

## ORIGINAL ARTICLE

# Molecular evidence of iron limitation and availability in the global diazotroph *Trichodesmium*

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**The activity of the N<sub>2</sub>-fixing cyanobacterial genus *Trichodesmium* is critical to the global nitrogen (N) and carbon (C) cycles. Although iron (Fe) has been shown to be an important element limiting the growth and N<sub>2</sub> fixation of *Trichodesmium*, there have been no specific data demonstrating the *in situ* affect of Fe on *Trichodesmium*. We surveyed *Trichodesmium* populations from the Atlantic and Pacific Oceans for Fe limitation using a novel quantitative reverse transcriptase-PCR (qRT-PCR) method monitoring the expression of an Fe limitation-induced gene, *isiB*. Here we report the first molecular evidence of *in situ* Fe limitation of *Trichodesmium* N<sub>2</sub> fixation, which was evident in samples from the Pacific Ocean, whereas limitation appeared minimal to nonexistent in Atlantic Ocean samples. As our method is *Trichodesmium* clade specific, we were also able to determine that representatives from the *Trichodesmium tenue* clade were the most biologically active group of *Trichodesmium* in the majority of our samples, which speaks to their dominance in open ocean regimes. Furthermore, comparisons of our field expression and chemical data with laboratory studies suggest that the majority of dissolved Fe in the open ocean is available to *Trichodesmium* colonies regardless of Fe complexation.**

*The ISME Journal* (2012) 6, 1728–1739; doi:10.1038/ismej.2012.13; published online 8 March 2012

**Subject Category:** geomicrobiology and microbial contributions to geochemical cycles

**Keywords:** nitrogen fixation; diazotrophs; iron; marine; Cyanobacteria

## Introduction

The N<sub>2</sub>-fixing cyanobacterial genus *Trichodesmium* is important to the global nitrogen (N) cycle by providing 'new' N to the system through N<sub>2</sub> fixation (Capone *et al.*, 1997). Data synthesized from multiple studies in the Atlantic Ocean show that diazotrophy (N<sub>2</sub> fixation) by *Trichodesmium* spp. can exceed mixing-driven NO<sub>3</sub> flux into the euphotic zone (Capone *et al.*, 2005), and it has been hypothesized that changes in N<sub>2</sub> fixation from diazotrophs can potentially influence CO<sub>2</sub> sequestration over geologic time scales (Capone *et al.*, 1997; Falkowski, 1997; Gruber and Sarmiento, 1997). Additionally, recent work in multiple laboratories has shown that N<sub>2</sub> fixation by *Trichodesmium erythraeum* IMS101 is predicted to increase concomitantly with atmospheric CO<sub>2</sub> (Hutchins *et al.*, 2007; Levitan *et al.*, 2007; Ramos *et al.*, 2007).

Therefore, defining the factors that affect *Trichodesmium* activity in the modern ocean is important for forecasting the biological removal of CO<sub>2</sub> from the atmosphere in the future.

The *Trichodesmium* genus comprises two major clades with cultured representatives (hereafter abbreviated *T. erythraeum* (Tery) and *Trichodesmium tenue* (Tten)), and although there are other *Trichodesmium* sequences that are episodically detected in field samples (Janson *et al.*, 1999; Orcutt *et al.*, 2002; Lundgren *et al.*, 2005; Hynes, 2009), members of these two major clades are the most commonly described (Benporath *et al.*, 1993; Carpenter *et al.*, 1993; Dyhrman *et al.*, 2006; Hynes, 2009; Hynes *et al.*, 2009). Despite the importance of *Trichodesmium*, the factors driving speciation in the genus have yet to be defined. In the last decade, a number of key laboratory discoveries have been made regarding the growth and N<sub>2</sub>-fixation rates of *Trichodesmium*, including: the CO<sub>2</sub> effects mentioned above, physical forcing from light (Breitbart *et al.*, 2008) as well as defining the temperature range of a representative of the Tery clade (Breitbart *et al.*, 2007). Additionally, other laboratory/field work (Berman-Frank *et al.*, 2001, 2007; Webb *et al.*, 2007; Jakuba *et al.*, 2008; Chappell

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Received 3 November 2011; revised 16 January 2012; accepted 16 January 2012; published online 8 March 2012

and Webb, 2010), chemical correlations in the field (Sanudo-Wilhelmy *et al.*, 2001; Kustka *et al.*, 2003b; Moore *et al.*, 2009) and community incubations (Mills *et al.*, 2004) show that the elements iron (Fe) and phosphorus (P) can influence *Trichodesmium* growth and N<sub>2</sub>-fixation capabilities. Because of its low solubility and comparatively high cellular requirement in *Trichodesmium* (Wu *et al.*, 2001; Kustka *et al.*, 2003b), Fe has been hypothesized to be an important limiting factor for N<sub>2</sub> fixation in much of the oceans that *Trichodesmium* inhabits (Berman-Frank *et al.*, 2001), potentially controlling large-scale distributions of N<sub>2</sub> fixation (Moore *et al.*, 2009). However, this predicted, widespread Fe limitation has yet to be directly demonstrated *in situ*, and it remains unclear what form of Fe is truly bioavailable for *Trichodesmium*.

Dissolved Fe (dFe) in sea water is operationally defined as the Fe that can flow through a 0.2 or 0.4 µm filter (Cutter *et al.*, 2010), and it is well established that in the surface oceans >99% of this Fe fraction is bound to organic ligands with undefined structures and uncertain bioavailability (Rue and Bruland, 1995). The remaining ≤1% of dFe (denoted Fe') is often presumed to be bioavailable and predominantly comprises Fe bound to inorganic ligands, and a small amount of the free ion (Fe<sup>3+</sup>) (Hudson and Morel, 1993). Potentially less bioavailable colloidal Fe (that is, Fe that is retained on a 0.02-µm filter) can also occur within the dFe fraction, but it has been suggested that most, if not all, noncolloidal forms are available to biology (Wu *et al.*, 2001). However, as the influence of organic complexation on Fe availability to phytoplankton is still uncertain, it is generally ignored in biogeochemical models (Moore *et al.*, 2004; Coles and Hood, 2007; Krishnamurthy *et al.*, 2007; Moore and Doney, 2007).

