The Relationship Between Iron and Nitrogen Fixation in *Trichodesmium* spp.

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ABSTRACT:

Trichodesmium spp. are considered the dominant nitrogen (N) fixing cyanobacteria in tropical and subtropical oceans, regimes frequently characterized by low iron (Fe). Limited information exists about what levels of Fe limit *Trichodesmium* N fixation. I developed a diagnostic for Fe limitation using quantitative reverse transcription PCR (qRT-PCR) of the Fe stress response gene *isiB*, which encodes for flavodoxin a non-Fe containing substitute for ferredoxin. I determined that high *isiB* gene expression corresponded to cell-specific reductions in N fixation rates in both phylogenetic clades of *Trichodesmium* grown on varying levels of Fe. Using these laboratory-determined thresholds, I assessed Fe limitation of *Trichodesmium* from the Sargasso Sea, equatorial Atlantic Ocean and Western Pacific Warm Pool in conjunction with other analytical measurements (N, phosphorus (P) and dissolved Fe (<0.4μm filtered)). I found widespread Fe limitation in *Trichodesmium* from the Pacific Ocean and minimal expression in the North Atlantic Ocean. I also found an inverse correlation between *isiB* expression and dissolved Fe:P ratios in seawater and data suggesting that most dissolved Fe in seawater, including organic ligand-bound Fe, is available to *Trichodesmium*. These data support and refine previous model predictions and demonstrate, *in situ*, the importance of Fe to the marine N cycle.

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Dedication

This thesis is dedicated to the memory of Benedicta Y Duterte and Celeste Fowler, two amazing women that taught me to strive for my dreams and never give up. My world was brighter for having you in it.

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CHAPTER 1. INTRODUCTION

1.1 INTRODUCTION

Primary producers are important to the oceanic food chain and the overall biogeochemistry of the ocean (Falkowski et al., 1998). Diazotrophic cyanobacteria impact the carbon (C) cycle directly through primary production and indirectly through their contribution of "new" nitrogen (N) to the euphotic zone via N_2 fixation (Capone et al., 2005). So far no eukaryotic phytoplankton have been found that are capable of fixing N_2 without a symbiont. Thus, the new N that diazotrophs provide to the surface ocean is vital to the N and C cycles both regionally (Capone et al., 1997; Zehr et al., 2001) and globally (Gruber and Sarmiento, 1997). Of the diazotrophs, *Trichodesmium* is of particular importance to the global N cycle where some estimates associate its biological N_2 fixation with up to 50 % of global N sequestration (Karl et al., 1997). Although we now know that there is a much greater diversity of diazotrophs actively fixing N in the oceans than previously believed (Zehr et al., 2001; Montoya et al., 2004; Grabowski et al., 2008), *Trichodesmium* is still considered to be one of the most important diazotrophs globally (LaRoche and Breitbarth, 2005).

An important step in determining the impact of *Trichodesmium* on the N and C cycles and how this might change in the future is elucidating the physical and chemical factors that control its distribution and activity *in situ*. *Trichodesmium* is typically found in oligotrophic tropical and subtropical environments in clear stable water columns with deep light penetration and a mixed layer depth of around 100 m (Capone et al., 1997). Recent work with *Trichodesmium erythraeum* cultures has determined that the temperature range for growth and N_2 fixation in the species is 22 – 34 °C, with an optimal temperature of about 28 °C (Breitbarth et al., 2007). Above this optimal temperature, there is a precipitous drop in N_2 fixation rates and a more gradual decline in growth rates (Breitbarth et al., 2007). In addition to these physical constraints, both iron (Fe) (Berman-Frank et al., 2001; Webb et al., 2001; Fu and Bell, 2003b; Kustka et al., 2003a; Kustka et al., 2003b; Berman-Frank et al., 2007; Shi et al., 2007; Kupper et al., 2008), phosphorus (P) (Hynes, In Press; Sanudo-Wilhelmy et al., 2001; Dyhrman et al., 2002;

Fu and Bell, 2003a; Fu et al., 2005; Moutin et al., 2005; Sohm and Capone, 2006; Webb et al., 2007; Sohm et al., 2008) or a combination of the two (Mills et al., 2004) have been implicated in controlling *Trichodesmium* abundance and productivity throughout the world's oceans.

There are currently six described species of *Trichodesmium*: *T. erythraeum*, *Trichodesmium thiebautii*, *Trichodesmium tenue, Trichodesmium hildebrandtii*, *Trichodesmium contortum* and *Trichodesmium spiralis* (formerly known as *Katagnymene spiralis*) (Orcutt et al., 2002; Lundgren et al., 2005). Early distinctions between the species were made using morphological differences, but the potential for overlapping morphological characteristics between species has led to the development of genetic tools that can be used to complement morphological distinctions (Orcutt et al., 2002; Lundgren et al., 2005). These genetic techniques have shown that there are two distinct clades of *Trichodesmium* (Orcutt et al., 2002), which split into the *T. erythraeum* strains in one group and *T. tenue*, *T. thiebautii*, *T. spiralis* and *T. hildebrandtii* in the other group. Recent work has shown that *T. contortum* is part of the *T. erythraeum* group (Annette Hynes personal communication). Though these two distinct clades of *Trichodesmium* are known to exist, not much is known about the potential for niche differentiation between the species and what impact this might have on estimates of N_2 fixation in the ocean. For example, all of the studies looking at Fe stress in *Trichodesmium* culture experiments have focused on one species, *T. erythraeum* (Berman-Frank et al., 2001; Webb et al., 2001; Fu and Bell, 2003b; Kustka et al., 2003b; Berman-Frank et al., 2007; Shi et al., 2007). Prior to the work presented in this thesis, there were no physiological data from laboratory cultures of the other clade at all, including the impact of Fe limitation on N_2 fixation.

It appears that the two main morphologies of *Trichodesmium*, puffs and tufts, may exist at different depths in the ocean (Post et al., 2002; Davis and McGillicuddy, 2006). A study in the Red Sea determined not only that puff forming colonies were more abundant deeper in the water column than tuft colonies, but that these deep water puff colonies contained more chlorophyll a and had higher carbon fixation rates per colony at

ambient light than the tuft colonies from higher in the water column (Post et al., 2002). Another study found consistent differences in nitrogen fixation rates, photosynthetic compounds and distribution of two morphologies of *Trichodesmium* in the ocean (Carpenter et al., 1993). It is important to note that these distinctions were made based on morphology, not genetic differences, which makes attributing them to one particular subset of *Trichodesmium* complicated. They do, however, support the theory that there are differences between the members of the genus, which should be explored if we are going to adequately model how N_2 fixation rates might be affected by changing dust deposition, temperature and carbon dioxide levels.

N_2-fixing cyanobacteria are believed to have evolved in an anoxic ocean where Fe was readily available, and they have Fe requirements 5 to 8 times higher than other phytoplankton when they are growing without a fixed nitrogen source (Berman-Frank et al., 2001; Kustka et al., 2003b). In the modern oxygenated ocean, dissolved Fe is not as prevalent because of the low solubility of its thermodynamically stable form, Fe^{3+} (Liu and Millero, 2002). There are many forms of Fe found in the oceans (*i.e.,* colloidal, ligand bound, dissolved, free ion, etc), and it is uncertain which forms are bioavailable to *Trichodesmium.* In most regions of the ocean, including the oligotrophic ocean gyres, dissolved (< 0.4 μm filtered) Fe has been found to be almost completely (>99%) complexed by organic molecules (Gledhill and van den Berg, 1994; Rue and Bruland, 1995; van den Berg, 1995; Wu and Luther, 1995). The role that these organic ligands play in controlling the distribution of the dissolved pool of Fe complicates our understanding of what is biologically available to organisms. It remains difficult to identify which organic molecules are acting as ligands in ocean water, and there is evidence that many marine phytoplankton, including *Trichodesmium*, are able to obtain Fe from some but not all of these organic complexes (Hutchins et al., 1999; Achilles et al., 2003).

While there have been many studies of organic complexation of Fe in the Atlantic Ocean (Witter and Luther, 1998; Boye et al., 2003; Powell and Wilson-Finelli, 2003; Cullen et al., 2006; Rijkenberg et al., 2008) and the Southern Ocean (Boye et al., 2001;

Croot et al., 2004; Boye et al., 2005; Gerringa et al., 2008), other areas of the ocean that are important habitats for *Trichodesmium* have been sparsely investigated. In the Pacific Ocean, for example, most Fe speciation studies have focused on the North Pacific (Rue and Bruland, 1995; van den Berg, 1995; van den Berg, 2006; Buck and Bruland, 2007; Kondo et al., 2007; Kondo et al., 2008) and Eastern Equatorial Pacific (Rue and Bruland, 1997), leaving vast portions of the ocean unstudied.

Prior to the work in this thesis, no studies had looked at Fe speciation in the South Western Pacific or the Western Pacific Warm Pool, a region of the Pacific Ocean that is defined by temperatures higher than 29°C, salinity below 35 and very low macro nutrient concentrations (Blanchot et al., 1997). In fact, few studies with any Fe measurements have been done in this part of the Pacific Ocean (Campbell et al., 2005; Obata et al., 2008). This region is of particular interest to studies of Fe chemistry because it has very low predicted dust deposition (Duce and Tindale, 1991; Jickells, 1999; Wagener et al., 2008). It is also of interest biologically because based on flow-cytometric and pigment analyses, cyanobacteria appear to be the dominant phytoplankton in this highly oligotrophic region (Blanchot et al., 1997; Neveux et al., 2006; Matsumoto and Ando, 2009) and blooms of *Trichodesmium* can be quite prevalent in the region close to New Caledonia (Dupouy et al., 1988).

In addition to there being regions of the oceans where there is a paucity of Fe measurements and incomplete information regarding Fe bioavailability, a further complication associated with connecting Fe levels in the ocean with Fe status of *Trichodesmium* is that they are capable of luxury uptake and storage of Fe during periods of high Fe abundance (Kustka et al., 2003b). This ability to store Fe is an important adaptation in areas of episodic Fe deposition, which can lead to confusing results when trying to assess Fe limitation in the field, as cells can be Fe replete when the Fe levels in the waters around them would suggest limitation. Attempts to assess the relationship between Fe and the global N cycle using analytical geochemical measurements, Fe quotas of different organisms and dust deposition models have made great strides in understanding the interconnectedness of these global cycles (Moore et al., 2004; Moore

and Doney, 2007). These studies have underscored the need for biological markers to assess *in situ* Fe stress. An *in situ* biological marker for Fe could be used to directly explore the linkages between Fe geochemistry, Fe status and N_2-fixation in natural populations of *Trichodesmium*, avoiding the difficulties associated with determining the best measurement of bioavailable Fe and complications relating to Fe storage capabilities.

The goals of this thesis were to address questions raised in the preceding paragraphs regarding distribution and speciation of Fe in the southwestern Pacific Ocean as well as to assess Fe limitation of *Trichodesmium* in different ocean regimes using a quantitative molecular method. Chapter 2 deals with measurements of dissolved Fe and Fe speciation in a region of the ocean where there have been few measurements made, the southwestern Pacific Ocean. Chapter 3 explores clade differentiation in *Trichodesmium* spp., specifically focusing on temperature optima and the response to Fe limitation. Chapter 3 also includes the development of clade-specific molecular markers for Fe limitation, which are calibrated to reductions in N_2 fixation rates in response to Fe limitation. Chapter 4 looks at Fe stress in field populations of *Trichodesmium* from both the Pacific and Atlantic Oceans using the molecular marker for Fe limitation developed in Chapter 3. The results from Chapter 4 validate model predictions of where Fe limitation of *Trichodesmium* is occurring with calibrated measurements of Fe limitation of N_2 fixation. The region where Fe limitation of *Trichodesmium* is most apparent is the Pacific Ocean. The dissolved Fe values from the field associated with Fe limitation are similar to those associated with Fe limitation in the lab, suggesting that much of the organically bound Fe in the open ocean is available to *Trichodesmium*. In addition, the correlation of expression of the Fe stress response gene, *isiB*, and the measured dissolved Fe/PO_4 ratio of seawater samples enables calculation of the critical Fe/PO_4 value associated with Fe limitation of *Trichodesmium*. The similarity between this calculated critical Fe/PO_4 value with the one used in some models to predict Fe limitation of *Trichodesmium*, serves as further validation of those models with empirical data. Overall, this thesis provides insight into how Fe controls N_2 fixation in *Trichodesmium*,

where in the ocean this control is important and what parameters are important to measure to determine the likelihood that *Trichodesmium* is experiencing Fe limitation.

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CHAPTER 2: DISTRIBUTION OF DISSOLVED FE AND ORGANIC FE BINDING LIGANDS IN THE WESTERN PACIFIC OCEAN

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2.1 Abstract

Dissolved iron (Fe) and the concentration of organic Fe-binding ligands were measured in the Western Pacific Ocean on a transect from Hawaii to Australia. Fe complexation was measured using competitive ligand exchange adsorptive cathodic stripping voltammetry (CLE-ACSV) with the competitive ligand 2,3-dihydroxynaphthalene (DHN). Dissolved Fe in surface samples ranged from 0.09 – 1.4 nM. Ligand concentrations ranged from 0.44 – 2.2 nM with binding constants ranging from $10^{11.6} - 10^{12.9}$. There appeared to be a linear relationship between Fe and ligand concentrations when [Fe] > 0.2 nM and a wide range of ligand concentrations when [Fe] < 0.2 nM. Our data supports the hypothesis that organic ligands are a ubiquitous part of Fe speciation in the oceans, even in regions of low dust deposition where Fe inputs are small.

2.2 Introduction

Iron (Fe) is an essential micronutrient for almost all organisms. Because of solubility constraints in its oxidized state (Liu and Millero, 2002), Fe can be low enough in surface waters to limit phytoplankton growth in the ocean (Martin et al., 1991; Morel et al., 1991; Falkowski et al., 1998; Boyd et al., 2007) even though it is the fourth most abundant element in the Earth's crust. The discovery of the role that Fe plays in controlling productivity in High Nutrient Low Chlorophyll (HNLC) regimes (Martin and Fitzwater, 1988; Martin et al., 1990) prompted significant research to determine what controls the bioavailability of Fe in the Ocean.

A major development in understanding Fe chemistry in the ocean was the discovery, using competitive ligand exchange adsorptive cathodic stripping voltammetry (CLE-ACSV), that organic ligands are ubiquitous and bind >99% of the dissolved Fe in the oceans (Gledhill and van den Berg, 1994; Rue and Bruland, 1995; Wu and Luther, 1995). Since those first studies determined that organic complexation is an important component of Fe speciation in the ocean, evidence of Fe-binding ligands has been found in every area of the ocean that has been studied (Hunter and Boyd, 2007), including the recent discovery that they are associated with hydrothermal vent plumes (Bennett et al., 2008).

While it remains unclear what the structures of these ligands are, there is evidence that the stability constants that have been measured are in line with the stability constants of known siderophores when analyzed in a seawater medium (Witter et al., 2000) as well as with unknown compounds with siderophore-like functional groups that were extracted from seawater (Macrellis et al., 2001). A recent study found evidence of two characterized sideophores in samples throughout the Atlantic Ocean (Mawji et al., 2008). It is known that marine bacteria and cyanobacteria are capable of making siderophore-like complexes (Wilhelm and Trick, 1994; Lewis et al., 1995; Soria-Dengg et al., 2001; Ito and Butler, 2005). It also appears that Fe bound to siderophores is available to at least some subset of phytoplankton (Hutchins et al., 1999; Maldonado and Price, 2001; Soria-Dengg et al., 2001; Achilles et al., 2003), although the type of chelator appears to be important in determining the bioavailability to different phytoplankton (Hutchins et al., 1999). Considering that this organically bound fraction may be bioavailable to some phytoplankton groups in the surface ocean, it is important to determine if there are areas where the amount of ligand is different in the ocean and potentially determine what controls this distribution.

There have been many studies looking at dissolved Fe and Fe speciation in the Atlantic Ocean (Witter and Luther, 1998; Powell and Donat, 2001; Boye et al., 2003; Cullen et al., 2006; Rijkenberg et al., 2008) and Southern Ocean (Boye et al., 2001; Croot et al., 2004; Boye et al., 2005; Gerringa et al., 2008). There have been fewer studies looking at Fe speciation in the Pacific Ocean and those have focused on the Northern Pacific Ocean (Rue and Bruland, 1995; van den Berg, 1995; van den Berg, 2006; Buck and Bruland, 2007; Kondo et al., 2007; Kondo et al., 2008) and the Eastern Equatorial Pacific Ocean (Rue and Bruland, 1997). Only two studies so far have looked at Fe in the open ocean of the South Western Pacific (Campbell et al., 2005; Obata et al., 2008). One was focused on the most western part of the basin and only looked at the total dissolvable fraction of Fe, which is measured on unfiltered seawater that has been acidified to pH 3.2 immediately following collection (Obata et al., 2008). The other study focused on total dissolved Fe (<0.22 μm filtered Fe) and looked at the region around New Caledonia and between New Caledonia and New Zealand (Campbell et al., 2005).

The data set presented in this work, which includes dissolved Fe and ligand concentrations, provides data on Fe geochemistry in the understudied region of the South Western Pacific Ocean, a region of very low dust inputs (Duce and Tindale, 1991; Jickells, 1999; Wagener et al., 2008). The transect includes the first measurements of Fe and Fe speciation through the hydrologic feature known as the Western Pacific Warm Pool (WPWP). The WPWP is a region defined by very warm surface temperatures (> 29°C), low surface salinity (<35) and low macronutrients (Blanchot et al., 1997). It is an oligotrophic region where the predominant phytoplankton are cyanobacteria (Blanchot et al., 1997; Neveux et al., 2006; Matsumoto and Ando, 2009). The cruise track also included a region of the ocean where there are reports of periodic blooms of the cyanobacterium *Trichodesmium* (Dupouy et al., 1988; Campbell et al., 2005; Rodier and Le Borgne, 2008). As cyanobacteria appear to be able to access Fe bound to some organic ligands (Hutchins et al., 1999; Achilles et al., 2003), it is important to determine the role of organic ligands in the Fe chemistry of the surface oceans in the region to understand Fe bioavailability to the predominant phytoplankton. In comparing the amount of organic ligand ([L]) present in each sample with various auxiliary chemical and biological parameters, the most striking relationship appeared to be between the amounts of dissolved Fe ([Fe]) verses [L] in a given sample, which is consistent with data from other regions of the ocean. The data we present is an addition to global datasets of dissolved Fe and Fe speciation in surface waters, covering an area where few values have been reported.

2.3 Methods

2.3.1 Sample Collection. Samples were collected aboard the R/V Kilo Moana as part of the Western Pacific Warm Pool (WPWP) cruise between January 12, 2007 and February 9, 2007 (Figure 1). Samples were collected using acid cleaned 5L Teflon-coated exterior spring niskin bottles (Ocean Test Equipment) mounted on a powder-coated rosette deployed on a Kevlar line. After recovery, the bottles were transferred into a trace metal clean "bubble" in the laboratory of the ship with positive pressure maintained by HEPA filtered air units. The headspace of each bottle was pressurized with 0.2 μm filtered ultra high purity (UHP) nitrogen pushing the water through a 142

mm 0.4 μm acid-cleaned polycarbonate filter held in a polycarbonate filter sandwich (Geotech Environmental Equipment, Inc.). Water for dissolved Fe analysis was collected in acid-cleaned 250 ml low density polyethylene (LDPE) bottles and acidified to pH 1.7 with concentrated HCl (Seastar). Water for speciation analysis was collected in 1 L acid-cleaned Teflon bottles and stored at 4°C until analysis.

2.3.2 Dissolved Fe Analysis. Fe in the seawater samples was determined using isotope dilution and magnesium hydroxide preconcentration followed by analysis using inductively coupled mass spectrometry (Wu and Boyle, 1998; Saito and Schneider, 2006). Roughly 13.5 ml of sample (exact volume determined gravimetrically) was poured into a 15 ml polypropylene centrifuge tube (Globe Scientific Inc.) and equilibrated with a ^{57}Fe spike (~0.4 nM) overnight. The following day, the $Mg(OH)_2$ and metals were precipitated out of the sample by the addition of a small amount (~100 μl) of high-purity ammonium hydroxide (Seastar Chemicals Inc.). Following ammonium hydroxide addition, the tubes were left undisturbed for 90 s and then they were inverted multiple times to fully mix them. After an additional 90 s, the tubes were centrifuged at 3000 x g for 3 minutes and the sample was decanted off. The tubes were then spun at 3000 x g for an additional 3 minutes forming a compact pellet, following which the remaining liquid was shaken off. The sample pellets were kept dry until the day of analysis (from a day to a week). On the morning of analysis, pellets were resuspended and dissolved in 1-2 ml 0.8 N Nitric Acid (Seastar). Samples were analyzed on a Thermo-Finnigan Element 2 (E2) inductively coupled mass spectrometer (ICP-MS) in medium resolution mode. A procedural blank was determined by processing 1 ml of low Fe seawater (which provides a negligible amount of Fe) and calculating its Fe value as though it were a 13.5 ml sample.

