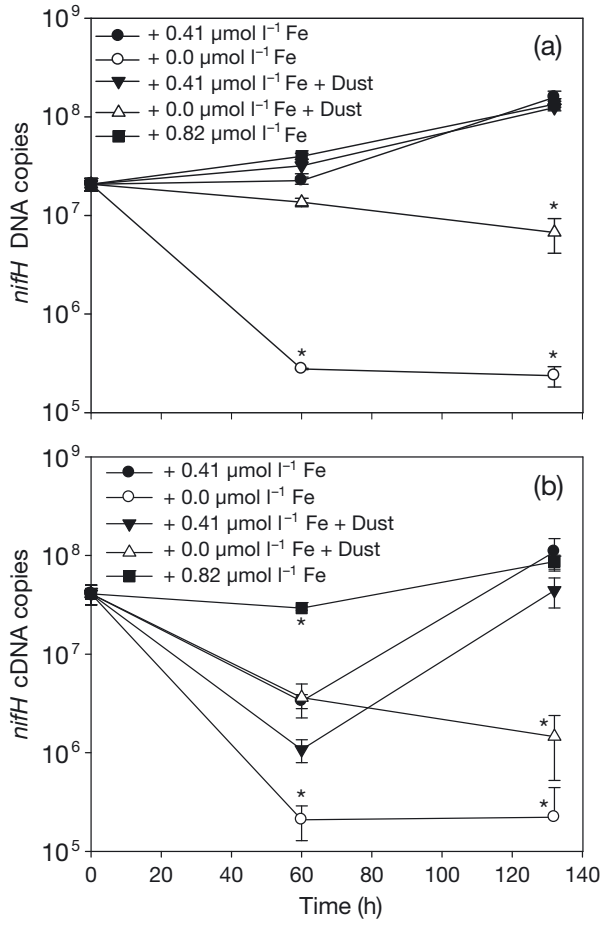


Fig. 6. *Trichodesmium erythraeum.* Changes in (a) *nifH* DNA and (b) *nifH* cDNA copy numbers during growth on dissolved iron and/or Saharan dust. Average (±SE) measurements from triplicate incubation bottles are shown. See 'Materials and methods' for detailed descriptions of treatments. *Values which are significantly different ($p < 0.05$) from all other treatments per time point

Effects of nutrient additions

A disparity was observed between the nutrient additions which resulted in increases in N_2 fixation rates and diazotroph phylotype abundances. In Expt C, N_2 fixation rates were significantly enhanced only by the addition of P or PFe, but maximum increases in phylotype abundances were seen with N and P additions. This could be explained by the role each nutrient plays in the cell. P is used by cells for growth and division and is found in nucleic acids, adenosine triphosphate, and phospholipids. Fe is a major component of enzymes, including nitrogenase. Increases in P and Fe could raise the potential for a diazotroph to carry out N_2 fixation. N is found in nucleic acids and metabolic compounds, which is important for growth and division. Previously published results from the same bioassay experiments showed that carbon fixation was N-limited but that cell growth was N and P co-limited (Davey et al. 2008). Perhaps the N and P additions stimulated the diazotrophic organisms in Expt C to either divide or make extra copies of their genomes, which would be detected as an increase in *nifH* abundances with qPCR. The combined P and Fe amendments stimulated diazotrophs to fix N_2, which would not necessarily correlate with an increase in *nifH* DNA, as the *nifH* DNA is just the molecular blueprint for N_2 fixation. It is more logical that *nifH* expression, which occurs during or just previous to N_2 fixation, and N_2 fixation rates could be correlated, but *nifH* cDNA was not looked at in this part of the study.

Cultured marine diazotrophs have relatively slow growth rates. Doubling rates ranging from 0.6 d^{-1} for *Crocosphaera* to 0.3 d^{-1} for *Trichodesmium*, or 40 to 80 h, respectively, have been published (Raven 2012). Although the length of the incubations was only 48 h, this was sufficient time for the cells to respond at the DNA replication level and for growth to occur. In many treatments, *nifH* copy numbers increased multiple-fold. DNA gene copy abundances are often used as a proxy for cell abundances, but some cells may have multiple copies of a gene per genome or have multiple genomes per cell, complicating the interpretation of qPCR-based abundance estimates. One situation that possibly occurred during the nutrient addition experiments is that many cells were in the Gap 1 phase and simultaneously entered the S phase, where DNA is replicated. Even though the biomass may not have increased significantly, *nifH* DNA copy numbers would have. It is also possible that growth rates based on laboratory cultures of diazotrophs growing on dissolved inorganic nutrients are not reflective of natural populations growing on a mix of organic and inorganic nutrients. More work is needed to determine the exact interpretation of gene copy abundances.