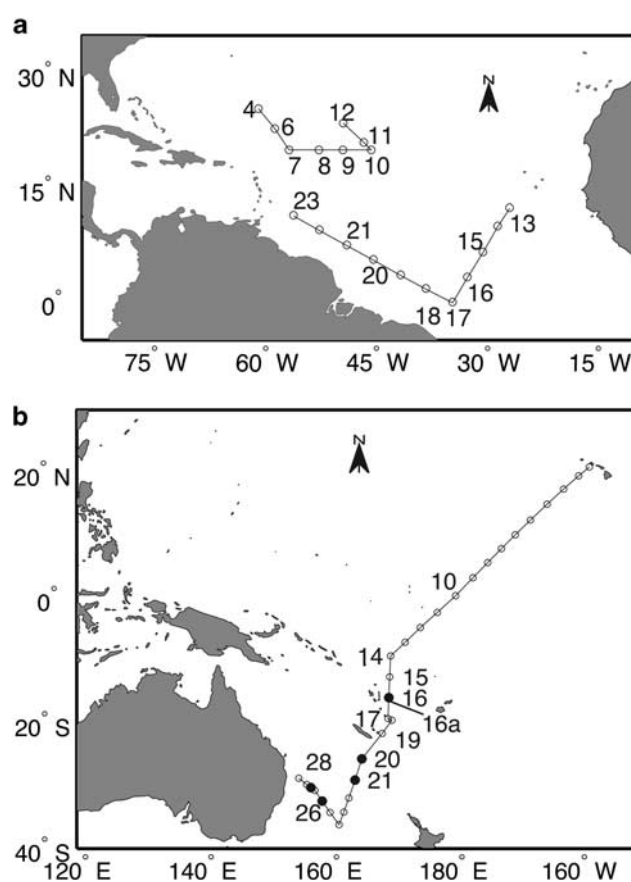
There are multiple methods to measure Fe stress and growth limitation in oceanic phytoplankton (Kolber *et al.*, 1994; Mills *et al.*, 2004; Rivers *et al.*, 2009). For example, the Fe stress-specific induction of flavodoxin has proven to be a valuable diagnostic in phytoplankton (LaRoche *et al.*, 1996). Building on this work, we developed an N<sub>2</sub>-fixation-rate-calibrated, clade-specific (that is, Tten and Tery), quantitative reverse transcriptase-PCR (qRT-PCR) method to evaluate Fe limitation of N<sub>2</sub> fixation in *Trichodesmium* spp. by monitoring the expression of the gene orthologs encoding flavodoxin, *isiB* (Chappell and Webb, 2010). We applied this assay on *Trichodesmium* colonies collected during daytime from three cruises in both the Atlantic and Pacific Oceans and analyzed the results in the context of *in situ* measurements of dFe and dissolved inorganic P (DIP). We chose sampling times and locations that were anticipated to cover a range of dFe and DIP concentrations, thus including regions where we expected to find Fe limitation as well as those where we hypothesized *Trichodesmium* would likely be P limited or Fe/P co-limited.

Our data show that Fe is indeed limiting to *Trichodesmium* in the ocean. Additionally, comparisons of our field expression data with laboratory results suggest that most, if not all, of the dFe is bioavailable to *Trichodesmium* regardless of complexation.

## Materials and methods

### Sample collection

Sampling took place on three cruises: in the Sargasso Sea on the R/V Oceanus (cruise OC399-4), 22–30 March 2004 (Figure 1a, stations 4–12), in the Equatorial Atlantic aboard the R/V Seward Johnson (cruise SJ0609), 12–24 July 2006 (Figure 1a, stations 13–21), and aboard the R/V Kilo Moana (cruise KM0701) as part of the Western Pacific Warm Pool cruise, 12 January to 9 February 2007 (Figure 1b). Samples for dFe and nutrients were collected using standard trace metal techniques similar to those described by Jakuba *et al.* (2008) using acid-cleaned 10 l Teflon-coated Go-Flo bottles (General Oceanics,



**Figure 1** Map of cruise tracks. (a) OC399-4 (stations 4–12) in the Sargasso Sea in March 2004. SJ0609 (Stations 13–21) east-to-west transect across the equatorial Atlantic Ocean in July 2006. (b) KM0701 north-to-south transect through the Western Pacific Warm Pool during January and February 2007. Open circles mark all stations from each cruise. Numbered stations represent where *Trichodesmium* colonies were collected. Filled circles represent where *isiB* expression was above threshold value indicative of Fe limitation.

Miami, FL, USA) or 5 l Teflon-coated exterior spring Niskin bottles (Ocean Test Equipment, Ft. Lauderdale, FL, USA) deployed on a Kevlar line. All sample processing was done under Class 100 cleanroom conditions either in a trace metal clean 'bubble' kept at positive pressure using HEPA (high-efficiency particulate arresting)-filtered air flow that was built on the ship or a trace metal clean van also supplied with HEPA-filtered air. Water for dFe analysis was 0.4- $\mu\text{m}$  filtered, collected in acid-cleaned 250 ml low-density polyethylene bottles and acidified to pH 1.7 with concentrated high-purity hydrochloric acid (Seastar, Sidney, BC, Canada). The value of dFe in the seawater samples was determined using isotope dilution and magnesium hydroxide pre-concentration followed by analysis using inductively coupled mass spectrometry (Saito and Schneider, 2006).

Water for nutrient analysis was 0.4- $\mu\text{m}$  filtered and then immediately frozen at  $-20^\circ\text{C}$  for later analysis. Analysis of DIP concentrations from the Sargasso Sea cruise was reported by Jakuba *et al.* (2008). Samples from SJ0609 and KM0701 were sent to the College of Oceanic and Atmospheric Sciences at Oregon State University where DIP was analyzed using a Technicon AutoAnalyzer II (SEAL Analytical Ltd, Fareham, UK) by J Jennings with a detection level of  $6\text{ nmol l}^{-1}$ .

*Trichodesmium* samples were collected using a 130- $\mu\text{m}$  phytoplankton net (Sea-Gear Corporation, Melbourne, FL, USA) that was hand towed at the surface for 10–20 min using a 30-m line. Immediately following the return to the surface, the contents of the tow were taken into the air-conditioned laboratory aboard the ship where the *Trichodesmium* colonies were separated from the other plankton using polypropylene bulb transfer pipettes. Colonies were transferred from the bulk solution into clean 0.4  $\mu\text{m}$ -filtered microwave-sterilized sea water, then they were filtered onto 5- $\mu\text{m}$  polycarbonate filters and stored in liquid  $\text{N}_2$  until RNA processing and analysis. Because of the relatively high *Trichodesmium* biomass observed on OC399-4, 200 ml of the bulk net tow was filtered onto 5- $\mu\text{m}$  polycarbonate filters and preserved in liquid  $\text{N}_2$  without separation and rinsing. On cruises OC399-4 and KM0701, samples were always taken between 0800 and 1000 h local time, which is consistent with the timing of sampling in our laboratory study (Chappell and Webb, 2010); on cruise SJ0609, samples were taken throughout the day and night (Supplementary Table S1), but only samples obtained during the day were included in later expression analyses.

#### RNA extraction and qRT-PCR

Upon return to the laboratory, samples were processed and analyzed using the previously described procedure for RNA extraction and qRT-PCR analysis of gene expression (Chappell and Webb, 2010). Separate primer sets for the Tery clade targeting *T. erythraeum* strains (IMS101 and GBRTLI101)

and *T. contortum* strain (21–74) and the Tten clade targeting *T. tenue* strains (Z-1 and H9-4) and *T. theibautii* strain (VI-I) were designed for orthologs of both our target Fe stress response gene, *isiB*, and a RNA normalization control gene, *rnpB*. The primers were originally described by Chappell and Webb (2010) and are relisted in Supplementary Table S2.