2.3.3 Organic Ligand Analysis. The titration of organic Fe complexing ligands was carried out in a manner closely following that described by van den Berg (van den Berg, 2006). Briefly, 20 ml of sample was added to a series of 30 ml preconditioned Teflon vials (Savillex Corporation). 10 μl of 1mM 2,3-dihydroxynaphthalene (DHN) (final concentration 0.5 μM) and Fe in a series of concentrations of 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 5 and 8 nM was added to each vial from an acidified Fe stock solution of 0.5 μM Fe. Samples were allowed to equilibrate overnight (17-24 hrs). After equilibration,

10 ml of each sample in order of increasing Fe was transferred to a polytetrafluoroethylene (PTFE) voltametric cell, 0.5 ml EPPS (3-(4-(2-hydroxyethyl)-1-piperazinyl)propanesulfonic acid)/bromate solution (final concentration 5 mM EPPS/20 mM bromate) was added, purging was initiated and reactive Fe was determined by cathodic stripping voltammetry (CSV). This was repeated with the second 10 ml of sample, and calculations were based on the results from the second scan. The scan conditions included a 5 minute purge of the sample with 0.2 μm filtered ultra high purity nitrogen gas, 90 s adsorption at -0.1 V, 10 s equilibration and a scan using sampled direct current (DC), step size 4 mV, frequency 10 s^{-1}.

Cathodic stripping voltammetry (CSV) was carried out using a Metrohm VA 663 Stand with a Hanging Mercury Drop Electrode (HMDE), glassy carbon working electrode, a double junction Ag/AgCl and a 3 M KCl reference electrode (Metrohm AG, Switzerland), which was connected to a μAutolab II potentiostat (Eco Chemie BV, The Netherlands). The μAutolab was connected to a laptop PC and was controlled using GPES software (Eco Chemie BV, The Netherlands).

Peak currents were plotted against $[Fe]_{total}$ for each sample (natural Fe in sample and added Fe) to get a titration curve. Linear regression of the final 3-5 points of a titration was used to obtain the sensitivity of the titration (the slope of that line, *S*). This calculated sensitivity and the α coeffecient for DHN complexation with Fe' of 0.5 μM DHN (166) previously determined (van den Berg, 2006) were then used to calculate [Fe'] and [FeL] for each sample in the titration.

Briefly, [FeDHN] is related to the current measured at individual point in the titration (i_p) and the sensitivity of the titration (*S*) and can be calculated using the equation: $i_p = S \times [FeDHN]$. [Fe'] is related to [FeDHN] by the α coeffecient for DHN complexation with Fe' and can be calculated using the equation: $[Fe'] = [FeDHN]/\alpha$. [FeL] is related to $[Fe]_{total}$, [Fe'] and [FeDHN] and can be calculated using the equation: $[Fe]_{total} = [Fe'] + [FeL] + [FeDHN]$.

[Fe'], [FeL] and [L] are in equilibrium in natural waters: Fe' + L ↔ FeL. The conditional stability constant for this equilibrium reaction, $K'_{FeL,Fe'}$, is defined by the equation: $K'_{FeL,Fe'} = ([FeL]/[Fe']) \times [L]$. To obtain values for [L] and $K'_{FeL,Fe'}$, we used our calculated values for [Fe'] and [FeL] and analyzed them using a non-linear fitting

program to fit the equation: $[FeL] = ([L] \times [Fe'] \times Fe'_{FeL,Fe'})/(1 + K'_{FeL,Fe'} \times [Fe'])$. This method has been described by Wilkinson (1961) (Wilkinson, 1961) and Gerringa et. al (Gerringa et al., 1995). [FeL] was also plotted against [FeL]/[Fe'] to evaluate whether or not there was a two-ligand system at any station, which is indicated by two lines with different slopes when [FeL] is plotted against [FeL]/[Fe']. Station 16a was the only station where a two-ligand analysis seemed necessary based on plotting [FeL] verses [FeL]/[Fe']. Calculations at this station were done using the Scatchard linearization (Ruzic, 1982; van den Berg, 1982), where the linear regression of each of the lines from the [FeL] vs [FeL]/[Fe'] plot has a x-intercept equal to [L] and a y-intercept equal to $[L] \times K'_{FeL,Fe'}$.

Once [L] and $K'_{FeL,Fe'}$ are determined either using the Scatchard linearization or the non-linear Wilkinson-Gerringa method, ambient [Fe'] and [FeL] from the initial sample can be calculated using the following two equations: $[Fe]_{natural} = [Fe'] + [FeL]$ and $K'_{FeL,Fe'} = ([FeL]/[Fe']) \times [L]$.

2.4 RESULTS

A map of the stations from the Western Pacific Warm Pool (WPWP) cruise is show in Figure 1. In order to be able to compare data from different groups that publish data from this cruise, we have left the station numbers the same even though we do not have ligand data from all stations along the cruise.

The values for dissolved [Fe] (0.4 μm filtered Fe concentration), [L] (calculated ligand concentration), $Log_{10} K_{FeL,Fe'}$ (binding constant of the ligand with respect to the inorganically bound fraction of Fe) and [Fe'] (calculated inorganically bound Fe concentration) for the surface samples (15 m unless otherwise noted) are listed in Table 1. [Fe] values ranged from 0.09 – 1.4 nM at the surface. Despite scrupulous cleaning of Niskin bottles prior to the beginning of the cruise, there is a possibility that the total Fe samples from the first four stations are erroneously high as a result of residual Fe leaching from the insides of the bottles. We believe this high Fe was washed out after the first few stations of the cruise. Because we suspect contamination of these samples, they have been left out of later analysis of the relationship between [Fe] and [L]. Station 17, which also had a high [Fe] of 0.95 nM at 15 m, was very close to one of the Vanuatu

islands and we believe that the high [Fe] value recorded there was a result of Fe input from the islands and not contamination. [L] values ranged from 0.4 – 2.02 nM. Log_{10} $K_{FeL,Fe'}$ values ranged from 11.6 – 12.9. At station 16a, there was evidence of two ligand classes with [L1] = 0.86 nM with a Log_{10} $K_{FeL,Fe'}$ = 12.6 and [L2] = 2.02 nM with a Log_{10} $K_{FeL,Fe'}$ = 11.6.

Depth profiles of [Fe] down to 500 m shown in Figure 2 for Station 14 (2A), Station 15 (2B) and Station 20 (2C) correspond to the values listed in Table 2. At all three stations, [Fe] is lowest in the surface waters above 100 m and then begins to rise around the 300 m sample. Figure 3 shows [Fe], [L] and [Fe'] (inorganically bound Fe) for station 14 to a depth of 100m. While [L] varies with depth more than [Fe], it is clear that it always exceeds [Fe].

Figure 4 shows the relationship between [L] and [Fe] for samples where [Fe] > 0.2 nM and [Fe] < 0.2 nM. The dashed line shows [Fe] = [L]. The solid line is the linear regression of [L] vs. [Fe] for the samples where [Fe] > 0.2 nM. The equation for the line including error associated with each parameter is [Fe] = (0.54 +/- 0.09) × [L] + (0.01 +/- 0.08). The R^2 value for the relationship is 0.75.

2.5 Discussion

Given the importance of Fe to phytoplankton productivity, knowing the distribution of Fe in the surface oceans is imperative to understanding global primary production. While their source and composition remains unclear, organic ligands appear to play a key role in stabilizing Fe in the ocean as it is apparent that organic ligands complex >99% of dissolved Fe in most of the ocean (Gledhill and van den Berg, 1994; Rue and Bruland, 1995; Wu and Luther, 1995). Because of the uncertainty regarding bioavailability of the Fe bound to these organic ligands (Hutchins et al., 1999; Maldonado and Price, 2001; Soria-Dengg et al., 2001; Achilles et al., 2003), it is important that we increase our understanding of ligand concentrations in the ocean. With this work, we have added to the growing dataset of oceanic ligand concentrations and dissolved Fe values, providing values for a region of the ocean that has been understudied with respect to trace metal geochemistry, the Western Pacific Warm Pool (WPWP).

Excluding potentially contaminated Fe samples, our measured WPWP surface [Fe] (dissolved < 0.4 μm Fe concentration) values ranging from 0.09 – 0.95 nM (Table 1) are within range of what has been seen in other regions of the ocean (Johnson et al., 1997). With a few notable exceptions, our surface values were low, between 0.1 – 0.4 nM (Table 1), and comparable with other recent measurements of dissolved Fe in surface samples from the South Pacific Ocean (R.F. Zhang and E. Boyle, personal communication). The exceptions to these low surface values are the first few stations of the transect after we left Hawaii (Stations 3-6), a station where we were in the midst of a surface slick of the N_2-fixing cyanobacterium *Trichodesmium* (Station 16a) and two stations in the islands close to New Caledonia (Stations 17 and 19). The higher surface values at the start of the transect, 0.6 – 1.4 nM for stations 3 – 6, could be the result of deep winter mixing and a recent rain event in this region close to Hawaii. Alternatively, and potentially more likely given that these are higher than values we would expect as a result of deep mixing and recent time-series data suggesting that January is a time of particularly low Fe deposition to this area (Boyle et al., 2005), these values might be high as a result of residual Fe contamination leaching from the inside of the Niskin bottles at the beginning of the cruise despite rigorous acid-cleaning of the bottles on land in between cruises. The high values in the midst of the surface slick of *Trichodesmium* could indicate that the slick was the result of a bloom caused by an input of Fe to an Fe-starved region. Alternatively, the surface slick could have been the result of a convergence of water masses bringing areas of moderate *Trichodesmium* biomass together, resulting in some accumulation of biomass near the surface where high light intensity bleached and killed the *Trichodesmium*. If the latter were the case, high Fe values could result from releases of cellular Fe as *Trichodesmium* cells burst. Another potential source of Fe could be the result of *Trichodesmium* biomass from a bloom, which may or may not have been caused by high Fe, being broken down by grazers and releasing cellular Fe. Both hypotheses involving cellular Fe release would also explain the ligand results from that station, which will be discussed later. The high Fe values close to the islands are in line with what others have found for the region (Campbell et al., 2005) and are potentially the result of river run-off and other coastal sources of Fe. Station 17, which had the higher Fe value, 0.95 nM, was also closer to an island than

Station 19. Station 17 was less than 4 km away from land and had a bottom depth of only 350 m. It is not as simple to attribute the slightly elevated value of 0.5 nM at Station 19 to coastal influences as the bottom depth was ~1400 m and the closest island was roughly 50 km away, though this is not of great concern as others have seen high values of Fe in oceanic surface samples from this region (Campbell et al., 2005). It appears surprising that Fe values in the surface waters of the southwestern Pacific Ocean are not that much lower than those observed in regions of the Atlantic Ocean given that the dust deposition to the Pacific Ocean is predicted to be so low (Duce and Tindale, 1991; Jickells, 1999; Wagener et al., 2008). Recent work looking at the annual cycle of Fe in surface waters near Hawaii indicates that Fe values in the surface of the Pacific might be high as result of a higher solubility of the dust deposited in the Pacific Ocean (Boyle et al., 2005), though at least during the period of peak dust deposition, this did not appear to be the case (Wu et al., 2007). Further study looking at the solubility of Fe in the South Pacific Ocean and the role that organic ligands play in controlling that solubility may help clarify questions about why Fe remains detectable in the surface waters despite low dust deposition.

Our profiles of [Fe] (Figure 2, Table 2) are typical of what is expected in the open ocean (Johnson et al., 1997). We found surface values around 0.2 nM with a small near-surface maximum in Fe that is potentially the result of atmospheric deposition of Fe that has not been completely been drawn down by scavenging and biological uptake (Bruland et al., 1994; Wu et al., 2001; Boyle et al., 2005). Below this near-surface Fe maximum, we find otherwise low Fe values in the upper 100 m. When we look at the region of our profiles below 100 m, we see that [Fe] rises in a typical "nutrient-type" distribution that has been observed in profiles from other areas of the Ocean (Bruland et al., 1994; Johnson et al., 1997). While it is apparent that Fe rises below the euphotic zone, it is no longer believed that there is one particular deep ocean value for Fe as was once hypothesized (Johnson et al., 1997). Our limited deep ocean data set has values that vary between 0.4 nM and 0.7 nM (Table 2), while other data from the North Pacific shows deep ocean dissolved Fe values around 0.4 nM (Bruland et al., 1994; Wu et al., 2001; Boyle et al., 2005). Profiles from a number of different ocean regions have shown that there is not just one deep ocean value for Fe, but rather the value can vary from 0.4 nM to

1 nM (Bruland et al., 1994; Wu et al., 2001; Boyle et al., 2005; Johnson et al., 2007) and is more likely influenced by the source of Fe to a given water mass.

Our results for ligand concentrations ([L]) ranging from 0.44 nM – 2.2 nM and binding constants of ligands with respect to the inorganically complexed Fe ($K_{FeL,Fe'}$) ranging from $10^{11.6} - 10^{12.9}$ are in line with the oceanic values that have been presented elsewhere in the literature, with [L] reported from 0.33 – 2.5 nM and $K_{Fe',L}$ from $10^{10.6}$ - $10^{13.9}$ (Rue and Bruland, 1995; van den Berg, 1995; Rue and Bruland, 1997; Boye et al., 2001; Powell and Donat, 2001; Boye et al., 2003; Croot et al., 2004; Boye et al., 2005; Cullen et al., 2006; van den Berg, 2006; Buck and Bruland, 2007; Kondo et al., 2007; Kondo et al., 2008; Rijkenberg et al., 2008). There was only one station where it appeared that we observed at a two-ligand system, 16a, which was in the middle of a surface slick of the N_2-fixing cyanobacterium *Trichodesmium*. There is considerable debate over the causes and status of *Trichodesmium* when they appear as these surface slicks, some of which were described above. Regardless of the cause, an accumulation of a large amount of biomass is likely to lead to a significant amount of grazing by copepods and viral or bacterial degradation of organic matter. It could be that a large amount of cell lysis in the patch resulted in the release of lower $K_{Fe',L}$ "L2-type" ligands, which have been surmised to be porphyrins based on their lower conditional stability constants (Witter et al., 2000) and their predominance in deeper waters where the majority of organic matter degradation takes place (Hunter and Boyd, 2007). Further studies of ligand composition during and after phytoplankton blooms or in senescent cultures of phytoplankton in the laboratory could help determine if cell lysis is a significant potential source of L2 ligands.

Some studies have found a linear relationship between [Fe] and [L1] (Boye et al., 2003; Buck and Bruland, 2007; Buck et al., 2007). These relationships seem to work best on samples with higher Fe values and appear to break down completely at [Fe] values below 0.2 nM (Buck and Bruland, 2007). When we exclude data from samples where Fe was below 0.2 nM and the L2 ligand contribution to [L] from station 16a, we also get a linear relationship between [L] and [Fe] in our samples (Figure 4). The slope of the relationship for our data, 0.54, is lower than that determined by Buck and Bruland (2007), 0.69, but the difference may have something to do with both our small data set and that

the range of [Fe] for our samples is not as large. Another dataset from three marginal seas on the western boundary of the Pacific Ocean, exhibits a relationship closer to and sometimes above the [Fe]:[L] = 1:1 relationship (Kondo et al., 2007). Kondo et al (2007) hypothesize that one of the main reasons that different studies end up with varying relationships for [Fe]:[L] could be the result of many diverse methods for determining [L], including the use of a variety of competitive ligands and quantitative analyses. Certainly, a comparison of different methodology would help determine if the varying linear relationships between [L] and [Fe] are the result of experimental differences or if they might be site specific. If these differences are site specific it could be indicative of varying ligand sources and should be studied further.

Recent reports demonstrate that there seems to be a significant amount of L1-type ligands (i.e., ligands with high binding constants) associated with the colloidal fraction of Fe (Wu et al., 2001; Boye et al., 2003; Cullen et al., 2006), but siderophores are generally low molecular weight molecules and as such would be expected to be associated with the 0.02 μm fraction of Fe. Hunter and Boyd (2007) in their review of oceanic speciation data conclude that if L1 consists mainly of siderophores, those siderophores must become closely associated with colloidal Fe to explain their distribution. This conclusion could be supported by the close relationship between [L1] and [Fe] when [Fe] > 0.2 nM (Buck and Bruland, 2007) (Figure 4) when viewed in light of recent work showing that most of the variation in dissolved Fe concentrations in the upper ocean is associated with the colloidal fraction of Fe and that while the < 0.02 μm fraction of Fe shows some variability, it is almost never above 0.4 nM (Bergquist et al., 2007). If the dissolved [Fe] > 0.2 nM is predominantly colloidal, as that work suggests, then the linear relationship between [L] and [Fe] at higher Fe values indicates that L1 is associated with colloids. It could be that the association with colloidal Fe is what keeps L1 ligands, whether they are siderophores or not, at such high values throughout the oceans, despite the fact that they might not be produced everywhere.

While our results are within the range of [L] values that have been observed in the open ocean, with most of our values below 1 nM they appear low when compared with the [L] values in the Atlantic Ocean (Witter and Luther, 1998; Powell and Donat, 2001; Boye et al., 2003; Cullen et al., 2006; Rijkenberg et al., 2008) or Northeastern Pacific

(van den Berg, 1995; van den Berg, 2006; Buck and Bruland, 2007). The lower values for [L] that we observed in the Pacific gyre could be a result of the low dust deposition, which is predicted for this area (Duce and Tindale, 1991; Jickells, 1999; Wagener et al., 2008). In fact, they are comparable to the values in other low dust deposition regions like the Southern Ocean (Boye et al., 2001; Boye et al., 2005; Gerringa et al., 2008) and other regions of the Pacific Ocean (Rue and Bruland, 1995; Rue and Bruland, 1997; Kondo et al., 2007; Kondo et al., 2008). The hypothesis that ligands are produced in response to Fe inputs is based on the number of studies that have shown an increase in ligands following mesoscale addition of Fe to surface waters (Rue and Bruland, 1997; Boye et al., 2005; Kondo et al., 2008). If ligands are produced in response to Fe inputs, then one of the lower dust input regions of the ocean would be expected to have low ligand production values. In addition to potentially low production values, another facet to consider is the destruction of ligands by UV light. While the photodestruction of many siderophores is generally accepted (Barbeau et al., 2003), there is some debate about the photoreactivity of all oceanic ligands (Barbeau, 2006). Two studies on ligands from natural waters show conflicting results, with one group seeing a reduction in ligands in response to UV light (Powell and Wilson-Finelli, 2003) and another seeing no reduction (Rijkenberg et al., 2006). A study looking at the speciation of Fe in samples of surface waters (< 2 m) saw a reduction in ligands in those waters that receive the most UV irradiation (Powell and Donat, 2001). A recent review of ligand photochemistry suggests that the reduction in siderophore-like ligands in the Rijkenberg study could have been masked by a large amount of colloidal Fe (Barbeau, 2006). Considering the dominance of colloidal Fe in the ocean and its association with L, it could be this protection from UV degradation that keeps L that is associated with colloidal Fe at high levels in the surface ocean. This also might explain why the fraction of L that is associated with lower Fe waters (<0.2 nM) is not related to Fe levels, because ligands that are not associated with colloidal Fe may be more susceptible to UV degradation. Future studies, looking at the effects of UV on the ligand composition of ultrafiltered (< 0.02 μm) seawater samples could help answer these questions. Alternatively, it could be that the siderophores that persist in areas of high UV penetration are those that are not susceptible to UV degredation, such as the ferrioxamine

siderophores (Barbeau et al., 2003), which appear to be dominant in the Atlantic Ocean (Mawji et al., 2008).

While questions remain regarding the source and composition of ligands in the open ocean, it is clear that organic ligands are ubiquitous and play a key role in stabilizing dissolved Fe in the ocean. In this study, we have shown that ligands are prevalent even in the low Fe waters of the Western Pacific ocean and have found that the relationship between [L] and [Fe] is similar to that of other regions of the open ocean. The data presented here is an addition to the growing datasets of dissolved Fe and Fe speciation in the ocean, covering a region where few measurements have been made.