Adaptations of diazotrophs to Fe and P stress

PFe additions enhanced N_2 fixation in Expts A and B, but fixation was also elevated in dust treatments. Quantification of the *nifH* gene revealed increases of more than 2 orders of magnitude with Saharan dust additions. Additions of N, P, and especially Fe also resulted in elevated *nifH* copy numbers, depending on the diazotrophic phylotype, but these increases were not of the same magnitude as observed with dust additions. Based on the increases in diazotrophic

phylotype abundances and N_2 fixation rates with additions of Saharan dust, it appears that the chemical composition of dust is a very good match for several of the nutrients and trace metal requirements of diazotrophs.

Saharan dust contains many elements including trace metals, the most biologically important being P and Fe (Baker et al. 2006). N can adsorb to dust particles as NO, NO_2, and NH_3 (Mills et al. 2004, Duce et al. 2008). Saharan dust also contains organic N, P (Mahowald et al. 2008), and C (Duarte et al. 2006). *Trichodesmium* spp. have genes coding for organic P utilization enzymes (e.g. alkaline phosphatase and C-P lyase), making dissolved organic P accessible, and have a higher affinity for organic P over PO_4, which may give them a competitive advantage over other organisms (Sohm & Capone 2006). However, not all diazotrophs have equal access to dissolved organic P: for example UCYN-B (*Crocosphaera*) does not have C-P lyase (Dyhrman et al. 2006) and was not enhanced by dust additions in the D1 treatment and only moderately enhanced in the D2 treatment. The inability of *Crocosphaera* to utilize phosphonates may explain the low abundances and very patchy distribution of this phylotype in the North Atlantic. Although the amount of P and Fe released during the bioassay experiments described above was at the nmol level (Mills et al. 2004), the diazotrophic community was clearly able to respond to this low level influx of nutrients.

Saharan dust is the major source of both Fe and P to the Atlantic Ocean providing up to 16 Tg Fe yr^{-1} (Jickells et al. 2005) and 1.15 Tg P yr^{-1}(Mahowald et al. 2008), or 82 and 83% of the total input, respectively, and it is potentially very favorable to growth of diazotrophic organisms due to the low N:P ratio (Krishnamurthy et al. 2010). The absolute amounts of each nutrient that may be obtained from average, and even extreme, dust events are minimal (Table 3). A quick calculation shows that an average deposition of 30 µg dust m^{-3} (Guieu et al. 2002) to a 10 m surface layer delivers roughly 2.6 nmol N m^{-3}, 1.0 nmol P m^{-3}, and 21.5 nmol Fe m^{-3}. However, if a cell or colony of cells were adept at obtaining nutrients directly from dust particles before they leached into the surface water layer, the benefits of these added nutrients in a concentrated form would be greatly increased for microorganisms inhabiting nutrient-poor, oligotrophic waters. *Trichodesmium* may have several mechanisms for effectively scavenging Fe, and potentially other nutrients, from dust particles (Carpenter et al. 1991). For instance, colonies of *Trichodesmium* collected in the field rapidly attached to dust particles, and scanning electron microscopy revealed dust particles among bundles of *Trichodesmium* filaments (Carpenter et al. 1991). These colonies are very effective at dissolving dust and oxides (Rubin et al. 2011). Changes in *Trichodesmium* morphology from single filaments to large colonies upon dust addition (regardless of the Fe concentration in the media) were observed in our culture experiments (Fig. 4). Diazotrophs may also be able to access Fe through siderophores, high affinity Fe-binding compounds. Genes for nonribosomal peptide synthetase (NRPS) systems, which can be used to produce siderophores, are found in both the *T. erythraeum* and *Crocosphaera watsonii* genomes (Hopkinson & Morel 2009). Field *Trichodesmium* colonies have been shown to take up siderophore-bound Fe and, interestingly, radial 'puff' colonies had higher uptake rates than lateral 'tuft' colonies (Achilles et al. 2003). It is possible that other diazotrophs may have similar strategies for acquiring P and Fe in oligotrophic environments.

Table 3. Estimated inputs of total P, Fe, and N in a 10 m surface layer from an average (30 µg m^{-2}) and large (100 µg m^{-2}) dust event (as defined by Guieu et al. 2002)

	Composition (%)	Average event Deposited (µg m^{-3})	Average event Conc. (nmol m^{-3})	Large event Deposited (µg m^{-3})	Large event Conc. (nmol m^{-3})
P	0.1[a]	0.03	1.0	0.1	3.2
Fe	4[a]	1.2	21.5	4	71.6
N	0.12[b]	0.036	2.6	0.1	9.6

[a]From Guieu et al. (2002); [b]from Mills et al. (2004)