#### Cloning

Prior laboratory work had shown that the clade-specific *isiB* and *rnpB* primers only amplified products from the expected isolates (Chappell and Webb, 2010), but to verify this finding with field samples, we cloned Tten RT-PCR products from two stations during the KM0701 cruise: Stations WP14 and WP16a. The PCR for cloning was done using an ABI9700 PCR System (Applied Biosystems Inc., Foster City, CA, USA), which has the same ramp settings as the 7500 Fast Real-Time PCR Machine using MasterTaq Taq DNA polymerase (Eppendorf AG, Hamburg, Germany), at a final concentration of 1.25 units per PCR reaction as per the manufacturer's instructions without additional  $\text{Mg}^{+2}$  ( $1 \times$ ) in a 20  $\mu\text{l}$  reaction with a final complementary DNA (cDNA) concentration of 1–2  $\text{nmol l}^{-1}$  and a final primer concentration of 200  $\text{nmol l}^{-1}$ . To mimic the qRT-PCR conditions, the PCR cycling conditions were  $50^\circ\text{C}$  for 2 min,  $95^\circ\text{C}$  for 10 min; 40 cycles of  $95^\circ\text{C}$  for 15 s and  $55^\circ\text{C}$  for 1 min; followed by a final extension at  $55^\circ\text{C}$  for 5 min. The RT-PCR products from Stations WP16a and WP14 amplified with Tten *isiB* and Tten *rnpB* primers were cloned directly using the TOPO TA Cloning Kit for Sequencing (Invitrogen Corporation, Carlsbad, CA, USA). A total of 48 clones from each reaction were incubated in  $2 \times$  LB for 36 h at  $28^\circ\text{C}$  in a shaking incubator (New Brunswick Scientific, Edison, NJ, USA) and extracted using the Montage Plasmid Miniprep<sub>96</sub> Kit (Millipore Corporation, Billerica, MA, USA) as per the manufacturer's guidelines and sequenced using M13 forward and reverse primers by Laragen Inc. (Los Angeles, CA, USA). Sequences were trimmed of vector and examined. As all *rnpB* sequences were identical to *T. tenue rnpB*, no further analysis was done. The *isiB* sequences were aligned with *Trichodesmium isiB* sequences from GenBank using the MUSCLE algorithm (Edgar, 2004) and then arranged into a phylogenetic tree using the maximum likelihood method (HKY85 substitution model, 1000 bootstraps, and transition/transversion of four) with PHYML in the Geneious software package (Biomatters, Auckland, New Zealand). As we rarely detected Tery in the field samples and there is reduced sequence diversity in this clade (Hynes *et al.*, 2012), the Tery primers were not similarly tested.

#### Restriction analysis

Using the Tten *isiB* sequences defined above from stations WP16 and WP14, we were able to screen our

RT-PCR products from all stations for the presence of uncultured, novel *Trichodesmium* Tten phylogenotypes in our amplicons from other stations via the presence of two separate, conserved restriction sites (Supplementary Figure S1). Briefly, cDNA was amplified from all KM0701 and SJ0609 stations (where prior qRT-PCR detected Tten *isiB*) using Tten *isiB* primers at a concentration of 200 nmol l<sup>-1</sup>, BIO-X-ACT Short Mix (Bioline, London, UK) at 1 × concentration in a 20 µl reaction with 1–2 nmol l<sup>-1</sup> template on a Mastercycler thermal cycler (Eppendorf AG). The PCR cycling conditions were: 50 °C for 2 min, 95 °C for 10 min; 40 cycles of 95 °C for 15 s, 55 °C for 1 min; followed by a final extension at 60 °C for 5 min. For the *HaeIII* restriction digest, 5 µl of PCR product was incubated with 25 units of *HaeIII* (New England Biolabs, Ipswich, MA, USA) and 1 × NEB4 buffer in a final volume of 47.5 µl for 3 h at 37 °C, followed by 20 min at 80 °C. For the *AcII* restriction digest, 5 µl of PCR product was incubated with 7.5 units of *AcII* (New England Biolabs) and 1 × NEB4 buffer in a final volume of 45 µl for 3 h at 37 °C. Uncut and cut PCR products were run side by side on a 1.5% agarose gel and imaged using GeneSnap imaging software on a ChemiGenius<sup>2</sup> gel documentation system (Synoptics Ltd, Cambridge, UK).

#### Nitrogen fixation analysis

The N<sub>2</sub>-fixation rates were measured on samples from two stations on cruise OC399-4 and six stations on KM0701 by the acetylene reduction assay described by Capone (1993) using the same methods described by Webb *et al.* (2007). The N<sub>2</sub>-fixation rates were normalized to fluorometrically measured chlorophyll *a* (Herbland *et al.*, 1985). Briefly, the protocol entailed incubating 10–20 colonies at temperatures similar to the sea water they were isolated from at ~75 µmol quanta m<sup>-2</sup> s<sup>-1</sup> in 75 ml polycarbonate bottles (Nalge Nunc International Corp., Rochester, NY, USA) filled with 30 ml presterilized filtered sea water in a Percival incubator (Percival Scientific, Inc., Perry, IA, USA). A total of 6 ml of acetylene gas was added to the headspace at the start of the incubation, and acetylene production was measured shipboard on a Shimadzu GC-8a (Shimadzu Scientific Instruments, Columbia, MD, USA) hourly for a maximum of 3 h.

## Results

#### Sampling and environmental parameters

A map of the sampling locations for all cruises is shown in Figure 1. Only stations where there was amplifiable *Trichodesmium* cDNA are numbered. Fitting with the winter season in the northern hemisphere, *Trichodesmium* was primarily detected in net tows during the southern portions of the WP cruise. Detailed information on station locations, sea surface temperature, dFe, DIP, % Tten clade *rnpB*

and Tten clade expression values are listed in Table 1. The dFe values were highest in the Sargasso Sea and lowest in the western Pacific Ocean, which is consistent with global atmospheric dust deposition data (Mahowald *et al.*, 2009). Conversely, DIP values were lowest in the Sargasso Sea and highest in the western Pacific Ocean. The Tten clade *rnpB* dominated most samples, and Tten *isiB* expression was lowest in the Sargasso Sea and highest in the western Pacific Ocean (Table 1).

#### Q-PCR primer efficiency and specificity

Clade-specific primers were tested with multiple representatives of each clade (*T. erythraeum* strains IMS101 and GBRTL101 for the Tery primers and *T. tenue* strains Z-1 and H9-4 and *T. thiebautii* VI-I for the Tten primers). The efficiencies of the Tten *isiB* and Tten *rnpB* primers were between 90% and 94% using DNA isolated from cultures of *T. tenue* and *T. thiebautii*. The qPCR efficiencies for all qRT-PCRs run were generally in the 91–93% range, with three at 96% and one at 90%.