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Table 1. Dissolved Fe, organic Fe-binding ligand concentrations, conditional stability constants and calculated free inorganic Fe for <0.4 μm filtered samples

Station	Depth (m)	Latitude	Longitude	[Fe] (nM)	Ligand Class	[L] (nM)	$\log_{10} K$	[Fe'] (pM)
3	15	19.53	-159.90	0.70 +/- 0.05 *				
4	15	17.38	-162.44	0.95 +/- 0.04 *	L1	1.06 +/- 0.02	12.5	2.64
5	15	14.96	-165.05	1.40 +/- 0.05 *	L1	1.40 +/- 0.06	12.9	12.09
6	15	12.43	-167.73	0.63 +/- 0.03*				
7	15	7.88	-172.34	0.16 +/- 0.02				
8	15	5.65	-174.53	0.41 +/- 0.03				
9	15	3.24	-176.88	0.21 +/- 0.02	L1	0.53 +/- 0.03	12.3	0.33
10	15	0.37	-179.64	0.11 +/- 0.04	L1	0.38 +/- 0.09	12.0	0.26
11	15	-2.30	177.44	0.11 +/- 0.04	L1	0.79 +/- 0.06	11.8	0.26
12	15	-4.72	174.73	0.28 +/- 0.11	L1	0.52 +/- 0.06	12.0	1.00
13	15	-7.07	172.31	0.31 +/- 0.11	L1	0.44 +/- 0.08	11.9	2.80
14	15	-9.25	170.00	0.20 +/- 0.04	L1	1.57 +/- 0.10	11.8	0.22
14	25			0.24 +/- 0.02	L1	0.59 +/- 0.06	12.1	0.56
14	50			0.18 +/- 0.01	L1	0.88 +/- 0.14	11.9	0.24
14	100			0.16 +/- 0.01	L1	0.57 +/- 0.10	11.6	1.04
15	15	-12.58	169.86	0.11 +/- 0.03	L1	1.07 +/- 0.10	11.7	0.20
16	15	-15.89	169.72	0.29 +/- 0.03				
16a	7	-15.98	169.77	0.63 +/- 0.02	L1	0.85 +/- 0.00	12.6	0.41
					L2	2.02 +/- 0.59	11.6	
17	15	-19.22	169.57	0.95 +/- 0.02	L1	1.52 +/- 0.06	12.5	0.55
19	15	-21.62	168.66	0.50 +/- 0.08	L1	1.06 +/- 0.09	12.5	0.28
20	15	-25.67	165.42	0.09 +/- 0.02	L1	0.38 +/- 0.04	11.7	0.69
21	15	-29.04	164.34	0.24 +/- 0.02	L1	0.62 +/- 0.03	12.8	0.10
22	15	-31.92	163.36	0.21 +/- 0.08				
23	15	-34.16	162.55	0.29 +/- 0.02				
24	15	-36.17	161.79	0.19 +/- 0.02				
25	15	-34.23	160.35	0.21 +/- 0.03	L1	0.93 +/- 0.04	12.3	0.31
26	15	-32.42	159.09	0.20 +/- 0.02	L1	0.86 +/- 0.03	12.6	0.07
28	15	-30.26	157.30	0.51 +/- 0.12	L1	0.81 +/- 0.04	12.2	0.96
29	15	-29.76	156.62	0.41 +/- 0.00				
30	15	-28.76	155.37	0.32 +/- 0.11	L1	0.49 +/- 0.03	12.8	0.33

*possible Fe contamination

Table 2. Dissolved (<0.4 μm filtered) Fe with depth

Station	Depth (m)	[Fe] (nM)	Standard Deviation
14	15	0.20	0.04
14	25	0.24	0.02
14	50	0.18	0.01
14	100	0.16	0.01
14	150	0.07	0.00
14	300	0.11	0.03
14	500	0.71	0.00
15	15	0.09	0.00
15	25	0.26	0.05
15	50	0.09	0.00
15	75	0.11	0.00
15	100	0.06	0.01
15	150	0.07	0.01
15	300	0.35	0.04
15	500	0.52	0.01
20	15	0.09	0.02
20	25	0.37	0.00
20	50	0.04	0.00
20	75	0.07	0.01
20	100	0.05	0.00
20	150	0.08	0.01
20	300	0.19	0.01
20	500	0.40	0.03

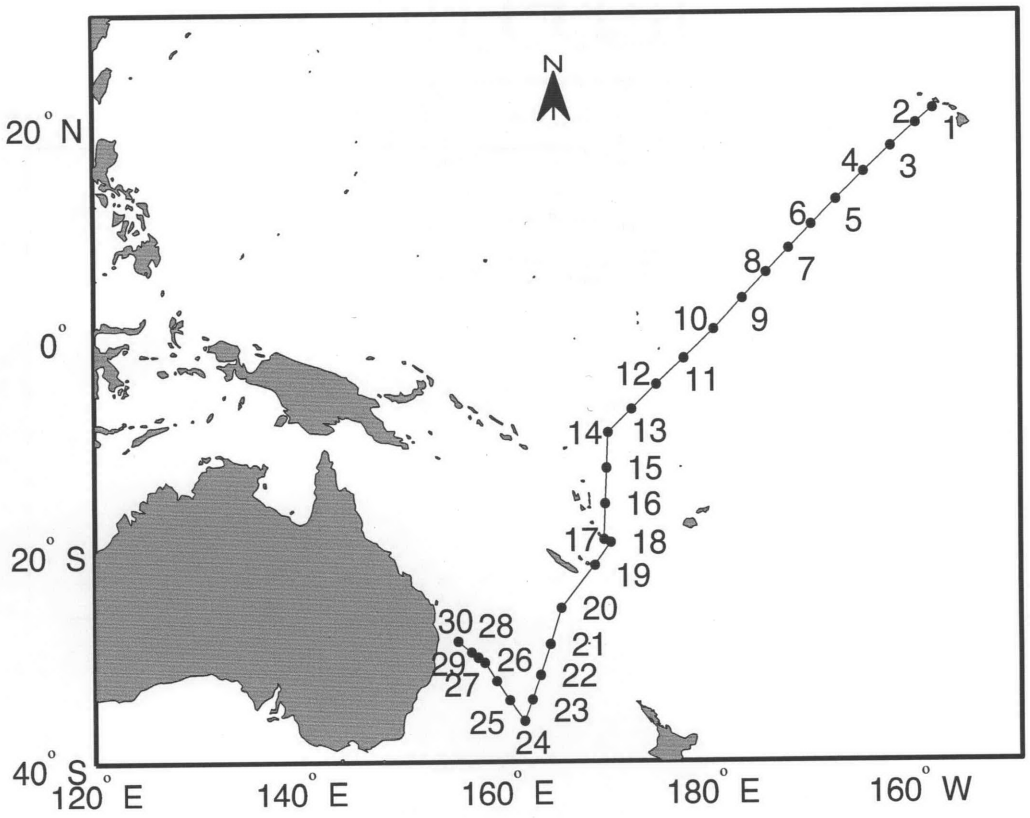

Figure 1. Map of sampling stations from the Western Pacific Warm Pool cruise (KM0701) on the R/V Kilo Moana.

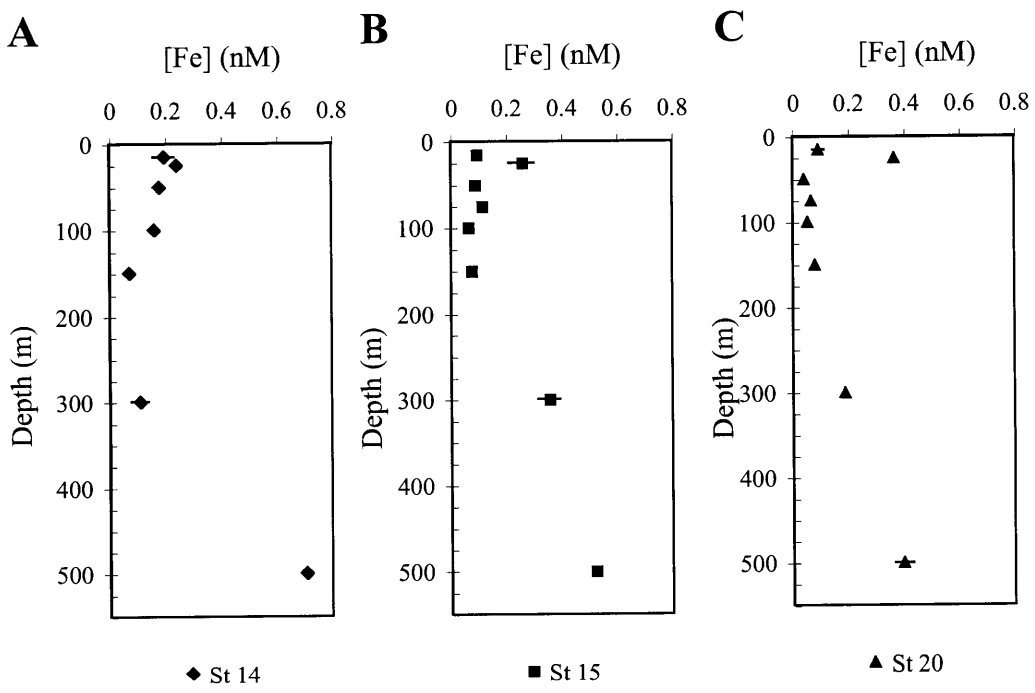

Figure 2. Dissolved Fe (< 0.4 μm filtered) depth profiles from three stations along the cruise track. (A) Station 14. (B) Station 15. (C) Station 20. Error bars are standard deviations of triplicate analyses.

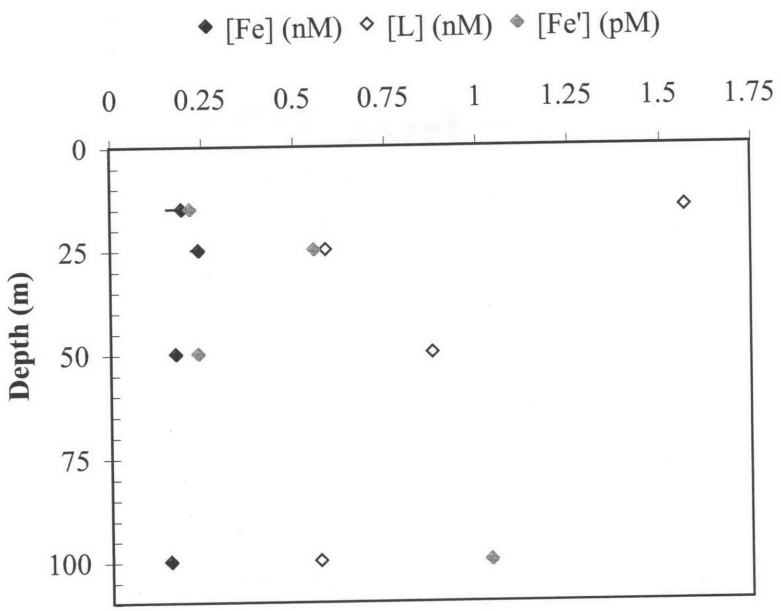

Figure 3. Dissolved Fe ([Fe]), inorganically bound Fe ([Fe'])and organic ligand ([L]) concentrations at Station 14 up to 100 m depth. Dissolved Fe and the organic ligand concentrations are plotted on a nM scale while inorganically bound Fe is plotted on a pM scale.

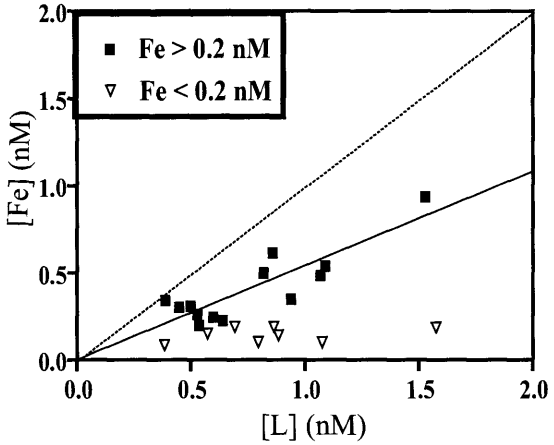

Figure 4. The concentration of dissolved Fe as it relates to ligand concentrations. The solid triangles are for samples where [Fe] > 0.2 nM and the open triangles are samples where [Fe] < 0.2 nM. The dashed line represents 1:1 = [Fe]:[L]. The solid line is the linear regression of [Fe] vs [L] when [Fe] > 0.2 nM (solid triangles).

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CHAPTER 3. A MOLECULAR ASSESSMENT OF THE IRON STRESS RESPONSE IN THE TWO PHYLOGENETIC CLADES OF *TRICHODESMIUM*

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3.1 ABSTRACT

Trichodesmium spp. play key roles in global carbon and nitrogen budgets and thus understanding what controls their activity is important for understanding climate change. While iron (Fe) availability has been shown to be an important chemical factor for controlling both growth and nitrogen fixation rates in *Trichodesmium*, all culture experiments to date have focused solely on representatives from one clade of *Trichodesmium*. Genomic sequence analysis determined that the *T. erythraeum* (IMS101) genome contains many of the archetypical genes involved in the prokaryotic iron stress response. Focusing on three of these genes, *isiB*, *idiA*, and *feoB*, we found that all three showed an Fe stress response in axenic *T. erythraeum* (IMS101), and their sequences were well-conserved across four species in our *Trichodesmium* culture collection (consisting of two *T. erythraeum* strains (IMS101 and GBRTRLI101), two *Trichodesmium tenue* strains (Z-1 and H9-4), *Trichodesmium thiebautii* and *Trichodesmium spiralis*). With clade-specific quantitative PCR (QPCR) primers for one of these genes, *isiB*, we found that high *isiB* expression at low Fe levels corresponded to specific reductions in N_2 fixation rates in both major phylogenetic clades of *Trichodesmium* (the *T. erythraeum* clade and *T. tenue* clade). With regard to the two clades, the most significant difference determined was temperature optima, while more subtle differences in growth, N_2 fixation rate and gene expression responses to Fe stress were also observed. However the apparent conservation of the Fe stress response in the *Trichodesmium* genus suggests that it is an important adaptation for success in the oligotrophic ocean.

3.2 INTRODUCTION

Primary producers play important roles in controlling both the oceanic food chain and the overall biogeochemistry of the ocean. Open-ocean diazotrophic cyanobacteria, such as *Trichodesmium* spp. and *Crocosphaera watsonii*, are of particular interest to researchers studying global biogeochemical cycles, due to their contribution to both the

carbon (C) cycle via primary production and to the nitrogen (N) cycle because of their ability to fix N_2 (Capone, 2001; Montoya et al., 2004). The "new" N, N that has not been regenerated from degradation of organic matter in the mixed layer, produced by these cyanobacteria is vital to the N and C cycles on regional (Zehr et al., 2001), and global scales (Capone et al., 1997; Gruber and Sarmiento, 1997), as well as potentially influencing CO_2 sequestration over geologic time-scales (Capone et al., 1997; Falkowski, 1997; Gruber and Sarmiento, 1997). N_2-fixing cyanobacteria are thought to have evolved in an anoxic ocean where iron (Fe) was readily available as Fe^{2+}, and despite changes in the availability of Fe in the modern ocean, these organisms still maintain high cellular Fe requirements, potentially because of the ancient evolution of N_2 fixation biochemistry (Berman-Frank et al., 2001; Kustka et al., 2003). This increased Fe requirement has been argued to be an important constraint on N_2 fixation in the modern ocean (Moore et al., 2004; Moore and Doney, 2007). Therefore, increased understanding of how diazotrophic cyanobacteria acquire Fe, what forms of Fe are bioavailable and how they respond to Fe deprivation is important for predicting potential feedbacks on climate change.

Genetic analyses of laboratory cultures have shown there are two distinct clades within the genus *Trichodesmium*, one consisting of strains of *Trichodesmium erythraeum* and *Trichodesmium contortum*, which we will refer to as the Tery clade, and the other consisting of *Trichodesmium tenue*, *Trichodesmium thiebautii*, *Trichodesmium spiralis* and *Trichodesmium hildebrandtii*, which we will refer to as the Ten clade (Hynes and Waterbury unpublished results; Orcutt et al., 2002). Multiple experiments have shown that Fe is an important micronutrient for the Tery clade (Berman-Frank et al., 2001; Fu and Bell, 2003; Kustka et al., 2003), providing valuable information about the role that Fe plays in controlling growth and N_2 fixation in *Trichodesmium*. Comparable data is not available for the Ten clade. Morphological data from the field suggests that representatives from the Ten clade might be more prevalent deeper in the water column (Carpenter et al., 1993; Post et al., 2002; Davis and McGillicuddy, 2006) and may even have different N_2 fixation rates than the Tery clade (Carpenter et al., 1993), though this has not been proven using genetic techniques that distinguish between the clades. Thus

understanding the impact that Fe has on N_2 fixation in the Ten clade of *Trichodesmium* is important to improve our understanding of the genus' role in global carbon and nitrogen cycling.

To constrain the relationship between Fe and N_2 fixation in *Trichodesmium*, we evaluated multiple indicators of Fe limitation including growth and N_2 fixation rates with cultures from both clades of *Trichodesmium* grown on varying levels of Fe. In addition, we designed a molecular gene expression-based assay to look at the cellular Fe stress response of genes believed to be part of the Fe-uptake machinery or shown to be Fe stress-induced in other organisms (Bibby et al., 2001; Katoh et al., 2001; Webb et al., 2001; Michel et al., 2003; Shi et al., 2007). We found that the Ten clade had a lower optimum temperature for growth and slightly reduced N_2 fixation rates at higher Fe values. The Ten clade also showed a lower basal expression of our molecular marker gene, *isiB*, but a more pronounced increase in gene expression as Fe became limiting. While there were slight differences in the individual Fe stress responses in the two clades, overall we observed similar trends in the Fe level associated with a significant increase in gene expression and concomitant reductions in N_2 fixation.

3.3 Results

3.3.1 Genomic Database Analysis. We screened the genome of *T. erythraeum* (IMS101) to evaluate the potential Fe stress response in the genus. A list of IMS101 genes predicted to be involved in Fe transport and homeostasis, their closest orthologs and their closest experimentally–characterized orthologs (genes that have been characterized using genetic and proteomic techniques) as determined by BLAST analysis are presented in Table 1. A schematic of both the predicted Fe uptake system and the fate of Fe within the cell with labels corresponding to the genes described in Table 1 is shown in Figure 1.

Sensing and responding to Fe availability has been shown to have a transcriptional component (Escolar et al., 1999). Fitting with these data, *Trichodesmium* is predicted to have three homologs encoding the ferric uptake regulator (Fur) protein. In

many microbes this protein has been shown to modulate the organism's response to Fe starvation (Andrews et al., 2003). Two of these *Trichodesmium* genes, YP_721684 and YP_722978, are highly similar to the *furA* gene of *Anabaena* PCC 7120, while the third gene, YP_721679, is most similar to the *furB* gene of *Anabaena* PCC 7120 and appears to be specific to cyanobacteria (Hernandez et al., 2004).

The IMS101 genome also contains genes with the potential to modulate Fe use and storage in response to changing environmental conditions. One such adaptation includes replacing the Fe-rich electron carrier ferredoxin with the flavin-containing electron carrier flavodoxin (IsiB) (Leonhardt and Straus, 1992; LaRoche et al., 1996). The IMS101 genome has two predicted IsiB homologs, encoded by genes YP_721410 and YP_722232. In addition to the ferredoxin/flavodoxin substitution, some gram-negative bacteria synthesize the IsiA protein in times of Fe deprivation, which forms a protective ring around photosystem I (Leonhardt and Straus, 1992; Bibby et al., 2001; Michel and Pistorius, 2004). The IMS101 genome has one predicted homolog for the IsiA protein encoded by gene YP_721411. Finally, the genome also shows evidence of Fe storage capabilities including a cytochrome b1 type bacterioferritin protein (Andrews et al., 1993; Keren et al., 2004) and a ferritin-like DPS protein (Michel et al., 2003; Castruita et al., 2006), predicted to be encoded by YP_722441 and YP_723752, respectively.