Saharan dust as a determinant for diazotroph distributions

Patterns in *Trichodesmium* abundance have been previously correlated with dust deposition (Langlois et al. 2008, Moore et al. 2008). Moore et al. (2009) provided a well-supported argument for the correlation of *Trichodesmium* and Saharan dust deposition. On a transect from the North Atlantic Ocean through the South Atlantic Ocean, both the distribution of *Trichodesmium* and peak N_2 fixation rates were aligned with maximum dissolved Fe and Al concentrations, the latter being a proxy for dust deposition, and reduced P concentrations. In the South Atlantic, there was a lack of *Trichodesmium* which correlated with decreased Fe and Al concentrations. Here integrated N_2 fixation was 16 µmol N d^{-1} m^{-2} and detected only in the <20 µm fraction; it is not likely to

be attributable to *Trichodesmium.* This result was recently confirmed by Fernandez et al. (2010) on 2 similar transects through the North and South Atlantic during the fall and spring. Additionally, a *Trichodesmium* bloom off western Florida, large enough to draw-down PO_4, occurred after a large deposition event (Lenes et al. 2001). *Trichodesmium* colony abundance at the Bermuda Atlantic Time-Series site was positively correlated to the magnitude of dust deposition; however, fixation rates did not increase with increasing dust deposition (Orcutt et al. 2001). Clearly, in the tropical North Atlantic Ocean, evidence is mounting to support the hypothesis that the deposition of desert dust originating from the African continent is a strong determinant for the distribution of *Trichodesmium.*

Saharan dust may not be beneficial to all marine organisms. A set of experiments performed in the Gulf of Aqaba demonstrated that Saharan dust was toxic, probably due to Cu bound to the dust, to picoeukaryotes and *Synechococcus*, but was not toxic to *Prochlorococcus* (Paytan et al. 2009). However, other studies have observed significantly lower Cu solubility for Saharan dust over the Atlantic (Sholkovitz et al. 2010) or no changes in seawater Cu speciation upon addition of Saharan dust (Heller & Croot 2011). In the bioassay experiments presented here, Saharan dust had variable effects on the picophytoplankton community (Fig. 4). Interestingly, the positive response of *Synechococcus* and *Prochlorococcus* was highest where the diazotroph response (specifically UCYN-A) was highest and decreased moving east, as did the positive response to dust of the phylotypes.

Regulation of N_2 fixation versus photosynthesis

N_2 fixation is an expensive process for cells, with respect to both energy and trace metal requirements. A phototrophic diazotroph requires 42 to 48 Fe atoms for both the nitrogenase enzyme and photosynthetic machinery (Shi et al. 2007). Diazotrophs, such as *Crocosphaera*, that fix nitrogen during the night are able to recycle Fe atoms between photosynthesis and nitrogenase enzymes (Raven 2012). Others, like *Trichodesmium*, that carry out photosynthesis and N_2 fixation simultaneously, need to find another solution. Under Fe stress, *Trichodesmium* cultures preferentially down-regulate N_2 fixation to conserve Fe for photosynthesis (Shi et al. 2007, Küpper et al. 2008). It is possible that open-ocean diazotrophs are in a permanent state of Fe stress when a balance between N_2 fixation and photosynthesis must be reached. Down-regulation of N_2 fixation in favor of photosynthesis may have occurred in the bioassay experiments and could explain the discrepancy between the increase in fixation rates and phylotype abundances. Photosynthesis may also have been preferentially up-regulated over *nifH* expression in the culture experiment. After 60 h, *nifH* expression decreased in all treatments except for the highest Fe concentration. The culture experiments also showed that *Trichodesmium* is able to at least partially fulfill its Fe requirements from atmospheric dust. Increases in *nifH* copy numbers were not higher with dust as an Fe source than that of dissolved Fe in the culture experiments compared to the field experiments, but cultured *Trichodesmium* is accustomed to high nutrient and Fe concentrations and may have been in a different physiological state than the oceanic diazotrophs. Rubin et al. (2011) also demonstrated that laboratory cultures of *T. erythraeum* were less efficient at obtaining Fe from dust particles than field communities. Only 2.4 nmol Fe was released from the dust into media without cells during lab experiments (P. Croot unpubl.). This is one-twentieth of the amount in the YBCII media. However, *Trichodesmium* colonies that are able to trap dust particles can potentially scavenge more iron directly without elevating bulk solution concentrations. In fact, when placed in media with atmospheric dust as the sole Fe source, the culture survived to the end of the experiment while the 0.0 µmol l^{-1} Fe control culture died, indicating that *Trichodesmium* was able to slowly extract Fe from the dust.

CONCLUSIONS

Aeolian dust deposition has long been suspected to provide Fe to *Trichodesmium* colonies in the Atlantic Ocean (Carpenter et al. 1991). Results of the bioassay experiments showed that dust additions clearly affected not only *Trichodesmium*, but other diazotrophic phylotypes abundances as well, indicating that atmospheric dust deposition may greatly influence the distribution of various diazotrophic groups. Culture experiments with *Trichodesmium* grown in Fe-free media and Saharan dust showed that it is able to access and use Fe from dust. Experiments with other cultured open-ocean diazotrophs need to be performed to see if this is a characteristic of all diazotrophs. A correlation between modeled annual aeolian dust deposition and *nifH* phylotype abundance has been observed in the Atlantic Ocean (Lang-

lois et al. 2008), supporting the results of the experiments presented here. Saharan dust deposition appears to play a major role in the distribution of diazotrophic organisms, and thus N_2 fixation, in the Atlantic Ocean.

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