Although we have previously shown that the *Trichodesmium rnpB* and *isiB* Q-PCR primers were clade specific with DNA and RNA from cultured isolates (Chappell and Webb, 2010), *Trichodesmium* sequences have been detected in environmental studies that are not represented in culture (Janson *et al.*, 1999; Lundgren *et al.*, 2005; Hynes, 2009). To eliminate concerns that these potential uncultured phylotypes were more numerically dominant and/or might have different physiologies than our cultured isolates, we tested the specificity of our primers to verify that they were only amplifying the expected targets. Cloning and sequencing of the Tten clade *rnpB* Q-PCR products amplified from field samples yielded only sequences identical to *T. tenue* H9-4 (EMBL accession HE608976-HE609006), whereas this analysis of Tten clade *isiB* (EMBL accession HE608683-HE608765) revealed sequence diversity and showed that the primers were amplifying uncharacterized, noncultured phylotypes of *Trichodesmium*. Figure 2 and Supplementary Figure S2 summarize this analysis and identify a well-supported *isiB* clade from Station WP16a that is not represented in our culture collection and has not been seen before in field samples (group I on the tree), as well as a sub-clade with slightly lower bootstrap support values (group II on the tree). Examination of the alignment revealed a conserved *HaeIII* cut site 66 base pairs into the novel *isiB* PCR product of the group I sequences, and a conserved *AcII* cut site 40 base pairs into the novel *isiB* PCR product of the group II sequences that were not present in the rest of the Tten sequences (Supplementary Figures S1 and S2). Restriction analyses verified that sequences matching the new clade from Station WP16a (group I in Figure 2) were only detected in samples from stations WP16a and WP10 (Figure 3a), whereas the novel clade, group II in Figure 2, was

**Table 1** Near-surface (10–15 m) data from stations on all three cruises where *isiB* expression of *Trichodesmium* was measured

Station	Lat	Long	Temp (°C)	Fe (nM)	PO <sub>4</sub> (μM)	Log ( <i>isiB/rnpB</i> ) <sup>a</sup>	%Tten
S4	25.40	-61.15	24.7	1.17 ± 0.04	0.0037 ± 0.0049 <sup>b</sup>	-1.96 ± 0.02	99.95
S6	22.80	-58.93	25.2	1.04 ± 0.08	<0.014 <sup>b</sup>	-2.30 ± 0.02	99.97
S7	20.00	-57.00	25.0	1.15 ± 0.09	0.0136 ± 0.0049 <sup>b</sup>	-2.34 ± 0.04	99.99
S8	20.00	-52.97	25.2	1.00 ± 0.12	0.0017 ± 0.0001 <sup>b</sup>	BDL	100.00
S9	20.00	-49.73	25.0	1.11 ± 0.04	0.002 ± 0.0004 <sup>b</sup>	-3.42 ± 0.09	100.00
S10	20.00	-45.90	24.6	1.01 ± 0.04	0.0058 ± 0.0049 <sup>b</sup>	BDL	100.00
S11	20.97	-46.90	24.8	0.94 ± 0.06	0.0016 ± 0.0004 <sup>b</sup>	-2.21 ± 0.02	99.76
S12	23.52	-49.68	25.4	0.82 ± 0.05	0.0033 ± 0.0004 <sup>b</sup>	-2.58 ± 0.09	100.00
E13	12.40	-27.20	26.9	0.74 ± 0.12	0.045 ± 0.000	-1.62 ± 0.02	100.00
E15	6.60	-30.80	28.4	0.61 ± 0.02	0.016 ± 0.002	-1.99 ± 0.02	100.00
E16	3.30	-32.90	27.8	0.09 ± 0.00	0.037 ± 0.002	-1.40 ± 0.04	100.00
E17	0.01	-34.90	26.8	0.14 ± 0.01	0.076 ± 0.004	-1.31 ± 0.02	100.00
E18	1.80	-38.50	28.2	0.10 ± 0.00	0.031 ± 0.009	-1.01 ± 0.02 <sup>c</sup>	100.00
E20	5.60	-45.60	28.5	0.67 ± 0.03	0.035 ± 0.010	-1.80 ± 0.04	99.48
E21	7.50	-49.20	28.7	1.89 ± 0.03	0.068 ± 0.009	-1.54 ± 0.04	99.86
E23	11.40	-56.40	28.4	1.17 ± 0.05	0.052 ± 0.00	-1.24 ± 0.02 <sup>c</sup>	NA
WP10	0.37	-179.64	29.3	0.11 ± 0.04	0.324 ± 0.000 <sup>d</sup>	0.33 ± 0.05 <sup>a,c</sup>	41.19
WP14	-9.25	170.00	30.6	0.20 ± 0.04	0.168 ± 0.000 <sup>d</sup>	-1.98 ± 0.02	100.00
WP15	-12.58	169.86	30.2	0.11 ± 0.03	0.133 ± 0.002 <sup>d</sup>	-1.72 ± 0.01	99.95
WP16	-15.89	169.72	28.8	0.29 ± 0.03	0.169 ± 0.000 <sup>d</sup>	-0.14 ± 0.04 <sup>a</sup>	99.61
WP16a	-15.98	169.77	28.5	0.63 ± 0.02	0.148 ± 0.003 <sup>d</sup>	-0.70 ± 0.05 <sup>a,c</sup>	83.06
WP17	-19.23	169.58	27.2	0.95 ± 0.02	0.137 ± 0.004 <sup>d</sup>	-1.66 ± 0.03	99.86
WP19	-21.62	168.66	26.8	0.50 ± 0.08	0.073 ± 0.000 <sup>d</sup>	-1.29 ± 0.02	98.87
WP20	-25.67	165.42	26.0	0.09 ± 0.02	0.102 ± 0.002 <sup>d</sup>	-0.56 ± 0.05 <sup>i</sup>	99.59
WP21	-29.04	164.34	24.5	0.24 ± 0.02	0.050 ± 0.002 <sup>d</sup>	0.13 ± 0.04 <sup>a</sup>	100.00
WP26	-32.42	159.09	24.5	0.20 ± 0.02	0.084 ± 0.002 <sup>d</sup>	-0.63 ± 0.03 <sup>a</sup>	99.98
WP28	-30.26	157.30	24.7	0.51 ± 0.12	0.061 ± 0.000 <sup>d</sup>	-0.75 ± 0.10 <sup>a</sup>	NA

Abbreviations: BDL, *isiB* expression was below detection level in the sample; Fe, iron; NA, not applicable; Tten, *Trichodesmium tenue*. 'S' stations are from cruise OC399-4 in the Sargasso Sea, 'E' stations are from cruise SJ0609 in the Equatorial region of the Atlantic Ocean and 'WP' station are from cruise KM0701 in the Pacific Ocean.

The s.d. of triplicate technical replicates are given for [Fe], the *isiB/rnpB* ratio, and [PO<sub>4</sub>] from OC399-4 (S) and duplicate technical replicates for [PO<sub>4</sub>] from SJ0609 (E) and KM0701 (WP).

%Tten = ((no. of copies of Tten clade *rnpB*)/(no. of copies of Tery + Tten clade *rnpB*)) × 100.

<sup>a</sup>Values above -0.84 ± 0.16 are indicative of Fe limitation.

<sup>b</sup>PO<sub>4</sub> values from Jakuba *et al.* (2008).

<sup>c</sup>Stations that were excluded from subsequent analysis because of primer specificity or time of sampling.

<sup>d</sup>PO<sub>4</sub> values from Hynes *et al.* (2009).

not significantly represented in the PCR products from any station (Figure 3b). As cultured representatives of these newly identified *Trichodesmium isiB* clades do not exist, we were not able to test their Fe stress response, and thus we excluded *isiB/rnpB* expression data of stations WP10 and WP16a from subsequent analyses.