Although there are no clear siderophore biosynthetic genes in the IMS101 genome, it is predicted to encode for the uptake of a variety of Fe forms. These include genes that may facilitate the uptake of siderophore-bound Fe, such as a cluster of two genes predicted to encode the intermembrane proteins ExbB and ExbD, genes YP_723908 and YP_723909, respectively, and a possible quite divergent TonB protein, encoded by gene YP_721313. TonB-ExbB-ExbD complexes have been shown to translocate the energy stored in the proton gradient across the inner bacterial membrane to the outer membrane to allow the transport of large molecules like siderophores into the periplasm of gram-negative bacteria (Braun, 1995). Genes YP_722814, YP_722813, YP_722952 and YP_723445 are all predicted to encode proteins involved in an ABC-

type Fe^{3+} transporter (Koster, 2001). YP_722814 is homologous to an inner membrane component of a binding protein dependent transport system and is clustered with a gene, YP_722813, which is homologous to an ATPase component of an ABC-type Fe^{3+} transporter (Katoh et al., 2001). The close proximity of the two genes and their related function, suggests that they may be part of the same operon. The IMS101 genome contains two potential periplasmic Fe^{3+} binding protein components of an ABC transport system (Koster, 2001), *idiA*, YP_722952, and a predicted hydroxamate-Fe-binding protein, YP_723445. The confirmed presence of a signal peptide region in the transcript of the *idiA* gene, which was not found for YP_723445, suggests that the IdiA protein is being processed through a membrane to outside of the cytoplasm and possibly moved into the periplasm, a step that is necessary in the maturation of a periplasmic protein (Fulda et al., 2000). However, it is important to note that our inability to discern a signal peptide region in the transcript of YP_723445 using SignalP 3.0 is not a definitive indication that the protein will not be found in the periplasm, as slight modification of the translational start codon has been shown to mask potential signal peptide regions (Fulda et al., 2000). Additionally *Trichodesmium* has the predicted ability to transport Fe^{2+} using the proteins FeoA and FeoB, encoded by genes YP_722525 and YP_722524, respectively (Kammler et al., 1993).

3.3.2 Sequence Analysis. Others have shown that there can be differences in nutrient scavenging strategies employed by closely related cyanobacteria (Martiny et al., 2006; Palenik et al., 2006; Rivers et al., 2009), therefore in order to determine how well conserved the Fe stress regulon was within the genus *Trichodesmium*, we sequenced genes predicted to be involved in Fe(III) uptake, Fe(II) uptake, and Fe quota reduction (*idiA* (YP_722952), *feoB* (YP_722524) and *isiB* (YP_721410), respectively) from isolates covering the range of *Trichodesmium* species in our culture collection. These results showed that all three genes are well conserved across the four species of *Trichodesmium* at the DNA level (Table 2), with the largest dissimilarity detected between the Tery clade (IMS101 and GBRTRLI101) and the three species of the Ten clade (93%-96% similarity between *T. erythraeum* and the other species, compared with

98%-100% similarity within the Ten clade). No obvious shared promoter regions could be defined for the three genes using alignments of the intergenic spacer regions preceding each gene in the *T. erythraeum* IMS101 genome.

3.3.3 Axenic IMS101 Nutrient Stress Experiment. To verify the annotation and test the hypothesis that the *idiA*, *feoB* and *isiB* genes were induced under Fe limitation, we monitored their expression using RT-PCR in Fe growth limitation experiments with axenic cultures of IMS101. At the onset of growth limitation (T1, Figure 2 A+B), RT-PCR showed that all three genes were expressed only in the Fe-omitted culture. At the point when the Fe-omitted culture was beginning to senesce and the replete treatment was late in the exponential growth phase (T2, Figure 2 A), all three genes were expressed in both the replete and Fe-omitted treatments, but not in the P-omitted culture (T2, Figure 2 B).

3.3.4 Physiological Growth Response of the Ten and Tery Clades. Optimal growth temperatures were determined for representatives from both phylogenetic clades. In replicated experiments performed at the same light level (140 $\mu Ein/m^2/s$), the Ten clade consistently had a lower optimal temperature (26°C) than the two Tery representatives tested (28°C) (Figure 3). The maximum growth rates were lower for the Ten clade representative (0.33 +/- 0.01 $\mu\ d^{-1}$) than GBRTRLI101 (0.60 +/- 0.01 $\mu\ d^{-1}$) and IMS101 (0.78 +/- 0.01 $\mu\ d^{-1}$). The error reported for growth rates is standard error calculated from triplicate biological replicates.

As Fe had been shown to be important for the growth of *Trichodesmium*, but its effect had only been studied in the Tery clade, we did growth limitation experiments using a range of Fe' (inorganically bound Fe) values ranging from 0.15 nM to 4.5 nM with representatives from both phylogenetic clades of *Trichodesmium* at their optimal growth temperature (the values of Fe' were calculated using VisualMintEQ as described in the Experimental Procedures). In both clades, growth rates increased as the concentration of inorganically bound Fe ([Fe']) in the medium increased (Figs. 4A+B). In the Ten clade, growth rates increased from 0.13 +/- 0.01 $\mu\ d^{-1}$ to 0.19 +/- 0.01 $\mu\ d^{-1}$ with the maximal growth rate achieved at [Fe'] of 0.54 nM. In the Tery clade, growth

rates increased from 0.12 +/- 0.00 μ d^{-1} to 0.21 +/- 0.00 μ d^{-1} with the maximal growth rate achieved at [Fe'] of 0.94 nM. N_2 fixation rates also increased concomitantly with [Fe'] in the medium, and continued to do so even after the growth rates had plateaued (Figs. 4A+B). In the Ten clade, N_2 fixation rates increased from 3.2 +/- 0.2 nmol N/hr/μg Chl to 10.1 +/- 0.3 nmol N/hr/μg Chl, with the maximal N_2 fixation rates achieved at [Fe'] of 4.5 nM. In the Tery clade, N_2 fixation rates increased from 3.6 +/- 0.3 nmol N/hr/μg Chl to 13 +/- 0.7 nmol N/hr/μg Chl with the maximal N_2 fixation rates achieved at [Fe'] of 4.5 nM. Despite these differences, when the N_2 fixation rates were normalized to growth rates a similar relationship was revealed for both phylogenetic clades (Figure 4C). To ensure that N_2 fixation rate differences were not the result of changes in Chl/DNA ratios in the different treatments, we evaluated the Chl/DNA ratio for samples from low and high Fe treatments in both clades and found no significant Fe-associated difference and a value of 1.6 x 10^{-8} +/- 3.0 x 10^{-9} μg chl/copy *isiB* (Figure 4D). Error reported for Tery clade growth and nitrogen fixation rates is standard error calculated from biological replicates (5-6 replicates at each Fe treatment). Error reported for Ten clade growth and nitrogen fixation rates is standard error calculated from triplicate biological replicates at each Fe treatment. Error reported for chl/copy DNA is standard error based on triplicate low and high Fe replicates from the Tery clade and duplicate low and high Fe replicates from the Ten clade (10 replicates total).

3.3.5 Quantified Fe stress response. Both clades of *Trichodesmium* show an inverse relationship between *isiB* expression and N_2 fixation rates in response to changing [Fe'] in the medium, with *isiB* expression increasing and N_2 fixation rates decreasing as the [Fe'] was reduced (Figure 5 A+B). In the Tery clade, the threshold associated with a 50% decrease in N_2 fixation rates occurs at an *isiB*/*rnpB* ratio of 1.4 +/- 0.5 and an [Fe'] of 0.54 nM (Figure 5A), while in the Ten clade, the same threshold occurs at an *isiB*/*rnpB* ratio of 0.063 +/- 0.02 at the same [Fe'] (Figure 5B). While the Ten clade shows a lower basal expression level than the Tery clade (Figure 5 A+B), it also shows a greater increase in expression at lower Fe conditions than the Tery clade (Figure 5C). The thresholds listed above are based on the actual values from these experiments, without

curve fitting. The error reported for each measurement is standard error of biological replicates (3 for the Ten clade and 6 for the Tery clade at each Fe treatment). We fit the expression data with an exponential decay model and found that for the Tery clade *isiB/rnpB*= $2.2 \times 10^{(1.9 \times [Fe'])}+0.44$ with an $R^2 = 0.61$ and for the Ten clade *isiB/rnpB*= $0.14 \times 10^{(1.5 \times [Fe'])}+0.011$ with an $R^2 = 0.49$. We fit the % maximum N_2 fixation data using a 2nd order polynomial model and found that for the Tery clade % Maximum N_2 Fixation= $24.9+ 50 \times [Fe'] - 7.4 \times [Fe']^2$ with an $R^2 = 0.75$ and for the Ten clade % Maximum N_2 Fixation = $29.4 + 32.2 \times [Fe'] - 7.41 \times [Fe']^2$ with an $R^2 = 0.93$. Using these equations, N_2 fixation is at 50% maximum in the Tery clade at [Fe'] = 0.56 +/- 0.14 nM and in the Ten clade at [Fe'] = 0.70 +/- 0.10 nM with corresponding *isiB/rnpB* expression values of 1.2 +/- 0.3 and 0.062 +/- 0.017, respectively. The error reported was calculated using error propagation analysis and the standard error of each regression.

In an experiment where we transferred Fe limited *T. erythraeum* (GBRTRLI101) into Fe replete medium, we found that the expression of *isiB* dropped to basal levels within 24 hours, while cultures that were transferred back into Fe-omitted medium retained expression above the threshold levels indicative of Fe limitation of N_2 fixation (Figure 6).

3.4 Discussion

Field studies indicate that *Trichodesmium* spp. are widely distributed and a significant source of new nitrogen in the tropical and subtropical Atlantic and Pacific Oceans (Capone, 2001). The high Fe requirement of N_2 fixing cyanobacteria like *Trichodesmium* spp. creates a biological linkage between the geochemistries of N and Fe (Kustka et al., 2003). Despite the defined important relationship between *Trichodesmium* and Fe availability, prior physiological data has been limited to laboratory studies using strains representative of only the Tery clade of *Trichodesmium* (Berman-Frank et al., 2001; Fu and Bell, 2003; Kustka et al., 2003; Berman-Frank et al., 2007). As others have seen large difference in the genomic capability of marine cyanobacteria to compensate

for Fe deprivation (Palenik et al., 2006; Rivers et al., 2009), the work described herein is both important and timely and represents the first physiological data comparing the response of the Tery and Ten clades of *Trichodesmium* to Fe deprivation.

3.4.1 Genomic Capabilities and Conservation Within the Genus.

Trichodesmium spp. inhabit environments typified by episodic inputs of Fe through dust deposition or mesoscale eddies, followed by long periods of deprivation. Thus it is not surprising that the IMS101 genome contains many genes predicted to encode for the uptake of different forms of Fe, Fe quota compensation, and Fe storage mechanisms (Table 1, Figure 1). To assess the importance of these adaptations throughout the genus as a whole, we evaluated the conservation of some of the genes involved in Fe uptake and quota compensation within the genus *Trichodesmium*.

The *Trichodesmium* IMS101 genome is predicted to encode the genetic capacity to transport both ferric (FeIII) and ferrous (FeII) Fe into the cell. However, since the oceans are oxidizing, Fe(III) is likely one of the main sources of Fe to open-ocean cyanobacteria. This is supported by the presence of a complete periplasmic binding protein-dependent ABC transport system for Fe (encoded by *idiA* (YP_722952), inner membrane channel (YP_722814), and ATPase (YP_722813)) and the knowledge that these types of systems have been shown to be critical for moving Fe through the periplasm into cytoplasm in bacteria and cyanobacteria (Koster, 2001). Although oceanic bulk water is oxidized, there are many redox microniches, including chemical environments that can become reducing (Shanks and Reeder, 1993; Azam, 1998; Moran et al., 2004). Consistent with these microniches, the IMS101 genome is also predicted to encode for genes of Fe(II) transport (*feoA* and *feoB*: YP_722525 and YP_722524, respectively). While *feoB* has been found in some freshwater cyanobacterial genomes (Katoh et al., 2001) and three strains of coastal marine *Synechococcus* (Palenik et al., 2006; Rivers et al., 2009), in open-ocean cyanobacteria it has only been identified in the genomes of the diazotrophs *T. erythraeum* (IMS101) and *C. watsonii* (WH8501). *Trichodesmium*'s apparent genetic capacity for acquiring Fe^{2+} may indicate that there is an indirect or direct Fe (III) reduction scheme involved in Fe uptake, either

extracellularly or within the periplasm. Others have shown that photolysis of Fe (III)-siderophore complexes could be a source of Fe^{2+} for the oceans (Barbeau et al., 2003), and this could be a passive Fe (II) resource to *Trichodesmium*. This reduction might also occur in the microcosms associated with colony formation. Since bacteria are known to commonly dispense with genes not required for success in their natural habitat (Teuber M., 1992), the presence of the *feoA* and *feoB* genes in the IMS101 genome indicates that *Trichodesmium* may be actively pursuing Fe^{2+} as a cellular Fe source.

In addition to genes associated with Fe uptake, the IMS101 genome also contains a gene involved in Fe quota reduction, *isiB* (YP_721410). During times of Fe deprivation, organisms that have the *isiB* gene are able to synthesize the flavin-containing protein flavodoxin, and use it to replace the Fe-rich electron carrier ferredoxin in the Z-scheme of photosynthesis (Leonhardt and Straus, 1992). The *isiB* gene that we sequenced, YP_721410, shows a greater similarity to flavodoxin genes that have been fully characterized and shown to be Fe stress induced in other organisms (Fillat et al., 1991) than the other putative flavodoxin-encoding gene, YP_722232.

Sequencing of *isiB*, *idiA*, and *feoB* from various *Trichodesmium* species revealed high similarity across the genus (Table 2), with the largest differences consistently occurring between species from the two different clades. This separation is consistent with previous work looking at the genetic characteristics of different species of *Trichodesmium,* where the groups that comprise the Ten and Tery clades were first defined with cultured isolates (Orcutt et al., 2002). The conservation of the components of the *Trichodesmium* Fe stress regulon studied herein stand in contrast to the variation in gene content that has been seen in genomes of the unicellular cyanobacteria (Palenik et al., 2006; Rivers et al., 2009). While our analyses do prove that all of these genes are used in *Trichodesmium*, the conservation of all three genes across the genus suggests that these Fe limitation compensation mechanisms are important for success in the oligotrophic ocean.

3.4.2 Axenic IMS101 Expression Analyses. Initial gene expression analysis with axenic batch cultures of *T. erythraeum* IMS101 grown under replete, Fe-omitted and

P-omitted conditions showed that all three genes (*idiA, feoB* and *isiB*) were expressed only under Fe limitation (Figure 2). Importantly, if the expression of any of the genes had been part of a generalized stress response of the organism, we would have expected to see expression in the P-omitted treatment as well. The expression that appears at the later time point, "T2," in the replete experiment was expected, based on chemical modeling of the medium that suggests the cells will experience Fe limitation before P limitation. In order for this to occur in an EDTA-buffered medium, the culture would have had to grow to a "blown buffer" cell density (Saito et al., 2008), where the demand for Fe from the accumulated biomass exceeds the amount supplied by the dissociation of Fe from the EDTA buffer, which results in the inorganically bound Fe ([Fe']) being lower than calculated based on equilibrium dynamics. As the expression occurs before growth limitation, it suggests that upregulation of the Fe stress regulon begins as soon as the organism begins to experience a decrease in cellular Fe availability. This hypothesis is corroborated by results from our Fe titration experiments with representatives from the two *Trichodesmium* clades, which show that increases in expression of the *isiB* gene and decreases in N_2 fixation rates occur throughout the range of Fe values we tested, while growth rates only decrease at the lowest Fe values.

3.4.3 Clade Differentiation. Our results show that the optimal temperature for growth for the Tery clade is 28°C, which is consistent with the results of Breitbarth and colleagues (Breitbarth et al., 2007), while the optimal temperature for growth for the Ten clade is 2°C lower (Figure 3). Similar to what Breitbarth and colleagues found with *T. erythraeum* (Breitbarth et al., 2007), we also saw a significantly reduced N_2 fixation rate when we grew *T. tenue* above its optimal temperature (data not shown). These data imply that there is a niche differentiation in the genus based on temperature, which could result in a differential depth distribution of the species, data that are consistent with the morphological distributions of *Trichodesmium* that have been described from the field (Post et al., 2002; Davis and McGillicuddy, 2006). Furthermore, our data suggest that temperature-based niche differentiation could lead to a species composition shift in the

oceans if anthropogenic climate change leads to a significant increase in sea surface temperature.

Tery and Ten clade representatives grown in varying levels of Fe showed a direct correlation between Fe and growth rates (Figure 4A) and Fe and N_2 fixation rates (Figure 4B). Both clades showed an eventual plateau in growth rates at high Fe values, which occurred at a slightly lower Fe value for the Ten clade than the Tery clade, [Fe'] of 0.54 nM and 0.94 nM, respectively. While this could suggest a lower Fe quota for the Ten clade, it could also be a by-product of imperfect culturing conditions, indicating that some other element or factor becomes limiting for the organism above this Fe value. In both clades of *Trichodesmium*, N_2 fixation rates continue to increase even after growth rates have reached their plateau. These data suggest that *Trichodesmium* fixes more N_2 than is strictly needed when growing in the presence of excess Fe. Alternatively, the reduction of N_2 fixation rates prior to a reduction in growth rates could be evidence of the organism's sacrificing the high Fe-requiring N_2 fixation in favor of carbon fixation at the onset of Fe stress (Kupper et al., 2008). If the former hypothesis is true, it could potentially explain the dissolved organic nitrogen (DON) excretions observed in many replete Fe culture experiments done with *Trichodesmium* (Capone et al., 1994; Mulholland and Capone, 2001). If Fe plays a role in excess N_2 fixation and DON excretion, it could indicate an increased importance for Fe in modulating the N cycle in the oligotrophic gyres and should be explored further.

At the highest values of Fe in the medium, the Ten clade had reduced chl-normalized N_2 fixation rates compared to the Tery clade. To ensure that this difference in N_2 fixation rates was not a byproduct of our normalization of the rates to chl, we tested the chl/DNA ratio of the cultures across the different Fe treatments and found that they were constant in the species and experimental Fe conditions (Figure 4D). Thus, there was a real difference in the N_2 fixation rates between the two clades. This could indicate that while the Ten clade does fix excess N_2 when abundant Fe is available, it does not do so to the extent that the Tery clade does. However, when the lower maximal growth rates in the Ten clade are taken into account, the difference in N_2 fixation rates appear to be a

factor of growth rate (Figure 4C). While the absolute amount of N_2 fixed by the two clades was different, the amount of [Fe'] associated with a 50% reduction in N_2 fixation rates was very similar for both clades (0.54 nM Fe without curve fitting and with curve-fitting: 0.56 +/- 0.14 nM for Tery and 0.70 +/- 0.10 nM for Ten), indicating a similar impact of Fe on N_2 fixation rates throughout the genus. These values are in line with what others have found for the critical [Fe'] value associated with a decrease in N_2 fixation with the *T. erythraeum* clade (Berman-Frank et al., 2001; Berman-Frank et al., 2007).

To determine whether the cellular response to Fe limitation was the same in the two *Trichodesmium* clades, we designed a qRT-PCR method to look at the expression of one of the Fe stress response genes. This approach allows for the comparison of data on the onset of Fe limitation of growth, N_2 fixation rates, and the cellular level Fe stress response. While all three genes showed the expected expression response with the axenic *Trichodesmium* cultures, we developed the qRT-PCR method with *isiB* because the role of *isiB* in the cell is well understood (Leonhardt and Straus, 1992) and it has been used as a marker for Fe stress in other phytoplankton (LaRoche et al., 1996; Bibby et al., 2001). Also, its role in photosynthesis suggests that it has the potential to be in high copy number when it is expressed, which will make it easier to detect using RT-PCR. In order to assess relative expression rates in non-axenic cultures, we normalized the number of *isiB* copies in a given sample to the number of copies of a constitutively expressed gene, *rnpB*, which we have chosen based on experiments comparing its stability in cDNA from cultures grown under different physical and chemical treatments (see experimental procedures). We designed primer sets for *isiB* and *rnpB* that able to distinguish between both clades of *Trichodesmium* (see experimental procedures) that can be used to generate *isiB* expression data that is normalized to *Trichodesmium* RNA (*rnpB*).