#### Fe stress in natural *Trichodesmium* samples

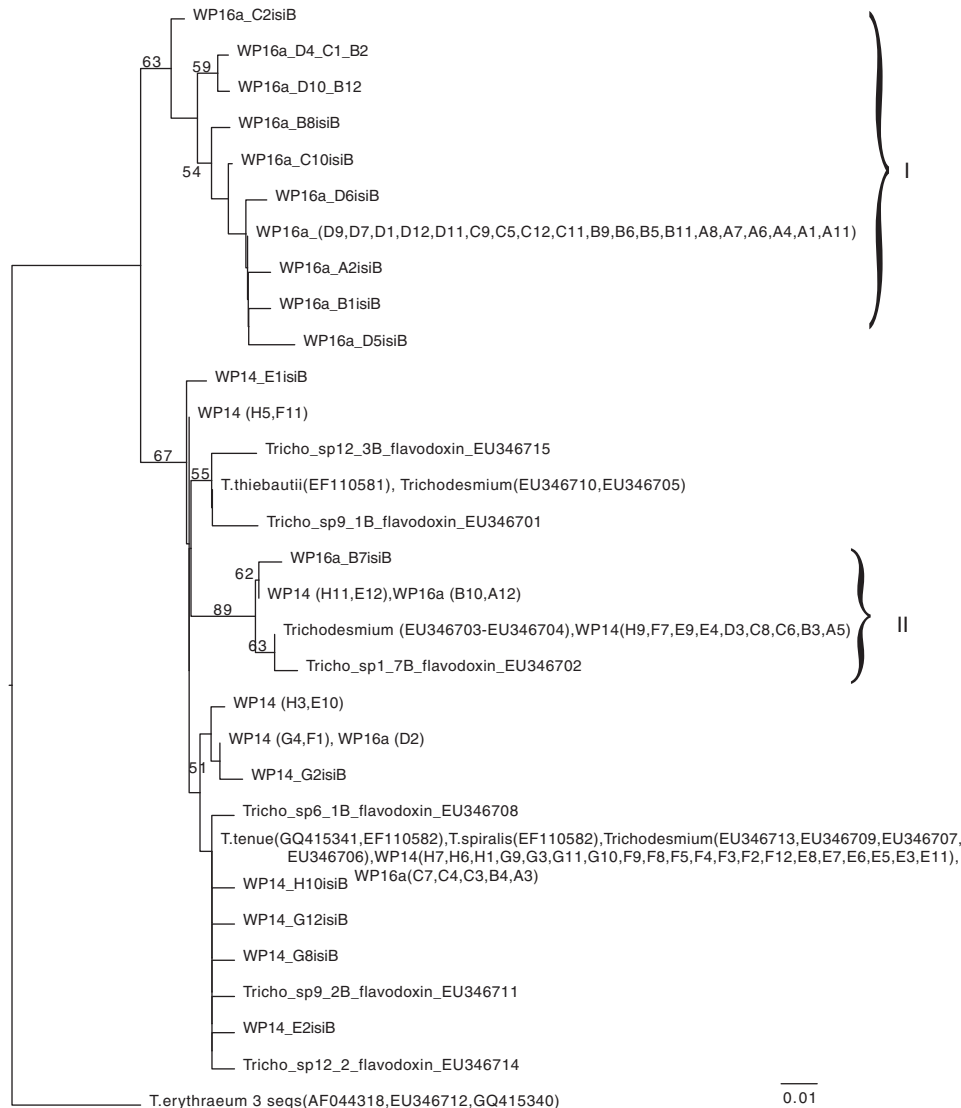
As mentioned above, there are many species of *Trichodesmium* in the ocean making up at least two clades (Janson *et al.*, 1999; Lundgren *et al.*, 2005; Hynes *et al.*, 2012). Herein, we only report Tten clade-specific *isiB* gene expression (relative to the Tten reference gene *rnpB*), as the majority of samples did not have a significant amount of Tery clade RNA (Table 1).

High expression of the Fe stress gene, *isiB*, was only detected in Tten colonies isolated from the Pacific and not from the Atlantic Ocean (Table 1). Comparisons of the Tten *isiB* expression data and dFe/DIP are depicted in Figure 4a and resulted in a line (equation:  $\log_{10} (isiB/rnpB) = (-0.60 \pm 0.14) \times \log_{10} ([Fe]/[PO_4]) + (-2.7 \pm 0.28)$ ) with an *R*<sup>2</sup> value

of 0.50. Comparisons of  $\log_{10} (isiB/rnpB)$  and total dFe resulted in a line (equation:  $\log_{10} (isiB/rnpB) = (-1.51 \pm 0.38) \times [Fe] + (-0.7 \pm 0.26)$ ), which has an *R*<sup>2</sup> value of 0.47. The relationship between *isiB* expression and N<sub>2</sub>-fixation rates from select stations in the Atlantic and Pacific Oceans is shown in Figure 5, and is described by a linear regression line (nmol N fixed per h per μg chl =  $(-0.37 \pm 0.13) \times (\log_{10} (isiB/rnpB)) + (0.05 \pm 0.46)$ ), which has an *R*<sup>2</sup> value of 0.62.

## Discussion

A number of studies have established the importance of Fe limitation to *Trichodesmium* N<sub>2</sub> fixation in the laboratory (Berman-Frank *et al.*, 2001; Webb *et al.*, 2001; Fu and Bell, 2003; Kustka *et al.*, 2003a,b; Berman-Frank *et al.*, 2007; Shi *et al.*, 2007; Kupper *et al.*, 2008; Chappell and Webb, 2010). Recently, these efforts have focused on developing molecular methods that can be used to evaluate *Trichodesmium* Fe limitation at the cellular level (Webb *et al.*, 2001; Shi *et al.*, 2007; Chappell and Webb, 2010). Global biogeochemical models can

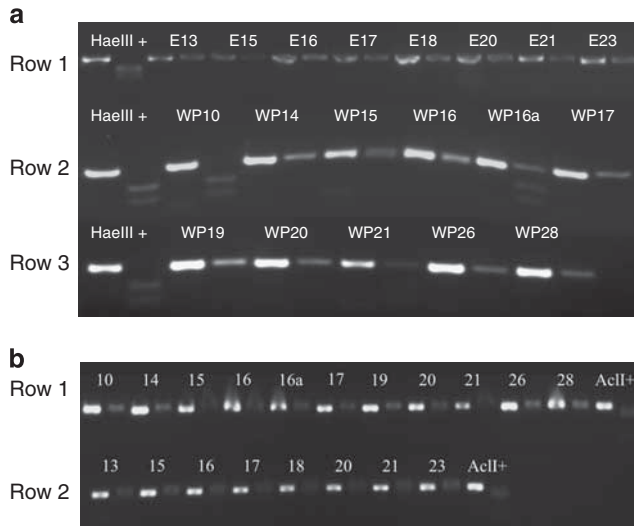


**Figure 2** Maximum likelihood tree of sequences from clones of *Tten isiB* RT-PCR products from two stations from the Pacific Ocean and *Trichodesmium isiB* sequences in Genbank based on a MUSCLE (Edgar, 2004) alignment done in Geneious (www.geneious.com) using the JC69 method of nucleotide substitution. Bootstrap values < 50 were removed from the tree for clarity. Sequences in the ‘I’ grouping have the *HaeIII* cut site. Sequences in the ‘II’ grouping have the *AcII* cut site.

be useful tools to predict the past, current, and future activity of key functional groups in the ocean (Moore *et al.*, 2004; Coles and Hood, 2007; Krishnamurthy *et al.*, 2007; Moore and Doney, 2007); however, empirical verification is required to prove where Fe is constraining *Trichodesmium* N<sub>2</sub> fixation and to determine what form of Fe (that is, total, free, or inorganically bound Fe) is bioavailable. The development of a calibrated molecular method to evaluate Fe limitation of N<sub>2</sub> fixation in *Trichodesmium* spp. (Chappell and Webb, 2010) provided a technique capable of achieving this goal. This study represents the first quantitative assessment of Fe limitation in field populations of *Trichodesmium* through areas predicted to be Fe limited, P limited, and/or Fe–P co-limited.