Both Tery and Ten cultures show an inverse relationship between *isiB* expression and N_2 fixation rates in response to changing [Fe'] with *isiB* expression highest at the lowest N_2 fixation and [Fe'] values (Figure 5A+B). The results from these experiments have provided us with valuable information on the threshold of expression that

corresponds to a meaningful decrease in N_2 fixation rates, which is 0.063 *isiB/rnpB* for the Ten clade and 1.2 *isiB/rnpB* for the Tery clade. Above these threshold *isiB* expression levels, N_2 fixation rates had decreased by at least 50% from the maximal levels measured in both clades of *Trichodesmium* (Figure 5). Interestingly, the [Fe'] value where this reduction in N_2 fixation rates and increase in *isiB* expression occurs is approximately the same [Fe'] in both clades of *Trichodesmium* (0.56 +/- 0.14 nM for Tery and 0.70 +/- 0.10 nM for Ten), suggesting that there is not significant niche differentiation between the clades based on Fe availability. These data differ from genomic and physiological work in other cyanobacterial groups, which has found that the Fe stress response is an area of significant deviation between clades represented in a genus (Brand, 1991; Palenik et al., 2006; Rivers et al., 2009). Furthermore, these results suggest that other factors (possibly temperature, etc) are more important than Fe for niche differentiation in the *Trichodesmium* genus. The conservation of the Fe stress response across the two clades further implies that Fe is a common and important stressor for the genus.

Using the information on threshold *isiB* expression of the Tery clade, we were also able to evaluate how quickly *Trichodesmium* is able to respond to an alleviation of Fe stress. We found that *isiB* expression dropped within 24-hours of cultures being returned to Fe replete medium (Figure 6). These results are similar to what has been seen with *idiA* gene expression (Shi et al., 2007) and much faster than what has been detected using IdiA protein analysis in another cyanobacteria, Synechococcus WH7803, where the protein remained detectable even three days after cultures were transferred to Fe replete medium (Webb et al., 2001). These results highlight one of the advantages and challenges of looking at RNA verses proteins; RNA is degraded much more rapidly than protein and thus reflects the immediate cellular status of the organism. Meanwhile, proteins can persist for some time after they have actively been translated, thus their presence does not always reflect the current status of the organism.

This study is the first demonstration of Fe limitation using representatives of the two major clades of *Trichodesmium*. In addition to traditional measurements quantifying

the impact that Fe has on productivity, namely growth rates and N_2 fixation rates, we have added a molecular assessment of Fe limitation for both clades of *Trichodesmium*. This calibrated clade-specific assay allows us to quantify the impact that Fe is having on N_2 fixation, which should be quite relevant for the development of a field assay for Fe limitation in *Trichodesmium* and thereby improve oceanographic models.

3.5 Experimental Procedures

3.5.1 Genomic Database Searching. Genes associated with the Fe scavenging and control of Fe homeostasis systems in *T. erythraeum* IMS101 were identified using the Oak Ridge National Laboratory (ORNL) annotation of the genome accessed through the Joint Genome Institute (JGI) Internet portal (http://genome.jgi-psf.org/finished_microbes/trier/trier.home.html). The closest experimentally-characterized homolog was determined using Basic Local Alignment Search Tool (BLAST) analysis against the GenBank NR database using the Integrated Microbial Genomes system of JGI (http://img.jgi.doe.gov/v1.0/main.cgi) (Altschul et al., 1990). When appropriate, the presence of a signal peptide region was determined using SignalP 3.0 (Bendtsen et al., 2004).

3.5.2 Bacterial Strains. The four *Trichodesmium* spp. used in this study were *T. erythraeum* (IMS101 and GBRTRLI101), *T. thiebautii* (II-3), *T. tenue* (Z-1 and H94) and *T. spiralis* (KAT) (all but GBRTRLI101 and H94 have been described in Orcutt, 2002). All species are currently maintained in both the University of Southern California and the Woods Hole Oceanographic Institution culture collections. All but GBRTRLI101 and IMS101 were isolated by Dr. John Waterbury (Paerl et al., 1994; Fu and Bell, 2003). GBRTRLI101 was generously provided by Dr. F.X. Fu. Cultures of IMS101 used in the initial nutrient stress experiment were verified to be axenic by direct microscopic observations and lack of heterotrophic growth in marine purity medium as described (Waterbury et al., 1986). The five other species and the IMS101 culture used in the Fe titration experiment were maintained as bacterized enrichment cultures.

3.5.3 Culture Conditions. With the exception of GBRTRLI101, stock *Trichodesmium* spp. used for sequencing were cultured in a 75% Sargasso seawater medium prepared in a similar manner to that described previously (Webb et al., 2001). Sargasso seawater, stored in the dark in acid-washed polycarbonate carboys, was filtered successively through 1.0 and 0.2 μm Millipore membrane filters and "Tyndalized" by heating to boiling in a microwave oven in Teflon containers. The "Tyndalized" Sargasso seawater was then diluted to 75% with steam-sterilized MilliQ-water (Millipore, Bedford, MA). The medium (PMP) was then prepared by addition of filter- or steam-sterilized nutrients and trace metals made from tissue-grade chemicals purchased from Sigma Chemical to the following concentrations: 5×10^{-7} M EDTA, 8×10^{-6} M phosphoric acid, 1×10^{-7} M Fe (ferric citrate), 1×10^{-5} M Citric Acid, 1×10^{-7} M $MnSO_4$, 1×10^{-8} M $ZnCl_2$, 1×10^{-8} M $NaMoO_4$, 1×10^{-10} M $CoCl_2$, 1×10^{-10} M $NiCl_2$, 1×10^{-10} M $NaSeO_3$, and 1.5 μg of vitamin B_{12}/liter. GBRTRLI101 stocks were grown in an artificial seawater medium YBCII (Chen et al., 1996) with Fe added as ferric citrate. All *Trichodesmium* stock cultures were grown in Nalgene® polycarbonate flasks or culture bottles (Nalge Nunc International Corporation, Rochester, NY) that had previously been cleaned with a 2% solution of Citranox® (Alconox, Inc. White Plains, NY), followed by rinses in hot tap water, MilliQ water and at least a 24-hour soak in 0.5 N trace metal grade HCl before finally being rinsed in MilliQ water and microwave sterilized with pH 2 trace metal grade HCl. Growth conditions typically consisted of a 14 hr:10 hr light:dark cycle using cool white fluorescent lamps at ~50 μEin/m^2/s and a temperature of 25°C unless stated otherwise. The cultures were kept gently shaking by placement on a model 3520 LabLine® Orbital Benchtop shaker (Barnstead International, Dubuque, IA) within a model I-36 Percival incubator (Percival Scientific Inc., Perry, IA).

3.5.4 Gene Sequencing. DNA for sequencing was extracted using a modified version of the xanthogenate DNA extraction protocol of Tillet and Neilan (Tillett and Neilan, 2000). Cultures were filtered onto 5-μm polycarbonate filters and then resuspended in 100 μl of TE buffer with 50μg/ml RNaseA (Qiagen, Valencia, CA). The only other departures from the Tillet and Neilan protocol were mixing the supernatant

following the ice incubation with 700 µl phenol:$CHCl_3$:isoamyl alcohol (25:24:1), retaining the top layer of that mixture following centrifugation, and two additional 70% ethanol wash steps at the end of the procedure before resuspending the final pellet in 100 µl sterile MilliQ water. The genes *isiB* (234 bp out of a 516 bp gene), *feoB* (995 bp out of a 1821 bp gene), *idiA* (520 bp out of a 1050 bp gene) and *rnpB* were amplified from the extracted DNA via PCR using iProof™ High-Fidelity DNA polymerase (Bio-Rad Laboratories, Hercules, CA) at a final concentration of 1.25 units/PCR reaction. The external primers used to amplify each of the genes from the different species of *Trichodesmium* were designed from the sequenced genome of *T. erythraeum* IMS101 and are listed in Table 3. The primers used to amplify the *rnpB* gene were the degenerate primers defined in Vioque (Vioque, 1997). Temperature gradient PCR was used to determine optimal annealing temperatures for amplifying *feoB*, *isiB*, *idiA* and *rnpB* from the *Trichodesmium* species: 55.7°C, 56.1°C, 56.1°C and 55°C, respectively. PCR reactions were carried out on a Mastercycler© thermal cycler (Eppendorf AG, Hamburg, Germany) with the following holds and cycles: 98°C for 1 min; followed by 35 cycles of 98°C for 15 seconds, annealing temperature (as listed above) for 30 seconds, 72°C for 45 seconds; and one dwell at 72°C for 10 min. Amplified products were purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA) and sequenced directly using the facilities and protocols of the Josephine Bay Paul Center of the Marine Biological Laboratory (Woods Hole, MA). Sequences were analyzed and assembled using Sequencher 4.1 (Gene Codes Corporation, Ann Arbor, MI). Alignments were generated with ClustalX (Thompson et al., 1997). Gene sequences determined in this study were submitted to GenBank with the following accession numbers (EF110575-EF110583).

3.5.5 Nutrient Stress Experiments with Axenic IMS101. Fe-limited, P-limited, and replete cultures were prepared using PMP medium as described above and omitting ferric citrate and phosphoric acid where appropriate. To generate the inocula, cells from a PMP-grown culture of IMS101 were filtered gently onto a 5-µm polycarbonate filter. The filter was then washed with 50 to 100 ml "Tyndalized" Sargasso seawater before the cells were resuspended in a small amount of Sargasso seawater and distributed evenly

among the various treatment media. For nutrient limitation experiments, cultures were grown in 250 ml of medium in 500 ml Nalgene® baffled polycarbonate flasks that were cleaned using the procedure described above. At least two replicate treatments were performed per limitation experiment, and the limitation experiments were repeated three times. The growth of the cultures was monitored throughout the experiment by removing aliquots and measuring *in vivo* fluorescence using an *Aqua*Fluor™ hand-held fluorometer (Turner Designs, Sunnyvale, CA). Cells from the culture experiments were collected via filtration onto 5-μm polycarbonate filters and frozen in liquid nitrogen for later RNA extraction.

3.5.6 Temperature Optimization. Non-axenic cultures of two *T. erythraeum* strains (IMS101 and GBRTRLI101) and one *T. tenue* strain (H9-4) were grown in triplicate on the modified YBCII medium (Chen et al., 1996) with Fe added as ferric citrate. Cultures were grown in 50 ml polycarbonate tubes with light levels ~140 $\mu Ein/m^2/s$ at 24°C, 26°C, 28°C and 31°C. Growth was monitored daily using a TD-700 fluorometer with an *in vivo* Chla filter set (Turner Designs, Sunnyvale, CA).

3.5.7 Culture experiment with different Fe levels. Cultures of two *T. erythraeum* strains (IMS101 and GBRTRLI101) and one *T. tenue* strain (H9-4) were grown on modified YBCII medium (Chen et al., 1996) with EDTA held constant and varying amounts of ferric citrate added (Berman-Frank et al., 2001). Media preparation and culture handling was carried out using trace-metal clean techniques under HEPA filtration and class 100 conditions. The ferric citrate additions were 0 nM, 10nM, 25nM, 50nM, 100nM and 250nM, which correspond to concentrations of inorganically complexed Fe ([Fe']) values of 0.15 nM, 0.31 nM, 0.54 nM, 0.94 nM, 1.8 nM and 4.5 nM. The Visual MintEQ program (available for free download at http://www.lwr.kth.se/English/OurSoftware/vminteq/) was used to complete calculations of Fe speciation in the media based on known chemical additions and careful adjustment of pH to 8.15 +/- 0.02. The experiments were done in triplicate for each treatment at the optimal temperature for each clade (26°C for Ten and 28°C for Tery). Culture growth was monitored daily between 2 and 3 hours after the lights turned on in the incubator by

pouring an aliquot of each well mixed culture into an acid cleaned 50 ml polycarbonate tube and monitoring fluorescence on a TD-700 fluorometer with an *in vivo* Chla filter set (Turner Designs, Sunnyvale, CA). Before the growth experiment started, cultures were acclimated in Fe adjusted media through at least one doubling of cells and then transferred into fresh media when growth was balanced. In some cases, multiple transfers were required before growth rates separated between the different treatments. Samples were filtered down and frozen in liquid nitrogen for later RNA analysis on the morning when all treatments were growing exponentially and growth rates had separated between the low and high Fe treatments. This filtering was done using 25 mm 5-μm polycarbonate filters ~3-4 hours after the lights turned on in the incubator. On that same day, 30 ml aliquots of the cultures were placed in 60 ml Nalgene® polycarbonate bottles (Nalge Nunc International Corporation, Rochester, NY) and N_2 fixation rates were measured using the acetylene reduction assay (Capone, 1993). N_2 fixation rate measurements were based on a linear regression of ethylene concentrations measured over three hours after acetylene addition. Results were normalized to Chl *a* (Herbland et al., 1985).

3.5.8 Chl *a*/DNA Normalization. To ensure that Fe limitation did not affect the Chl *a*/DNA ratio of the cultures, we filtered 15 mls in triplicate from three low Fe and three high Fe cultures of GBRTRLI101 (18 filters total) and two low Fe and two high Fe cultures of H9-4 (12 filters total). Two of the 15 ml filters were used to determine the average Chl *a*/ml of each samples/condition. DNA was extracted from the remaining duplicate filters from each biological replicate using the DNeasy Plant Kit (QIAGEN Inc., Valencia, CA) and the number of copies of *isiB*/extraction was determined using the standard curve qPCR protocol described below with the DNA extractions added at a 1:10 dilution. The number of copies of *isiB*/ml culture was determined after taking into account all dilution steps involved in the extraction procedure. This value was then compared with the Chl *a*/ml value to determine Chl *a*/copy of DNA.

3.5.9 RNA Extraction and cDNA Synthesis. RNA was extracted using the Ribo-Pure^{TM}-Bacteria kit (Ambion Inc., Austin, TX) including the optional DNase-I

treatment. Total RNA extracts were quantified using a NanoDrop® ND-1000 Full Spectrum UV/Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Normalized quantities of total RNA extracts were then converted into cDNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). As a negative control for later RT-PCR reactions, normalized total RNA was also put through the iScript cDNA synthesis without the addition of the reverse transcriptase enzyme (later referred to as noRT).

3.5.10 RT Analysis of Gene Expression Axenic IMS101 Nutrient Experiment. PCR reactions were performed on cDNA extracts using internal primers for each gene designed from the sequenced genome of *T. erythraeum* (IMS101) (Table 3). The 15 µl reactions were run on a Mastercycler© thermal cycler (Eppendorf AG, Hamburg, Germany) with MasterTaq© Taq DNA polymerase (Eppendorf AG, Hamburg, Germany) at a final concentration of 1.25 units/PCR reaction per manufacturer's instructions without additional Mg^{+2} (1x). Template cDNA was added to a final concentration of 0.32 ng/µl and primers were added at a final concentration of 1µmol/L. The PCR reactions had the following conditions for each gene: *idiA* (95°C for 5 min; 30 cycles of 95°C for 1 min, 56.1°C for 1 min, 72°C for 30 sec; and 72°C for 10 min), *isiB* (95°C for 5 min; 35 cycles of 95°C for 1 min, 57.8°C for 1 min, 72°C for 30 sec; and 72°C for 10 min), and *feoB* (95°C for 5 min; 40 cycles of 95°C for 1 min, 56.1°C for 1 min, 72°C for 30 sec; and 72°C for 10 min).

3.5.11 Quantitative PCR Analysis of Gene Expression from Fe Titration Experiment. Separate qPCR primer sets for the *T. erythraeum* clade and the *T. tenue* clade were designed using AlleleID® (PREMIER Biosoft International, Palo Alto, CA) based on alignments made from our sequencing efforts (Table 3). The primers were tested and found to be specific for only the targeted clade, equally efficient across multiple representatives from each targeted clade and mixtures of DNA from target and non-target clades did not result in inhibition (data not shown). *rnpB* was determined to be the most stable housekeeping gene with *T. erythraeum* grown under different Fe, light and temperature conditions using geometric averaging of multiple candidate control

genes using the GeNorm method (Vandesompele et al., 2002). The GeNorm method calculates the most stable gene pair for a given set of data, and comparisons of our various conditions determined that the ranking of the normalization genes from best to worst was: *rnpB*, 16s, *glyA* and *recF*. Relative expression of *isiB* verses *rnpB* was determined using absolute quantification of each gene and dividing the number of copies of the *isiB* gene determined per sample by the number of copies of the *rnpB* gene determined per sample (Applied Biosystems User Bulletin #2: http://dna-9.int-med.uiowa.edu/RealtimePCRdocs/Compar_Anal_Bulletin2.pdf) (Larionov et al., 2005). The standards used for absolute quantification were cloned PCR products prepared as described (Zinser et al., 2006) using the TOPO TA Cloning® Kit for Sequencing (Invitrogen Corporation, Carlsbad, CA). Once cloned, the plasmids were extracted with QIAGEN Mini Prep kit (QIAGEN Inc., Valencia, CA), linearized using PstI (New England Biolabs® Inc., Ipswich, MA) and quantifed with Quant-iT™ PicoGreen® (Invitrogen Corporation, Carlsbad, CA). qPCR reactions were done on a 7500 Fast Real-Time PCR System (Applied Biosystems Inc., Foster City, CA) using *Power*SYBR® Green PCR Master Mix (Applied Biosystems Inc., Foster City, CA) at 1x concentration in a 20 μl reaction with a final cDNA concentration of 1-2 nM and a final primer concentration of 200nM. Cycler conditions were 50°C for 2 min, 95°C for 10 min; 40 cycles of 95°C for 15 sec, 55°C for 1 min with fluorescence being read at 55°C followed by dissociation curve analysis from 60°C to 95°C.

3.5.12 Alleviation of Fe Limitation Experiment. Following sampling for N_2 fixation and *isiB* expression, the remaining ~100 ml of two Fe limited cultures of GBRTRLI101 were each split into three aliquots and used to inoculate two culture flasks containing replete ([Fe'] = 4.5 nM) YBCII medium and one culture flask containing Fe omitted ([Fe'] = 0.15 nM) YBCII medium. This generated four +Fe treatments and two –Fe treatments. Samples for *isiB* expression analysis were taken immediately following inoculation and 24 hours later and processed as described in sections 3.5.9 and 3.5.11.