Quantitative analyses of cDNA revealed that *Tten* clade representatives were the predominant species

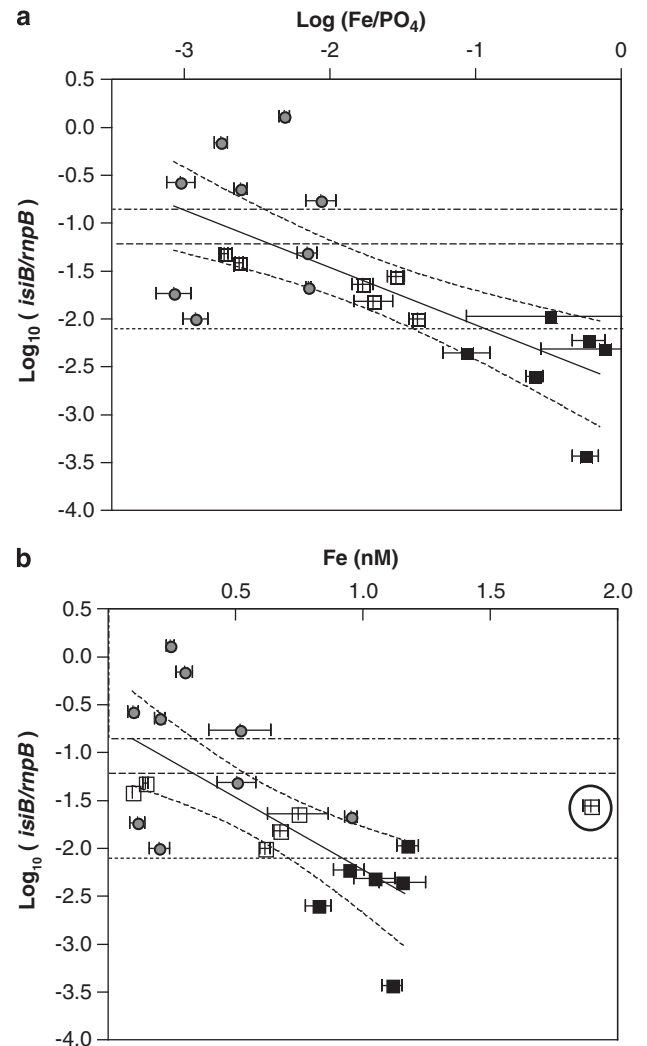
of *Trichodesmium* detected in colonies in the field (Table 1, %*Tten* column). These results are consistent with previous morphological studies that found that *T. thiebautii*, a representative from the *Tten* clade, is the dominant *Trichodesmium* in the open ocean (Carpenter and Price, 1977; Sohm *et al.*, 2008). The qPCR analysis of DNA from bulk surface water samples from the CTD also supports our conclusion that the *Tten* clade dominated the surface waters of our sampling sites (Hynes, 2009). Only two stations had a significant amount of *Tten* cDNA: stations WP10 and WP16a. Station WP10 appeared to be influenced by equatorial upwelling based on high DIP values (Supplementary Table S3) and Station WP16a was a sample from a *Trichodesmium* bloom that was entrained in ‘normal’ water chemistry conditions (that is, we were unable to chemically identify the cause of the bloom). In addition to



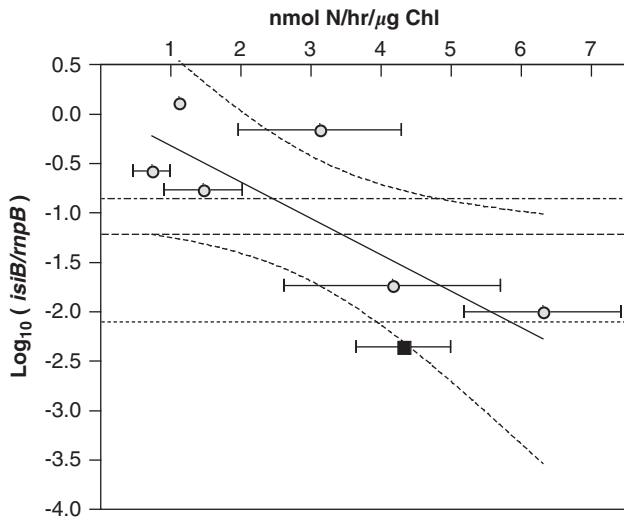
**Figure 3** (a) Image of a 1.5% Agarose gel of *isiB* RT-PCR products from field samples and *HaeIII* restriction digests. The left-most pair of each row is the *isiB* PCR product of a *HaeIII*-positive *isiB* clone and the result of the *HaeIII* restriction digest of that PCR product. Row 1 shows the results from the cDNA from the equatorial Atlantic transect (SJ0609) and rows 2 and 3 are the results from the cDNA from the western Pacific transect (KM0701). (b) Image of a 1.5% Agarose gel of *isiB* RT-PCR products from field samples and *AcII* restriction digests. The right-most pair of each row is the *isiB* PCR product of an *AcII*-positive *isiB* clone and the result of the *AcII* restriction digest of that PCR product. Row 1 shows the results from the cDNA from the western Pacific transect (KM0701) and row 2 shows the results from the cDNA from the equatorial Atlantic transect (SJ0609).

having a significant amount of Tery clade cDNA, both of these stations also showed evidence of a third group of *Trichodesmium*, the sub-clade of Tten that was revealed by our cloning efforts (Figure 3a), indicating that both stations exhibited a high overall diversity of *Trichodesmium* species. As there was a significant amount of Tten cDNA at all stations, *isiB* expression refers specifically to Tten *isiB* expression throughout the discussion.