3.6 ACKNOWLEDGEMENTS

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Table 1. *Trichodesmium* Fe Acquisition, Storage, Regulation and Quota Reduction Genes

Number in Figure - Gene Name	*Trichodesmium* Gene			Nearest Ortholog			Nearest Described Ortholog[i]	
	Protein Length	Accession #	Source	Accession #	Identities	Source	Accession #	Identities
1 - ABC-Type Fe^{3+} Transporter permease component	555	YP_722814	6803[ii]	BAA10029	328/528		N/A	
2 - ABC-Type Fe^{3+} Transporter ATP-ase component	378	YP_722813	8501[iii]	ZP_00516660	212/362	6803	BAA18213	201/345
3 - ABC-Type Fe^{3+} Transporter periplasmic component (*idiA*)	349	YP_722952	BP-1[iv]	BAC08065	210/352	6803	BAA16842	206/356
4 - ABC-Type Fe^{3+}-hydroxamate transport periplasmic component	309	YP_723445	29413[v]	ABA22556	188/306		N/A	
5 - Ferrous iron transport protein B (*feoB*)	606	YP_722524	6803	BAA17208	331/604		N/A	
6 - Ferrous iron transport protein A (*feoA*)	89	YP_722525	6803	BAA17207	39/75		N/A	
7 - ExbB Proton Channel	261	YP_723908	8501	ZP_00515768	139/205	6803	BAA16958	135/201
8 - ExbD	207	YP_723909	6803	BAA16959	93/205		N/A	
9 - TonB family protein	537	YP_721313	8106[vi]	ZP_01624500	63/235		N/D	
10 - Bacterioferritin-like protein	159	YP_722441	29413	ABA21384	112/143	7120[x]	BAB75639	111/143
11 - Ferritin-like DPS protein	180	YP_723752	8501	ZP_00514985	115/169	7120	BAB75507	120/173
Flavodoxin	182	YP_722232	8501	ZP_00515759	130/174	6803	BAA17947	70/170
Flavodoxin (*isiB*)	171	YP_721410	7120	BAB74104	103/143		N/A	
isiA	344	YP_721411	7120	BAB75700	261/341		N/A	
12 - Ferric uptake regulator (*fur*)	170	YP_721684	7806[vii]	AAT44865	125/172	7120	BAB78057	115/150
13 - Ferric uptake regulator (*fur*)	131	YP_721679	7601[viii]	AAT41916	78/124	7120	BAB74172	78/126
14 - Ferric uptake regulator (*fur*)	174	YP_722978	101[ix]	YP_721684	105/161	7120	BAB78057	89/150

Abreviations: N/A not applicable; N/D none determined; [i] if different than nearest ortholog; [ii]6803=*Synechocystis* PCC6803; [iii]8501 = *Crocosphaera watsonii* WH8501; [iv]BP-1 = *Thermosynechococcus elongatus* BP-1; [v]29413= *Anabaena variabilis* ATCC29413; [vi]8106 = Lyngbya sp. PCC 8106; [vii]7806 = *Microcystis aeruginosa* PCC7806; [viii]7601 = *Calothrix* PCC7601;[ix]101 = *Trichodesmium erythraeum* IMS101; [x]7120 = *Anabaena* PCC7120

Table 2 % Identity for Sequences

***idiA* (520 bp out of a 1050 bp gene)**

Taxa	*T. erythraeum**	*T.thiebautii*	*T. spiralis*	*T. tenue***
T. erythraeum	100			
T. thiebautii	93	100		
T. spiralis	94	98	100	
T. tenue	94	99	99	100

***feoB* (995 bp out of a 1821 bp gene)**

Taxa	*T. erythraeum*	*T.thiebautii*	*T. spiralis*	*T. tenue*
T. erythraeum	100			
T. thiebautii	95	100		
T. spiralis	95	98	100	
T. tenue	96	98	98	100

***isiB* (234 bp out of a 516 bp gene)**

Taxa	*T. erythraeum*	*T.thiebautii*	*T. spiralis*	*T. tenue*
T. erythraeum	100			
T. thiebautii	93	100		
T. spiralis	94	99	100	
T. tenue	94	99	100	100

* *T. erythraeum* includes IMS101 and GBRTRLI101 strains

** *T. tenue* includes Z-1 and H9-4 strains

Table 3. Primers used in Sequencing (External) and Gene Expression Experiments (Internal and QPCR)

Primer Name	5' Primer	3' Primer	Target Size (BP)
idiA External	AATCTCTATTCTTCCCGTCAC	GCTTCTGGACTAACTAAATGTTC	770
idiA Internal	TCCAGCTAACCTCCGC	AATGCCAGCCGCAAC	312
isiB	CAAGTCCCGAAGATTTTGATGG	CATAACCCTCTGTAGACCAAGACCC	264
isiB QPCR (Ten)	AAGTGACTGGGCTGGTTTC	CAATAGTAGTACCTCCTTTCTCAG	167
isiB QPCR (Tery)	AAAGTGACTGGAGTGGTTTC	GTAGTACCTCCAAGCCCA	163
feoB External	ATTTCTCTGAAGGTTCTTAAATG	TTATCAACTTAAAGCCAAAGCTC	1983
feoB Alternate	TGGAATTATTAGATGAGCTTTTCA	GCTCCTTGGTAAAAAATGAAAC	1917
feoB Internal	TCCCAACCTACTAATGCCACA	CTTCGGAAAAACCATTGAAA	217
rnpB QPCR (Ten)	GAATCTATGAACGCAACGGAAC	ACCAGCAGTGTCGTGAGG	102
rnpB QPCR (Tery)	ACCAACCATTGTTCCTTCG	CAAGCCTGCTGGATAACG	199
rnpB degenerate (Vioque, 1997)	GRTYGAGGAAAGTCCGGRCT	RTAAGCCGGRTTCTGT	~324

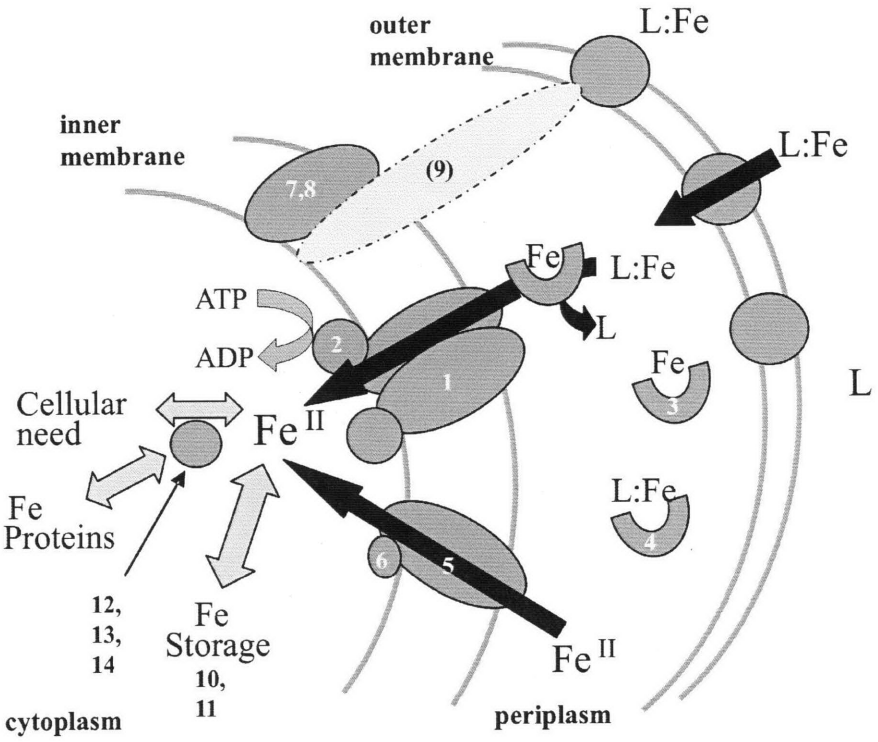

Figure 1: Schematic of iron (Fe) acquisition and cellular fate of Fe for *T. erythraeum* IMS101 as predicted from genome analysis. The numbers correspond to the proteins described in Table 1. Parentheses indicate that the corresponding protein in *T. erythraeum* IMS101 is divergent from experimentally described proteins. L represents Fe-binding ligands.

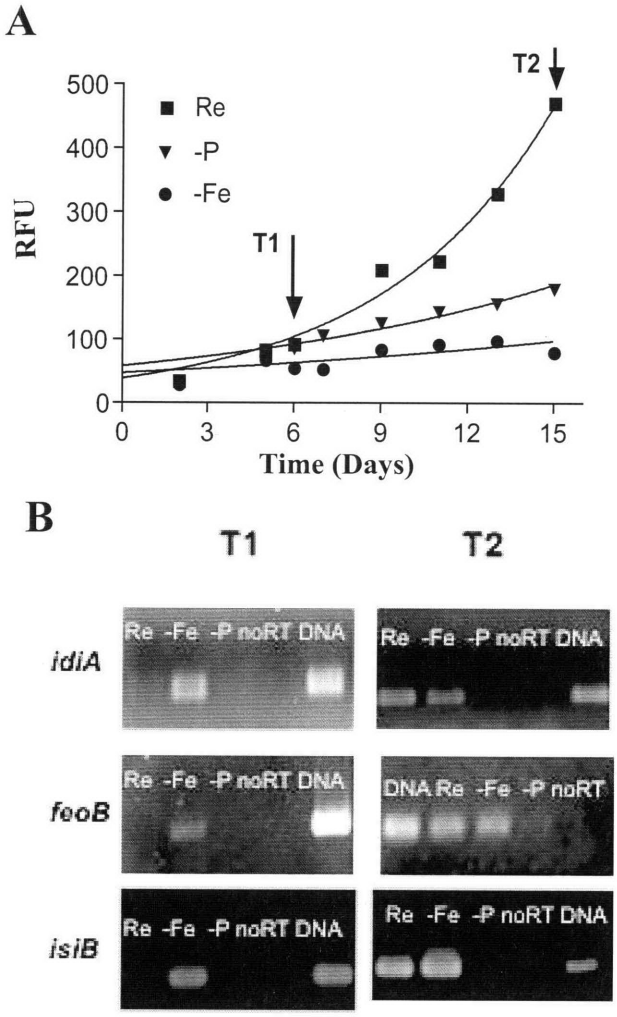

Fig. 2. Representative growth curve of nutrient limitation culture experiment with *T. erythraeum* IMS101 (A). RFU stands for relative fluorescence units and measures the orange fluorescence of the cultures. Arrows T1 and T2 indicate where on the growth curve samples were removed for gene expression analysis. (B) Agarose gel images showing expression of *idiA*, *feoB* and *isiB* as determined by RT-PCR at T1 and T2 in replete culture (Re), Fe limited culture (-Fe), P limited culture (-P). Genomic IMS101 DNA was used as a positive control (DNA). Absence of genomic DNA contamination in RNA preparation was confirmed by completing the reverse transcription reaction without reverse transcriptase (noRT) followed by RT-PCR.

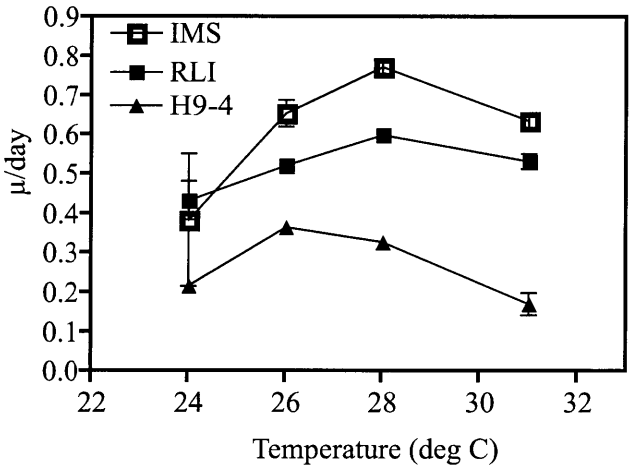

Fig. 3. Growth rate response of different *Trichodesmium* species grown at varying temperatures.

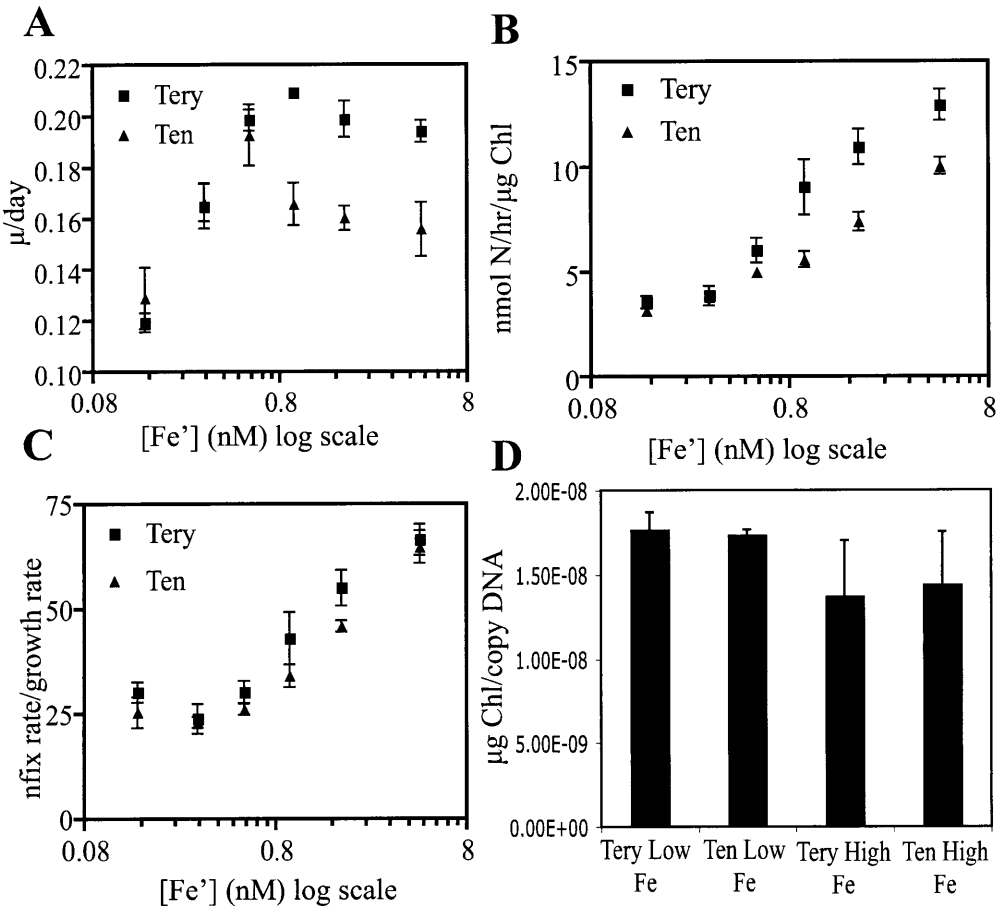

Fig. 4. Physiological responses to Fe limitation in two clades of *Trichodesmium*. (A) Growth rates of representatives of both clades of *Trichodesmium* grown on varying amounts of [Fe']. The [Fe'] values were 0.15 nM, 0.31 nM, 0.54 nM, 0.94 nM, 1.8 nM and 4.5 nM. (B) Nitrogen fixation rates determined by acetylene reduction normalized to μg chl of culture from representatives of both clades of *Trichodesmium* grown in the same media. (C) The relationship of nitrogen fixation rates defined above with culture growth rates. (D) The ratio of chl/DNA in samples from low and high Fe treatments in both clades of *Trichodesmium*. In all graphs error bars represent standard error of biological replicates.

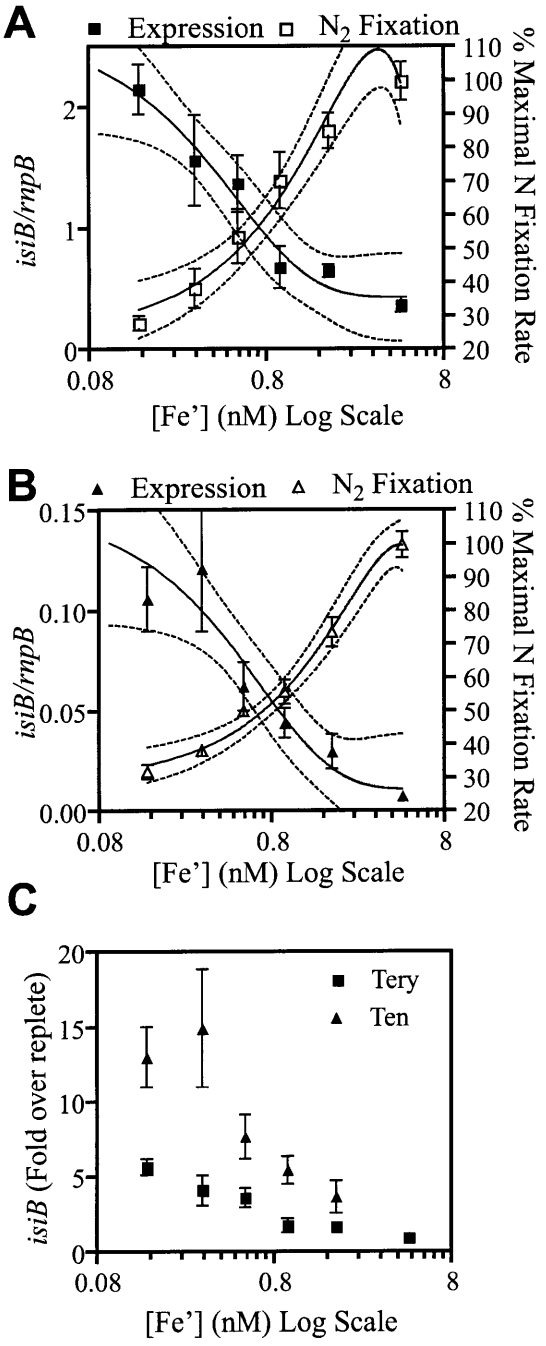

Figure. 5. Changes in *isiB* gene expression and N_2 fixation in response to growth on different Fe levels in representatives of both phylogenetic clades of *Trichodesmium*. In both panels A and B, gene expression is shown as a ratio of *isiB* gene transcripts divided by the number of *rnpB* gene transcripts in the closed symbols and % maximal N_2 fixation rates are shown in the open symbols. (A) The relationship between *isiB* expression and N_2 fixation rates in six *T. erythraeum* cultures (three IMS101 and three GBRTRLI101) grown at each [Fe'] treatment. (B) isiB expression and N_2 fixation rates measured in three *T. tenue* H9-4 cultures grown at each [Fe'] treatment. (C) The fold increase in expression of isiB relative to expression at replete [Fe'] of 4.5 nM is shown for both clades. In all three graphs, error bars are standard error based on biological replicates.

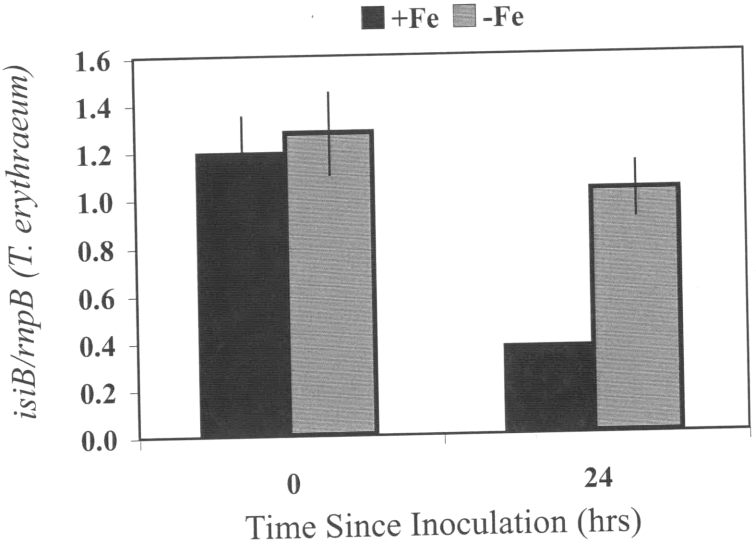

Figure 6. Response of *isiB* expression in *T. erythraeum* to Fe addition. *T. erythraeum isiB/rnpB* is shown for cultures that had been growing under Fe limited conditions immediately following inoculation into +Fe or -Fe medium and 24 hours later. Error bars respresent standard error of duplicate biological replicates for the -Fe treatment and quadruple biological replicates for the +Fe treatment.

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CHAPTER 4: A MOLECULAR DEMONSTRATION OF *TRICHODESMIUM*-SPECIFIC FE LIMITATION OF N_2 FIXATION IN THE PACIFIC AND ATLANTIC OCEANS

4.1 ABSTRACT

Diazotrophic cyanobacteria (*i.e.*, *Trichodesmium*) are important contributors to global carbon and nitrogen cycles. Understanding the environmental factors that control their growth and ability to fix N_2 is key to developing accurate global ecosystem models to predict the effects of climate change. Iron (Fe) has been shown to be an important element for limiting the growth and N_2 fixation of *Trichodesmium* in the laboratory, but there has been limited work assessing where this factor limits *Trichodesmium in situ*. We surveyed *Trichodesmium* populations in both the Atlantic and Pacific Oceans for Fe limitation using a recently developed molecular method involving quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) of the *isiB* gene, encoding flavodoxin, in conjunction with measurements of dissolved Fe and PO_4. Fe limitation of *Trichodesmium* was widespread in the Pacific Ocean and minimal to nonexistent in the Atlantic Ocean. We found an inverse correlation between expression of the *isiB* gene and Fe/PO_4 that allowed for the calculation of a critical Fe/PO_4 value associated with Fe limitation of N_2 fixation by *Trichodesmium*, which closely resembled values used in predictive global ecosystem models (where Fe speciation was ignored). In addition to validating previous models with quantitative evidence of Fe limitation of N_2 fixation in the field, the data presented suggests that the majority of dissolved Fe in the open ocean is available to *Trichodesmium* regardless of speciation.

4.2 INTRODUCTION

Diazotrophic cyanobacteria (e.g. *Trichodesmium*) are not only important contributors to primary production in the upper ocean, but they are also important in the nitrogen (N) cycle by providing "new" N to the system through N_2 fixation (Capone et al., 1997; Montoya et al., 2004). In certain regions of the ocean, it is believed that

diazotrophy accounts for up to 50% of the new N that enters the system (Karl et al., 2002). The N from N_2 fixation is understood to be critical to the carbon (C) and N cycles both regionally (Zehr et al., 2001) and globally (Capone et al., 1997; Gruber and Sarmiento, 1997). It is also thought that changes in N_2 fixation rates can potentially influence CO_2 sequestration over geologic time-scales (Capone et al., 1997; Falkowski, 1997; Gruber and Sarmiento, 1997).

For many decades, *Trichodesmium* was believed to be the only free-living cyanobacterial N_2 fixer in the open ocean (Mulholland, 2007). While recent work has revealed that unicellular cyanobacteria capable of fixing N can be quite prevalent in the ocean (Zehr et al., 2001; Montoya et al., 2004; Grabowski et al., 2008), *Trichodesmium* is still believed to be a major contributor to marine N_2 fixation (LaRoche and Breitbarth, 2005). Despite the importance of *Trichodesmium*, we still have very limited information about the factors that control its N_2 fixation rate and distribution, and how N fixed by *Trichodesmium* transfers through the food web (Mulholland, 2007). Culture work has shown that physical factors such as light (Breitbarth et al., 2008) and temperature (Breitbarth et al., 2007) are important for *Trichodesmium* growth and N_2 fixation rates. In addition to these physical factors, culture work, field correlations and qualitative molecular assays have shown that *Trichodesmium* N_2 fixation can be Fe-limited (Berman-Frank et al., 2001; Webb et al., 2001; Fu and Bell, 2003; Kustka et al., 2003b; Berman-Frank et al., 2007; Shi et al., 2007; Kupper et al., 2008), phosphorus (P) limited (Hynes, In Press; Sanudo-Wilhelmy et al., 2001; Dyhrman et al., 2002; Fu et al., 2005; Sohm et al., 2008) or Fe-P co-limited (Mills et al., 2004). What we lack is a quantitative assessment of how these factors might be limiting *Trichodesmium* N_2 fixation and a better understanding of where in the ocean these chemical factors are important.

In the modern ocean, Fe is not as biologically accessible as it was when cyanobacteria evolved because the thermodynamically stable redox state, Fe(III), has both a low solubility (Liu and Millero, 2002) and is strongly complexed by organic ligands that may render it unavailable to phytoplankton (Rue and Bruland, 1995; Rue and Bruland, 1997). Consequently, Fe is thought to limit primary production in large areas of

the oceans, leading to the development of high nutrient low chlorophyll (HNLC) regions (Martin and Fitzwater, 1988; Martin et al., 1991; Coale et al., 1996). Diazotrophic cyanobacteria have a high cellular Fe-requirement associated with the biochemistry of N_2 fixation that is hypothesized to be the result of their originating in an Fe-rich anoxic ocean (Kustka et al., 2003a; Kustka et al., 2003b). Biogeochemical models that incorporate measurements of global ocean circulation, dust transport and empirically determined physiological data for Fe limitation of phytoplankton have predicted that Fe limits diazotrophs, like *Trichodesmium,* in large areas of the ocean (Moore et al., 2004; Moore and Doney, 2007). Using the information that Fe and PO_4 can both limit N_2 fixation, a new plan to mitigate rising CO_2 proposes using inputs of Fe and PO_4 from the deep ocean to stimulate N_2 fixation (Karl and Letelier, 2008). Even as these CO_2 mitigation strategies are proposed, we still lack an understanding of how *Trichodesmium* acquires Fe, what forms of Fe are bioavailable, how they respond to Fe deprivation and validation of where they are experiencing Fe limitation, findings that are important steps to predicting potential feedbacks on climate change.

Bottle enrichments, fluorescent staining, and chemical quota correlations have been used to make predictions of the factors limiting diazotrophic activity in the oceans (Sanudo-Wilhelmy et al., 2001; Dyhrman et al., 2002; Kustka et al., 2003b; Mills et al., 2004). These studies suggest that the two primary elements limiting *Trichodesmium* N_2 fixation in the oceans are Fe and P. For example, in the Sargasso Sea *Trichodesmium* is thought to be predominantly PO_4 stressed (Sanudo-Wilhelmy et al., 2001; Dyhrman et al., 2002; Sohm and Capone, 2006), findings that agree well with models of the region (Moore et al., 2004; Coles and Hood, 2007). In the equatorial North Atlantic Ocean, models disagree whether PO_4 alone (Moore et al., 2004) or Fe-PO_4 co-limitation (Coles and Hood, 2007) are controlling *Trichodesmium* N_2 fixation. Field assessments of *Trichodesmium* through this region also report a mixture of PO_4 limitation (Sanudo-Wilhelmy et al., 2001; Sohm and Capone, 2006; Sohm et al., 2008) or Fe-PO_4 co-limitation of N_2 fixation (Mills et al., 2004). Similar uncertainty exists in the Western Pacific Ocean (including the Western Pacific Warm Pool) as this region of the ocean has

been understudied with respect to diazotroph assemblages and productivity (Campbell et al., 2005; LaRoche and Breitbarth, 2005). Pigment and flow-cytometric data suggests that cyanobacteria are important phytoplankton throughout the region (Blanchot et al., 1997; Neveux et al., 2006; Matsumoto and Ando, 2009) and there are reports of periodic blooms of *Trichodesmium* that can be seen via satellite (Dupouy et al., 1988). Model data suggests that Fe is likely to be the most important limiting nutrient for diazotrophs in the region (Moore et al., 2004), though local to New Caledonia it appears that PO_4 limitation may be an important factor to consider as well (Van Den Broeck et al., 2004; Moutin et al., 2005; Rodier and Le Borgne, 2008). Clearly we need more empirical quantitative data to determine which of these factors is controlling N_2 fixation by *Trichodesmium* in the field.

To get a better understanding of *in situ* Fe limitation of *Trichodesmium* N_2 fixation, we used a recently developed calibrated molecular method looking at expression of the gene encoding flavodoxin, *isiB*, (Chappell and Webb, submitted) to assess Fe limitation in field samples from around the globe in conjunction with measurements of PO_4 and Fe. In addition to looking at locations where we expected to find Fe limitation, we also explored areas presumed to be PO_4 limited and Fe-PO_4 co-limited. In general, we found a much higher prevalence of Fe limitation of *Trichodesmium* in the Pacific Ocean verses the Atlantic Ocean, with only one sample from the equatorial Atlantic showing evidence of *isiB* expression above the threshold expression level determined in the laboratory. When we compared the expression data from all the samples with the dissolved [Fe] and [PO_4] values from those stations, we found a threshold value of [Fe] above which there is no evidence of *Trichodesmium* Fe limitation similar to the value of [Fe'] associated with Fe limitation in laboratory cultures, indicating that most of the dissolved Fe in the open ocean is available to *Trichodesmium* regardless of ligand speciation. The relationship we determined between *isiB* expression and the [Fe]/[PO_4] ratio, enabled us to calculate an *in situ* critical [Fe]/[PO_4] ratio for Fe limitation, providing vetted values that will be useful for improved models.

4.3 METHODS

4.3.1 Dissolved Fe Sampling Procedure. Sampling took place on three cruises, one aboard the R/V Oceanus (cruise OC399-4) between March 22, 2004 and March 30, 2004 (Figure 1A, Stations 4-12), one aboard the R/V Seward Johnson (cruise SJ0609) between July 12, 2006 and July 24, 2006 (Figure 1A, Stations 13-21) and one aboard the R/V Kilo Moana (cruise KM0701) as part of the Western Pacific Warm Pool cruise between January 12, 2007 and February 9, 2007 (Figure 1B). Samples for dissolved Fe and nutrients were collected either using acid cleaned 10-L Teflon-coated Go-Flo bottles (General Oceanics) deployed directly on a Kevlar line (OC399-4) or acid cleaned 5L Teflon-coated exterior spring Niskin bottles (Ocean Test Equipment) deployed either directly on a Kevlar line (SJ0609) or mounted on a powder-coated rosette that was deployed on a Kevlar line (KM0701). After recovery, the bottles were transferred to a trace metal clean "bubble" kept at positive pressure using HEPA filtered air flow in the laboratory of the ship (KM0701) or to a trace metal clean van also supplied with HEPA filtered air on the deck of the ship (OC399-4 and SJ0609). The headspace of each bottle was pressurized with 0.2 μm filtered ultra high purity (UHP) nitrogen pushing the water through a 142 mm 0.4 μm acid-cleaned polycarbonate filter held in a polycarbonate filter sandwich (Geotech Environmental Equipment, Inc.). Water for dissolved Fe analysis was collected in acid-cleaned 250 ml low-density polyethylene (LDPE) bottles and acidified to pH 1.7 with concentrated high purity HCl (Seastar). Water for nutrient analysis was collected in 10% HCl cleaned high-density polyethylene (HDPE) bottles (OC399-4) or 10% HCl cleaned polypropylene 50 ml tubes (SJ0609 and KM0701) and immediately frozen at -20°C for later analysis. Analysis of the PO_4 concentrations from the Sargasso Sea cruise was reported in (Jakuba et al., 2008). Samples from SJ0609 and KM0701 were sent to the College of Oceanic and Atmospheric Sciences, Oregon State University and dissolved inorganic phosphorus (DIP) was analyzed using a Technicon AutoAnalyzer II by J. Jennings with a detection level of 6 nmol L^{-1}.

4.3.2 Dissolved Fe Analysis. Fe in the seawater samples was determined using isotope dilution and magnesium hydroxide preconcentration followed by analysis using

inductively coupled mass spectrometry (Wu and Boyle, 1998; Saito and Schneider, 2006). Roughly 13.5 ml of sample (exact volume determined gravimetrically) was poured into a 15 ml polypropylene centrifuge tube (Globe Scientific Inc.) and equilibrated with a ^{57}Fe spike (~0.4 nM) overnight. The following day, the $Mg(OH)_2$ and metals were precipitated out of the sample by the addition of a small amount (~100 μl) of high-purity ammonium hydroxide (Seastar Chemicals Inc.). Following ammonium hydroxide addition, the tubes were left undisturbed for 90 s and inverted multiple times to fully mix them. After an additional 90 s, the tubes were centrifuged at 3000 x g for 3 minutes and the sample was decanted off. The tubes were then spun at 3000 x g for an additional 3 minutes forming a compact pellet, following which the remaining liquid was shaken off. The sample pellets were kept dry until the day of analysis (from a day to a week). On the morning of analysis, pellets were resuspended in 1-2 ml 0.8 N Nitric Acid (Seastar). Samples were analyzed on a Thermo-Finnigan Element 2 (E2) inductively coupled mass spectrometer (ICP-MS) in medium resolution mode. A procedural blank was determined by processing 1 ml of low Fe seawater (which provides a negligible amount of Fe) and calculating its Fe value as though it were a 13.5 ml sample.

4.3.3 Collection of *Trichodesmium* Samples. A 130 μm-phytoplankton net (Sea-Gear Corporation, Florida) was towed using a 30 m line at the surface for 10-20 minutes. Immediately following the return to the surface, the contents of the tow were taken into the air-conditioned laboratory aboard the ship so that the *Trichodesmium* colonies could be separated from the other plankton using polypropylene bulb transfer pipettes. Colonies were transferred from the bulk solution into clean 0.4 μm filtered microwave-sterilized seawater, then they were filtered onto 5 μm polycarbonate filters and stored in liquid N_2 until RNA processing and analysis. On OC399-4, 200 ml of the bulk net tow was filtered onto 5 μm polycarbonate filters and preserved in liquid N_2 without separation and rinsing.

4.3.4 Nitrogen Fixation Measurements. We measured N_2 fixation rates on samples from two stations on cruise OC399-4 and five stations on KM0701. Net tows to collect samples were performed immediately before time zero of each N_2 fixation

incubation experiment, which were targeted for 1100, 1200 and 1300 hrs local time. 10-20 colonies were placed in 30 ml of filtered seawater in 75 ml Nalgene® polycarbonate bottles (Nalge Nunc International Corporation, Rochester, NY). N_2 fixation rates were measured using the acetylene reduction assay (Capone, 1993) using a Shimadzu GC-8A gas chromatograph with ethylene peaks integrated by a Shimadzu CR8A Chromatopac. N_2 fixation rate measurements were based on a linear regression of ethylene concentrations immediately following acetylene addition and measurements made at one and two hours after addition. Two to three replicate bottles were used for each incubation experiment and two replicate samplings of the headspace of each bottle was used for each time point. Results were normalized to Chl *a* measured using standard techniques (Herbland et al., 1985).

4.3.5 RNA Extraction and cDNA Synthesis. RNA was extracted using the Ribo-Pure^{TM}-Bacteria kit (Ambion Inc., Austin, TX) including the optional DNase-I treatment. Total RNA extracts were quantified using a NanoDrop® ND-1000 Full Spectrum UV/Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Normalized quantities of total RNA extracts were then converted into cDNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). As a negative control for later RT-PCR reactions, normalized total RNA was also put through the iScript cDNA synthesis without the addition of the reverse transcriptase enzyme (later referred to as noRT).

4.3.6 Quantitative PCR Analysis of Gene Expression. Separate qPCR primer sets for the *Trichodesmium erythraeum* clade (Tery) and the *Trichodesmium tenue* clade (Ten) designed and tested previously (Chappell and Webb, submitted) are relisted here (Table 1). qPCR primers were designed for our target Fe stress response gene, *isiB*, and a RNA normalization control gene, *rnpB*. Because the majority of samples did not have a significant amount of *T. erythraeum* clade RNA according to *rnpB* analysis of the cDNA (Table 2), we only report expression results for the *T. tenue* clade. % Tery = [(# copies of Tery clade *rnpB*)/(# copies of Tery + Ten clade *rnpB*)] × 100. Relative expression of *isiB* verses *rnpB* was determined using absolute quantification of each gene and dividing the

isiB gene copy number by the *rnpB* gene copy number determined for each cDNA sample (Applied Biosystems User Bulletin #2: http://dna-9.int-med.uiowa.edu/RealtimePCRdocs/Compar_Anal_Bulletin2.pdf) (Larionov et al., 2005). The standards used for absolute quantification were cloned PCR products prepared as described in chapter two of this thesis and (Zinser et al., 2006) using the TOPO TA Cloning® Kit for Sequencing (Invitrogen Corporation, Carlsbad, CA). qPCR reactions were done on a 7500 Fast Real-Time PCR System (Applied Biosystems Inc., Foster City, CA) using *Power*SYBR® Green PCR Master Mix (Applied Biosystems Inc., Foster City, CA) at 1x concentration in a 20 µl reaction with a final cDNA concentration of 1-2 nM and a final primer concentration of 200nM. Cycler conditions were 50°C for 2 min, 95°C for 10 min; 40 cycles of 95°C for 15 sec, 55°C for 1 min with fluorescence being read at 55°C followed by dissociation curve analysis from 60°C to 95°C.

4.3.7 DNA qPCR Test for Specificity of Primers. Where the biomass on the sample was high enough that the entire filter was not used for RNA extraction, DNA was extracted from a portion of the filter used for RNA extraction (OC399-4). Otherwise, DNA was extracted from an alternate bulk filter collected at the same station (SJ0609 and KM0701). DNA was extracted using the Mo Bio PowerPlant™ DNA extraction kit (Mo Bio Laboratories, Inc. Carlsbad, CA) following manufacturers guidelines including the optional DNA Clean-Up protocol. Following dilution of the DNA samples to ~0.5 ng/µl, 2 µl of DNA was run in triplicate 20 µl qPCR reactions with both sets of primers (six reactions total) to ensure that the ratio of *isiB* to *rnpB* in the DNA was the same as that found for cultured representatives (*i.e.*, 1:1). This control was used to ensure that our primers were equally efficient with field populations of *Trichodesmium* as they had been with laboratory cultures.

4.4 Results

4.4.1 Sampling Locations. Figure 1A shows the cruise track and station locations where *Trichodesmium* colonies were present for the two Atlantic Ocean cruises, OC399-4 (stations 4-12) and SJ0609 (stations 13-21). The station numbers for OC399-4

are a subset of the stations referred to in Jakuba et al (2008) and retain the same numbering scheme. SJ0609 was the second half of a two-leg transit across the equatorial Atlantic Ocean and retains the numbering scheme of the entire cruise. The cruise track for the Western Pacific Warm Pool cruise is shown in Figure 1B. The station numbering is the same as that of Hynes et al (in press) and Chapter 2 of this thesis. In all cases, the only numbered stations are the ones where expression data is reported. With the exception of OC399-4, where there was a large portion of the cruise that was out of the temperature range for *Trichodesmium* that has been completely left off the map, all stations from each cruise are marked along the cruise-track by a point even if they are not numbered.

4.4.2 Fe, PO_4 and Expression Data. The station location, total dissolved [Fe], [PO_4], [Fe]/[PO_4], the expression ratio *isiB/rnpB* and percent *T. erythraeum* for all stations where detectable levels of *Trichodesmium* were found are listed in Table 2. [Fe] and [PO_4] values are reported for the surface sample from each station, which was taken at a depth of 10 m for OC399-4 and 15 m for the other two cruises. The [PO_4] values from OC399-4 are reprinted from Jakuba et al (Jakuba et al., 2008). A subset of these PO_4 values were also reported in Dyhrman et al (Dyhrman et al., 2006). The [PO_4] values from KM0701 are the same as those graphed in Hynes et al (in press). OC399-4 stations are abbreviated with S for Sargasso Sea, SJ0609 stations are abbreviated with E for the Equatorial Atlantic Ocean and KM0701 stations are abbreviated with WP for the Western Pacific Ocean. The [Fe] and [PO_4] values are also shown in two bar graphs, one for the two Atlantic cruises, OC399-4 (S) and SJ0609 (E), (Figure 2A), and one for the Pacific cruise, KM0701, (Figure 2B). The cruise where [Fe] was most variable was in the Equatorial Atlantic Ocean. SJ0609 had the station with the highest [Fe] value of 1.89 nM on the western side of the basin at Station E21 where surface salinity measurements indicate that we were sampling in the Amazon River plume. At E21, the surface salinity measured by the CTD was 32.5, which rose to 36 by a depth of 50 m. With the exception of Station E20, which also had a small lens of low salinity water rising from 33.0 to 35.8 by a depth of 17 m, surface salinities for the remainder of the transect were between 35.5

and 36.1. SJ0609 also had one of the two stations with the lowest [Fe] of 0.09 nM, which was by the equator. The Western Pacific Ocean also had a range of [Fe], with a high value of 0.95 nM close to the islands approaching New Caledonia, but otherwise low [Fe] ~ 0.2 nM. The Sargasso Sea had consistently high [Fe] values ranging from 0.82 nM to 1.17 nM. The $[PO_4]$ values from the three cruises spanned a very large range, from below 1.4 nM in the Sargasso Sea to 324 nM in the Western Pacific, which resulted in a range of $\log_{10}$ $[Fe]/[PO_4]$ from -3.5 in the Western Pacific to -0.13 in the Sargasso Sea.

Analysis of the copy numbers of *rnpB* from the two clades in each cDNA sample (clade specific *rnpB*/total *Trichodesmium rnpB*), showed that the Ten clade dominated the cDNA in our samples (Table 2). As Tery cDNA was rarely detectable, we only report *isiB* expression data for the Ten clade. The Ten clade *isiB* expression, which is listed as $\log_{10}$ (*isiB/rnpB*), also showed a large range from -3.4 in the Sargasso Sea to 0.33 in the Western Pacific. Many of the stations from the Western Pacific and one station from the Equatorial Atlantic had expression values above the value associated with a 50% reduction in N_2 fixation in cultured *T. tenue* of *isiB/rnpB* = 0.062 +/- 0.017 or $\log_{10}$ (*isiB/rnpB*) = -1.24 +/- 0.1 (Chappell and Webb, submitted).

We plotted total dissolved [Fe] verses $\log_{10}$ (*isiB/rnpB*) (Figure 3A) and $\log_{10}$ ($[Fe]/[PO_4]$) verses $\log_{10}$ (*isiB/rnpB*) (Figure 3B). In each plot, the dashed line at $\log_{10}$ (*isiB/rnpB*) = -1.2 shows the critical value for $\log_{10}$ (*isiB/rnpB*) associated with a 50 % reduction in N_2 fixation rates for the Ten clade (Chappell and Webb, submitted). As shown in Figure 3A, the highest [Fe] value associated with expression above this threshold is 0.63 nM +/- 0.02 nM. A linear relationship was defined by plotting $\log_{10}$ ($[Fe]/[PO_4]$) verses $\log_{10}$ (*isiB/rnpB*) (Figure 3B). The equation for this line is $\log_{10}$ (*isiB/rnpB*) = (-0.67 +/- 0.13) × $\log_{10}$ ($[Fe]/[PO_4]$) + (-2.7 +/- 0.27), which has an R^2 value of 0.57. According to this equation, the critical $[Fe]/[PO_4]$ value associated with a 50 % reduction in N_2 fixation by *Trichodesmium* in the field is 0.005 +/- 0.007 mol/mol or ≤ 0.012 mol/mol.