During the Atlantic cruises, there was no indication of Fe limitation of  $N_2$  fixation based on *isiB* expression (that is, expression was never above the threshold value  $\log_{10} (isiB/rnpB) = -1.20 \pm 0.1$  corresponding to a 50% reduction in  $N_2$  fixation; Chappell and Webb, 2010; Table 1 and Figure 4). Basal *isiB* expression in all samples from the Sargasso Sea was consistent with low DIP and high dFe (Table 1 and Supplementary Figure S3). Although *isiB* expression was not above the threshold expression value in any of the Atlantic Ocean samples, there was an increase in expression above the basal expression value in some samples from the Equatorial Atlantic cruise indicative of growing Fe stress (Figure 4). Overall, the data from the two Atlantic cruises are consistent with previous work suggesting that the diazotrophs in the North Atlantic Ocean are predominantly P stressed



**Figure 4** Relationships between  $\log_{10} (isiB/rnpB)$  of the Tten clade and  $\log_{10} ([Fe]/[PO_4])$  (a), and dissolved [Fe] (b). Three dashed horizontal lines are shown on each plot. The bottom line is at the value  $\log_{10}(isiB/rnpB) = -2.09$ , which is the value associated with basal expression in the Tten clade from previous work (Chappell and Webb, 2010). The middle line at the value  $\log_{10}(isiB/rnpB) = -1.20$ , which is the value associated with a 50% reduction in  $N_2$  fixation in the Tten clade from previous work that we use as a threshold value to indicate Fe limitation of  $N_2$  fixation in Tten (Chappell and Webb, 2010). The top line is at the value  $\log_{10}(isiB/rnpB) = -0.84$ , which is the highest expression value measured in Tten laboratory cultures (Chappell and Webb, 2010). Values from the Sargasso Sea are plotted as filled squares, values from the Equatorial Atlantic are plotted as open squares and values from the Pacific are plotted as open circles. The solid line in (a) represents the linear regression of  $\log_{10} ([Fe]/[PO_4])$  versus  $\log_{10} (isiB/rnpB)$  with the dashed curves on either side representing the 95% confidence intervals. The equation for this line is  $\log_{10} (isiB/rnpB) = (-0.60 \pm 0.14) \times \log_{10} ([Fe]/[PO_4]) + (-2.7 \pm 0.28)$ , which has an  $R^2$  value of 0.50. The solid line in (b) represents the linear regression of [Fe] versus  $\log_{10} (isiB/rnpB)$  excluding the data point that is circled, which was influenced by the Amazon River Plume (Station E21). The dashed curves on either side represent the 95% confidence intervals for this regression. The equation for this line is  $\log_{10} (isiB/rnpB) = (-1.51 \pm 0.38) \times [Fe] + (-0.7 \pm 0.26)$ , which has an  $R^2$  value of 0.47. Error bars represent the s.d. of triplicate analyses.



**Figure 5** Relationships between  $\log_{10}(isiB/rnpB)$  and  $N_2$ -fixation rates at select stations. Three dashed horizontal lines are shown on the plot. The bottom line is at the value  $\log_{10}(isiB/rnpB) = -2.09$ , which is the value associated with basal expression in the Tten clade from previous work (Chappell and Webb, 2010). The middle line at the value  $\log_{10}(isiB/rnpB) = -1.20$ , which is the value associated with a 50% reduction in  $N_2$  fixation in the Tten clade from previous work that we use as a threshold value to indicate Fe limitation of  $N_2$  fixation in Tten (Chappell and Webb, 2010). The top line is at the value  $\log_{10}(isiB/rnpB) = -0.84$ , which is the highest expression value measured in the Tten clade from previous work (Chappell and Webb, 2010). Values from the Sargasso Sea are plotted as filled squares and values from the Pacific are plotted as open circles. The solid line represents the linear regression of  $N_2$ -fixation rates versus  $\log_{10}(isiB/rnpB)$  with the dashed curves on either side representing the 95% confidence intervals. The equation for this line is:  $\text{nmol N fixed per h per } \mu\text{g chl} = (-0.37 \pm 0.13) \times (\log_{10}(isiB/rnpB)) + (0.05 \pm 0.46)$ , which has an  $R^2$  value of 0.62.

(Sanudo-Wilhelmy *et al.*, 2001; Webb *et al.*, 2007), but that parts of the equatorial North Atlantic have the potential to shift between P and Fe limitation (Mills *et al.*, 2004).

In contrast to the North Atlantic, measured levels of *isiB* expression in Tten colonies from the Pacific Ocean indicated that Fe limitation was common over much of that transect where *Trichodesmium* was detected (Figure 1 and Table 1). These data are consistent with model predictions suggesting that Fe limits  $N_2$  fixation in this region (Moore *et al.*, 2004; Krishnamurthy *et al.*, 2007; Moore and Doney, 2007). There was some evidence of P stress detected during cruise KM0701 toward the western side of the Pacific Basin (Hynes *et al.*, 2009), which is consistent with a previous study indicating that parts of the southwestern Pacific Ocean can be transiently P stressed (Moutin *et al.*, 2005). Although the qualitative nature of the P-stress assay employed by Hynes *et al.* (2009) limits our ability to directly address Fe–P co-limitation during KM0701, our quantitative *isiB* expression data from *Trichodesmium* demonstrate for the first time the importance of Fe in these sectors of the Pacific Ocean.

Comparison of environmental chemical concentrations and *Trichodesmium isiB* expression data revealed an inverse linear relationship between *isiB* expression and dFe/DIP ( $R^2 = 0.50$ ; Figure 4a). Given the importance of both Fe and P as potential limiting nutrients for *Trichodesmium* (Berman-Frank *et al.*, 2001; Sanudo-Wilhelmy *et al.*, 2001; Kustka *et al.*, 2003b), it is reasonable that there could be an association between *isiB* expression values and the dFe/DIP ratio. In a low dFe and DIP regime, *Trichodesmium* may be growing below its optimal rate because of P limitation, and thus may be able to satisfy its Fe requirement to support that slow growth without any induction of *isiB* (that is, no Fe limitation). If DIP increases but dFe remains low and constant, growth rates and Fe demand would increase, leading to Fe limitation and *isiB* expression. We are mindful that *Trichodesmium* can also access P from the dissolved organic P pool under conditions of P stress (Dyhrman *et al.*, 2006; Sohm *et al.*, 2008; Hynes *et al.*, 2009). As the enzymes associated with dissolved organic P utilization are only significantly expressed when cells are DIP limited, we do not believe that dissolved organic P utilization would affect the relationship between *isiB* expression and dFe/DIP. In addition, there was no significant correlation between *isiB* expression and DIP ( $R^2 = 0.25$ , data not shown). Linear regression analyses suggest that the dFe/DIP value associated with Fe limitation of  $N_2$  fixation in field populations of *Trichodesmium* is  $0.004 \text{ mol mol}^{-1}$ , with a range from 0 to  $0.011 \text{ mol mol}^{-1}$  (propagation of error discussed in Supplementary Information). This range of values is similar to the dFe/DIP value of  $0.008 \text{ mol mol}^{-1}$ , which can be derived from the ratio of the half-saturation constants for Fe and DIP uptake by *Trichodesmium* used to estimate the transition between Fe and P limitation in diazotrophs in the model of Krishnamurthy *et al.* (2007). Also, consistent with the model, we did not observe *isiB* expression above our threshold value indicative of Fe limitation at any station where the dFe/DIP ratio was  $>0.008 \text{ mol mol}^{-1}$ . Thus, our data can be interpreted as an empirical validation of the model parameters and predictions.

There was also an inverse linear relationship between Tten *isiB* expression and dFe ( $R^2$  value of 0.47) when Station E21, a station influenced by the Amazon River plume, was excluded from the analysis (Figure 4b). The influence of the Amazon River on Station E21 was evident in the low surface salinity (32.7 p.s.s.) and high dFe ( $2 \text{ nmol l}^{-1}$ ). The elevated *isiB* expression at this station was surprising given this high dFe value. It is likely that the dFe at this station was bound by organic ligands that include a significant terrestrial component, as has been reported in other river plumes with similar salinities (Batchelli *et al.*, 2010). The dFe complexes with such ligands may differ substantially in their bioavailability to *Trichodesmium*. Indeed, recent evidence suggests that fulvic acid isolated from the



Suwanee River forms Fe complexes that are not bioavailable to another cyanobacterium, *Synechococcus* (Hassler and Twiss, 2006). Future work needs to examine *isiB* expression over a range of dFe concentrations in surface waters influenced by rivers. Using the equation derived from the linear regression of *isiB* expression and dFe, the critical dFe value associated with Fe limitation in the open ocean is  $0.33 \pm 0.22 \text{ nmol l}^{-1}$ . This concentration is equivalent to the [Fe'] associated with *Trichodesmium* Fe limitation of N<sub>2</sub> fixation and high *isiB* expression in prior EDTA-buffered culture experiments (Chappell and Webb, 2010), which is significant because Fe complexed to the synthetic ligand EDTA is not available to phytoplankton (Anderson *et al.*, 1978). Therefore, in EDTA-buffered cultures the concentration of the small inorganic Fe fraction (predominantly Fe-hydroxy complexes) is the bioavailable form. Characterization of organic complexation in the samples collected on cruise KM0701, indicated that the [Fe'] was  $\leq 1 \text{ pmol l}^{-1}$  (Chappell, 2009), several orders of magnitude below the [Fe'] threshold associated with Fe limitation and *isiB* expression in the laboratory (Chappell and Webb, 2010). The similarity between the thresholds (dFe in the field and Fe' in EDTA-buffered cultures) and high *isiB* gene expression suggest that most of the naturally occurring Fe complexes in sea water are bioavailable to *Trichodesmium* colonies, even though Fe complexes with EDTA are not, a conclusion that is supported by the consistency of our data with past models (Krishnamurthy *et al.*, 2007), noted above.

Many bacteria use siderophores (Fe-chelating organic molecules) to assist in Fe acquisition (Andrews *et al.*, 2003). No obvious siderophore biosynthetic genes have been identified in the *Synechococcus*, *Prochlorococcus* and *Trichodesmium* genomes (Rivers *et al.*, 2009; Chappell and Webb, 2010). *Crocospaera watsonii* WH8501 does have a large number of polyketide synthases and nonribosomal peptide synthetase genes indicative of secondary metabolite production (Ehrenreich *et al.*, 2005), but none of these genes have been implicated in siderophore production. These data imply that open ocean cyanobacteria might use fundamentally different mechanism(s) to acquire organically complexed Fe. There is some genomic evidence for *Trichodesmium* acquiring siderophore-bound Fe using a TonB-ExbBD protein complex, although the outer membrane Fe/siderophore receptors required for high-affinity transport have not been identified (Chappell and Webb, 2010). There are also field data showing that siderophore-bound Fe can be available to *Trichodesmium* colonies (Achilles *et al.*, 2003). Other potential mechanisms for Fe acquisition include the photochemical release of Fe from ligands (Barbeau *et al.*, 2003), cell-surface reduction of ligand-bound Fe (Maldonado and Price, 2001), and interactions between *Trichodesmium* and the microbial consortium associated with its colonies.

Fitting with our data, others have also recently shown that ferrihydrite Fe contained in dust is rapidly dissolved and rendered bioavailable by *Trichodesmium* colonies (Rubin *et al.*, 2011); however, the mechanism used is currently undefined. Regardless of the uncertainty associated with the cellular mechanisms that *Trichodesmium* employs for Fe acquisition (either dissolved or mineral), our data suggest that *Trichodesmium* is able to access most if not all of the Fe in the total, dissolved pool of Fe in the upper oligotrophic ocean including Fe that is bound to organic ligands.

Select measurements of N<sub>2</sub> fixation in *Trichodesmium* colonies obtained from different Fe regimes verified that high *isiB* expression correlated with low N<sub>2</sub>-fixation rates (Figure 5). These data corroborate our laboratory study (Chappell and Webb, 2010) and show that *isiB* expression is a valid marker for Fe limitation of *in situ* *Trichodesmium* N<sub>2</sub> fixation. Frequently, the scarcity of *Trichodesmium* biomass in low Fe regimes precludes the implementation of rate measurements that would test for Fe limitation of N<sub>2</sub> fixation, whereas our *isiB* expression method, which requires less material, can still probe the *in situ* Fe status of *Trichodesmium* in these low-biomass regimes. We are unable to directly comment on the validity of using *isiB* as an *in situ* marker for growth limitation of *Trichodesmium* as we did not measure C fixation on the cruises and culture data show that increases in *isiB* expression and decreases in N<sub>2</sub> fixation can occur before *Trichodesmium* growth rates decrease significantly (Chappell and Webb, 2010). However, the *isiB* expression threshold level chosen for this field study was only observed in growth-limited laboratory cultures (Chappell and Webb, 2010), which suggests that *Trichodesmium in situ* growth was also limited by Fe in the Pacific Ocean stations (Figure 4).

Because of the direct role of *Trichodesmium* in CO<sub>2</sub> and N<sub>2</sub> fixation and its indirect role in providing 'new' N to other phytoplankton, *Trichodesmium* is a biogeochemically essential organism (Capone *et al.*, 1997); thus, defining where and when *Trichodesmium* activity is reduced or enhanced is important for current estimates and future forecasts of both the global C and N cycles. By comparing our *Trichodesmium*-specific *isiB* expression data with chemical measurements, this study provides empirically tested thresholds for predicting where Fe is the nutrient limiting *Trichodesmium* N<sub>2</sub> fixation when only the dFe/DIP ratio or dFe are available. Furthermore, as our data imply that all of the dissolved Fe forms found in the open ocean are available to *Trichodesmium* colonies, extrapolation of thresholds based on total dFe to other oceanic regimes is much simplified. Such extrapolation can explain why *Trichodesmium* spp. are so abundant in the Western North Atlantic (Capone *et al.*, 2005) and the Australian Shelf-Coral Sea (Capone *et al.*, 1997), while being almost completely absent in the South

Atlantic (Moore *et al.*, 2009) and South Pacific Gyre (Bonnet *et al.*, 2008).

## Acknowledgements

This work was supported by National Science Foundation Grants OCE-0623499 and OCE-0220945. Funding for PDC was supplied by the Woods Hole Oceanographic Institution Academic Programs Office, the Center for Environmental Bioinorganic Chemistry at Princeton University, and a National Defense Science and Engineering Graduate Fellowship. We thank the captain and crew of the R/V Oceanus cruise OCE399-4 and chief scientists J Zehr and J Montoya, the captain and crew of the R/V Seward Johnson cruise SJ0609 and chief scientists Z Johnson and E Zinser and the captain and crew of the R/V Kilo Moana cruise KM0701. We thank T Goepfert, R Jakuba, W Krey, D Ohnemus, A Rose, B Wilson and F Valois for assistance with sample collection and technical support in the laboratory. We also thank E Boyle, D Capone, S Doney, J Sohm and J Waterbury for reviewing an earlier version of this manuscript.

## Author contributions

EAW, JWM and PDC conceived of the study, and PDC, EAW, JWM and AMH performed the research and wrote the manuscript.

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