We observed a linear relationship between $\log_{10}$ (*isiB/rnpB*) and N_2 fixation rates from the subset of stations on KM0701 and OC399-4 where both parameters were

measured (Figure 4). There was only one station from the cruise in the Sargasso Sea where we had measurable *isiB* expression and measured N_2 fixation rates. There was a second station with *isiB* expression that was below detection, which we gave the value for $\log_{10}$ (*isiB/rnpB*) = -3.42, which appears to be the basal expression of *isiB* in the field. Excluding the data from the Sargasso Sea (SS) does not considerably alter the slope of the line associated with this relationship and only the line inclusive of the SS data is plotted (Figure 4). The linear relationship with the SS stations included is: nmol N fixed/hr/μg chl = (-1.73 +/- 0.41) × ($\log_{10}$ (*isiB/rnpB*)) + (1.41 +/- 0.77), which has an R^2 value of 0.77. The linear relationship with just the samples from KM0701 is: nmol N fixed/hr/μg chl = (-2.00 +/- 0.79) × ($\log_{10}$ (*isiB/rnpB*)) + (1.35 +/- 0.95), which has an R^2 value of 0.68.

4.5 Discussion

A number of studies have established the importance of Fe limitation to *Trichodesmium* N_2 fixation in the laboratory (Chappell and Webb, submitted; Berman-Frank et al., 2001; Webb et al., 2001; Fu and Bell, 2003; Kustka et al., 2003a; Kustka et al., 2003b; Berman-Frank et al., 2007; Shi et al., 2007; Kupper et al., 2008). Recently these efforts have focused on developing molecular methods that can be used to evaluate Fe limitation at the cellular level (Chappell and Webb, submitted; Webb et al., 2001; Shi et al., 2007). The information available on the factors (i.e., Fe) controlling *Trichodesmium* N_2 fixation and growth has proved invaluable for modeling regions of the ocean (Moore et al., 2004; Coles and Hood, 2007; Moore and Doney, 2007). Though models can be useful tools, empirical verification is required to prove where Fe is important in controlling *Trichodesmium* N_2 fixation and determine what parameter of Fe (*i.e.,* total, free, inorganically-bound Fe) is important to measure for predicting where Fe limitation might be occurring. The development of a calibrated molecular method to evaluate Fe limitation of N_2 fixation in *Trichodesmium* spp. (Chappell and Webb, submitted) 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, PO_4 limited and Fe-PO_4 co-limited.

4.5.1 Ten clade predominance in open ocean populations of *Trichodesmium*. Representatives from the Ten clade were the principal component of *Trichodesmium* cDNA at most stations (Table 2). These results are consistent with reports based on morphology from previous field studies that *Trichodesmium thiebautii*, which is a representative from the Ten clade, is the dominant *Trichodesmium* in the open ocean (Carpenter and Price, 1977; Sohm et al., 2008). While our results indicate that cDNA was mostly from the Ten clade, this does not necessarily mean that representatives from the Tery clade were not present at some or even many of the stations. We looked at cDNA, so there is the possibility that there was Tery clade DNA present. If the Tery cells were dormant or growing slower for some reason, it would result in a much smaller amount of Tery clade cDNA, which might have been below the detection limit of our assay. Also, we were focusing on picked colonies not free trichomes. There is the possibility that we might have missed some Tery clade cDNA if the organisms were living as free trichomes and not colonies. While we may have missed some of the diversity of the *Trichodesmium* in the field, the net tow allowed us to get a concentrated sample of *Trichodesmium* to ensure that we had enough biomass for our downstream analyses. Given the dominance of the Ten clade and that Tery clade cDNA was not detected in most of our samples, the remainder of the paper deals only with Ten clade.

4.5.2 Basin-wide differences in Fe limitation of *Trichodesmium*. Most of the stations exhibiting Fe limitation were in the Pacific Ocean (Table 2, Figure 3). Low *isiB* expression in samples from the Sargasso Sea cruise is understandable, given the very low values of [PO_4] combined with the very high values of [Fe] measured (Table 2, Figure 2A). These results agree with previous work suggesting that *Trichodesmium* in this region are PO_4 stressed (Sanudo-Wilhelmy et al., 2001; Dyhrman et al., 2002; Sohm and Capone, 2006). The only station in the North Atlantic where we found *Trichodesmium isiB* expression levels indicative of Fe limitation of N_2 fixation was EA18, which was a station close to the equator where [Fe] was low compared to other parts of the cruise

(Table 2, Figure 2A). The high [PO_4] and low [Fe] at this station could have been the result of equatorial upwelling having brought nutrient rich water to the surface ocean, which resulted in a draw down of surface [Fe] by biological activity. If upwelling is what caused high [PO_4] and low [Fe] at stations 16, 17 and 18 (Figure 2A), it may not have been recent, as the surface temperatures were not low (all three stations had near surface temperatures between 27 ° C and 28.5 ° C), which is consistent with the rest of the cruise and does not suggest active upwelling of colder deep water. The data from this cruise is in good agreement with previous work suggesting that the equatorial North Atlantic is predominantly PO_4 stressed (Sanudo-Wilhelmy et al., 2001; Sanudo-Wilhelmy et al., 2001; Dyhrman et al., 2002; Dyhrman et al., 2002; Sohm and Capone, 2006; Sohm and Capone, 2006; Webb et al., 2007; Sohm et al., 2008; Sohm et al., 2008). The evidence we have showing Fe limitation near the equator supports the hypothesis that parts of the North Atlantic have the potential to shift between PO_4 and Fe limitation (Mills et al., 2004; Coles and Hood, 2007). Further study in this region using a combination of the technique used in this study and a complimentary one for PO_4 stress of *Trichodesmium* could prove to be a useful way to help determine what drives this shift. Even without such a metric, the data we have at hand shows that Fe limitation of N_2 fixation by *Trichodesmium* can occur in the North Atlantic Ocean.

The level of *isiB* expression we measured in the Pacific Ocean was generally higher than that of the Atlantic and above the Fe limitation threshold value for the Ten clade (Figure 3), indicating that the Ten clade of *Trichodesmium* was experiencing Fe limitation over much of that cruise. This is in agreement with model data looking at what controls diazotroph growth in the oceans (Moore et al., 2004). There is some data suggesting that parts of the southwestern Pacific Ocean are PO_4 stressed (Moutin et al., 2005). This hypothesis is based on PO_4 turnover rates, low seasonal PO_4 concentrations and a study of bloom dynamics in the coastal region of New Caledonia (Van Den Broeck et al., 2004; Moutin et al., 2005; Rodier and Le Borgne, 2008). Our data do not completely rule out the possibility that PO_4 may be playing a role in this region, but suggest that this effect might be more local to the region immediately surrounding New

Caledonia and that in the region between New Caledonia and Australia Fe limitation or Fe-PO_4 co-limitation is occurring. The especially high Fe/P ratio of the terrigenous inputs to the coastal area immediately off of New Caledonia as a result of the lateritic soils would be a very good explanation of this phenomenon (Tenorio et al., 2005). During the WP cruise, there was some evidence of P stress using enzyme-labelled fluorescence (ELF), which targets alkaline phosphatase activity of *Trichodesmium* colonies though the labeling was minimal in comparison to other regions (Hynes, In Press). There is the possibility of Fe-PO_4 co-limitation of *Trichodesmium* at two of these stations, WP21 and WP26, where there was some evidence of ELF labeling and *isiB* expression was above the threshold indicative of Fe limitation of N_2 fixation. While at station WP17, close to the islands of Vanuatu, there was no evidence of Fe limitation, but there was ELF labeling of *Trichodesmium*. Taken together these data suggest that Fe and P both have the potential to be stressors of *Trichodesmium* in the Pacific Ocean. However, one problem in comparing results from the ELF assay with our *isiB* expression data is that the ELF assay is not quantitative. Thus, while it indicates that some portion of the *Trichodesmium* population in a given sample is P stressed, it cannot be used as a metric for P limitation in the same way as our *isiB* expression assay can be used as a metric for Fe limitation of N_2 fixation. While the non-quantitative nature of the ELF assay limits our ability to say anything conclusive about Fe-PO_4 co-limitation in the area, our *isiB* expression data for *Trichodesmium* clearly points to the importance of Fe in the region.

4.5.3 Relationship between *isiB* expression and dissolved Fe. Looking at the relationship between *isiB* expression and total dissolved Fe (Figure 3A), we see that there is no evidence of *isiB* expression above the Fe limitation threshold when [Fe] (total dissolved < 0.4 μm filtered Fe) is greater than 0.63 nM +/- 0.02 nM. This value is within error of the value of [Fe'] (total inorganically bound Fe) that was associated with Fe limitation in cultured populations of *Trichodesmium*, 0.7 nM +/- 0.1 nM (Chappell and Webb, submitted), without adjusting for ligand-specific Fe speciation effects. When Fe speciation effects are taken into account, estimates of [Fe'] are ~ 3 orders of magnitude

below [Fe] in the western Pacific transect (Chapter 2, this thesis). This implies that most if not all of the dissolved Fe in the surface oceans is available to *Trichodesmium*, even that bound to organic ligands. We do acknowledge certain concerns associated with extrapolating data from laboratory culture studies to the behavior of an organism in the field. For example, laboratory cultures are grown at nutrient and biomass levels much higher than those found in the open ocean. However, we took care to avoid a "blown buffer" scenario, which happens when culture biomass is high enough that the Fe needed is higher than that released by dissociation with EDTA and results in [Fe'] being lower than what would be calculated based on equilibrium dynamics (Saito et al., 2008). Thus, we believe that the EDTA buffer system we used kept the [Fe'] in the steady state at biologically relevant levels and as such it is reasonable to extrapolate to the field. Furthermore, these data are not completely unexpected as there is genomic evidence to support that *Trichodesmium* has the ability to take up siderophore-bound Fe using a TonB-ExbBD protein complex (Chappell and Webb, submitted) as well as field data showing that certain types of Fe:ligand complexes (*i.e.,* siderophores) are available to *Trichodesmium* colonies (Achilles et al., 2003). Our results cannot ascertain the mechanism by which this Fe is available to *Trichodesmium*. For example, we cannot rule out the possibility that the availability of this ligand-bound Fe is controlled by photochemical release of Fe from ligands (Barbeau et al., 2003; Barbeau, 2006) instead of cellular uptake of the ligand bound Fe. Additionally it is also possible that cell-surface reduction of ligand-bound Fe is involved (Maldonado and Price, 2001), though this mechanism has yet to be confirmed in *Trichodesmium*. Finally, it is also possible that interactions between *Trichodesmium* and the microbial consortium associated with its colonies could be facilitating the uptake of organically bound Fe. Regardless of the uncertainty associated with the cellular mechanism, our data show that total dissolved Fe measurements provide useful information on where *Trichodesmium* is Fe limited in the field.

4.5.4 Relationship between *isiB* expression and Fe/P. Even more striking than the relationship between *isiB* expression and [Fe], which is not as robust at both high Fe

and low Fe values, is the relationship between *isiB* expression and the [Fe]/[PO_4] ratio. The relationship between these two parameters is linear on a log-log plot throughout the entire range of samples (Figure 3B). Given the importance of both Fe and PO_4 as potential limiting nutrients for *Trichodesmium* (Berman-Frank et al., 2001; Fu and Bell, 2003; Kustka et al., 2003b; Fu et al., 2005), it is not surprising that the ratio of [Fe]/[PO_4] would play a role in determining whether or not *Trichodesmium* was Fe limited, PO_4 limited, or potentially co-limited. While we acknowledge that an R^2 value of 0.57 is not the strongest correlation, there are a variety of reasons to explain why cellular level Fe limitation of N_2 fixation could be offset from measured [Fe]/[PO_4] values. These discrepancies are part of the reason that a molecular diagnostic for Fe limitation in *Trichodesmium* is so important. We know that *Trichodesmium* has the capacity to store Fe (Castruita et al., 2006), thus a low Fe value or low Fe/PO_4 value may not be associated with a high expression value if the *Trichodesmium* has stored Fe and the extracellular Fe levels just dropped. A higher expression level than would be predicted based on Fe/PO_4 could be a result of a recent Fe deposition event that the organism has not had the ability to respond to by turning off the expression of the Fe stress genes. With those caveats aside, when we use the linear regression to determine the [Fe]/[PO_4] that is associated with Fe limitation in field populations of *Trichodesmium*, we determine that Fe limitation is likely to occur below a dissolved [Fe]/[PO_4] ratio of 0.005 +/- 0.007 mol/mol. We acknowledge that there is a large amount of error associated with this value, which propagates from our extrapolation of three different regressions: [Fe'] verses % maximal N_2 fixation rates (culture data), [Fe'] verses *isiB* expression (culture data) and *isiB* expression verses [Fe]/[PO_4] (field data). However, we feel that we can at least place an upper limit for the critical dissolved [Fe]/[PO_4] value of 0.012 mol/mol, a value that is only slightly lower than the critical Fe/PO_4 value associated with a transition between Fe and PO_4 limitation in diazotrophs in the Moore et al model of 2004. In the model, the limiting nutrient is determined by comparing the predicted concentration of various nutrients with the half-saturation constants for uptake of each nutrient. Whichever nutrient is in lowest concentration with respect to the half saturation constant is

determined to be the limiting nutrient at that point. The critical value for Fe/PO_4 is, in essence, the ratio of the half-saturation constants for Fe and PO_4, 0.0133 mol/mol (Moore et al., 2004), which is close enough to our calculated upper bound of the critical Fe/PO_4 ratio of 0.012 mol/mol that our data can be interpreted as an empirical validation of the model parameters.

4.5.5 Relationship between *isiB* expression and N_2 fixation rates. We found an inverse relationship between *isiB* expression and N_2 fixation rates in the field (Figure 4). This shows that *isiB* expression is a good marker for Fe limitation of *in situ* *Trichodesmium* N_2 fixation. We are unable to 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 cruise and our culture data shows that increases in *isiB* expression and decreases in N_2 fixation occur before *Trichodesmium* growth rates are significantly impacted (Chappell and Webb, submitted). However, the correlation between *isiB* expression and N_2 fixation rates in the field enables us to translate *isiB* expression values into N_2 fixation rates and is supported by previous data linking *isiB* expression and N_2 fixation rates in laboratory culture experiments (Chappell and Webb, submitted).

4.6 Conclusion

The data presented in this chapter validates model predictions and demonstrates that Fe is an important limiting nutrient for N_2 fixation of *Trichodesmium* in the Pacific Ocean while Fe limitation is minimal in the North Atlantic Ocean. The relationship between *isiB* expression and $[Fe]/[PO_4]$ we observed allows us to determine a critical $[Fe]/[PO_4]$ value that is associated with a shift to Fe limitation, which additionally validates the parameters used in model predictions. The close relationship between both the [Fe] value in the field and the [Fe'] value from the laboratory experiments associated with the onset of Fe limitation suggests that most if not all of the dissolved Fe in the open ocean is available to *Trichodesmium* regardless of whether or not it is bound to organic ligands. These results mark an improvement of our understanding of what form of Fe is

controlling *Trichodesmium* N_2 fixation in the open ocean as well as provide the first empirical data for where Fe limitation of *Trichodesmium* N_2 fixation is occurring.

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Table 1. Primers used in Gene Expression Experiments

Primer Name	5' Primer	3' Primer	Target Size (BP)
isiB QPCR (Ten)	AAGTGACTGGGCTGGTTTC	CAATAGTAGTACCTCCTTTCTCAG	167
isiB QPCR (Tery)	AAAGTGACTGGAGTGGTTTC	GTAGTACCTCCAAGCCCA	163
rnpB QPCR (Ten)	GAATCTATGAACGCAACGGAAC	ACCAGCAGTGTCGTGAGG	102
rnpB QPCR (Tery)	ACCAACCATTGTTCCTTCG	CAAGCCTGCTGGATAACG	199

Table 2. Near surface (10-15m) data from stations on all three cruises where *isiB* expression of *Trichodesmium* was measured. Standard deviation of triplicate technical replicates are given for [Fe] and and the *isiB/rnpB* ratio and [PO_4] from OC399-4 (S) and duplicate technical replicates for [PO_4] from SJ0609 (E) and KM0701 (WP). BDL = *isiB* expression was below detection level in the sample.

Station	Lat	Long	Fe (nM)	PO4 (μM)	Log (Fe/P)	Log (*isiB/rnpB*)	% Tery
S4	25.40	-61.15	1.17 +/- 0.04	0.0037 +/- 0.0049[ii]	-0.50 +/- 0.57	-1.96 +/- 0.02	0.05
S6	22.80	-58.93	1.04 +/- 0.08	<0.014[i]	-0.13 +/- 0.43	-2.30 +/- 0.02	0.03
S7	20.00	-57.00	1.15 +/-0.09	0.0136 +/- 0.0049[ii]	-1.07 +/- 0.16	-2.34 +/- 0.04	0.01
S8	20.00	-52.97	1.00 +/- 0.12	0.0017 +/- 0.0001[i]	-0.23 +/- 0.06	BDL	0.00
S9	20.00	-49.73	1.11 +/- 0.04	0.002 +/- 0.0004[i]	-0.26 +/- 0.09	-3.42 +/- 0.09	0.00
S10	20.00	-45.90	1.01 +/- 0.04	0.0058 +/- 0.0049[ii]	-0.76 +/- 0.37	BDL	0.00
S11	20.97	-46.90	0.94 +/- 0.06	0.0016 +/- 0.0004[i]	-0.23 +/- 0.11	-2.21 +/- 0.02	0.24
S12	23.52	-49.68	0.82 +/- 0.05	0.0033 +/- 0.0004[i]	-0.61 +/- 0.06	-2.58 +/- 0.09	0.00
E13	12.40	-27.20	0.74 +/- 0.12	0.045 +/- 0.000	-1.79 +/- 0.07	-1.62 +/- 0.02	0.00
E15	6.60	-30.80	0.61 +/- 0.02	0.016 +/- 0.002	-1.41 +/- 0.06	-1.99 +/- 0.02	0.00
E16	3.30	-32.90	0.09 +/- 0.00	0.037 +/- 0.002	-2.63 +/- 0.03	-1.40 +/- 0.04	0.00
E17	0.01	-34.90	0.14 +/- 0.01	0.076 +/- 0.004	-2.74 +/- 0.02	-1.31 +/- 0.02	0.00
E18	1.80	-38.50	0.10 +/- 0.00	0.031 +/- 0.009	-2.49 +/- 0.13	-1.01 +/- 0.02	0.00
E20	5.60	-45.60	0.67 +/- 0.03	0.035 +/- 0.010	-1.71 +/- 0.13	-1.80 +/- 0.04	0.52
E21	7.50	-49.20	1.89 +/- 0.03	0.068 +/- 0.009	-1.56 +/- 0.06	-1.54 +/- 0.04	0.14
WP10	0.37	-179.64	0.11 +/- 0.04	0.324 +/- 0.000[iii]	-3.48 +/- 0.16	0.33 +/- 0.05	58.81
WP14	-9.25	170.00	0.20 +/- 0.04	0.168 +/- 0.000[iii]	-2.93 +/- 0.09	-1.98 +/- 0.02	0.00
WP15	-12.58	169.86	0.11 +/- 0.03	0.133 +/- 0.002[iii]	-3.08 +/- 0.12	-1.72 +/- 0.01	0.05
WP16	-15.89	169.72	0.29 +/- 0.03	0.169 +/- 0.000[iii]	-2.76 +/- 0.05	-0.14 +/- 0.04	0.39
WP16a	-15.98	169.77	0.63 +/- 0.02	0.148 +/- 0.003[iii]	-2.37 +/- 0.02	-0.70 +/- 0.05	16.94
WP17	-19.23	169.58	0.95 +/- 0.02	0.137 +/- 0.004[iii]	-2.16 +/- 0.02	-1.66 +/- 0.03	0.14
WP19	-21.62	168.66	0.50 +/- 0.08	0.073 +/- 0.000[iii]	-2.17 +/- 0.07	-1.29 +/- 0.02	1.13
WP20	-25.67	165.42	0.09 +/- 0.02	0.102 +/- 0.002[iii]	-3.04 +/- 0.10	-0.56 +/- 0.05	0.41
WP21	-29.04	164.34	0.24 +/- 0.02	0.050 +/- 0.002[iii]	-2.32 +/- 0.04	0.13 +/- 0.04	0.00
WP26	-32.42	159.09	0.20 +/- 0.02	0.084 +/- 0.002[iii]	-2.623 +/- 0.05	-0.63 +/- 0.03	0.02

[i]PO4 values from Jakuba *et al* 2008, PO_4 values in Dyhrman *et al* 2006 and Jakuba *et al* 2008, [iii]PO_4 values from Hynes *et al*, in press.

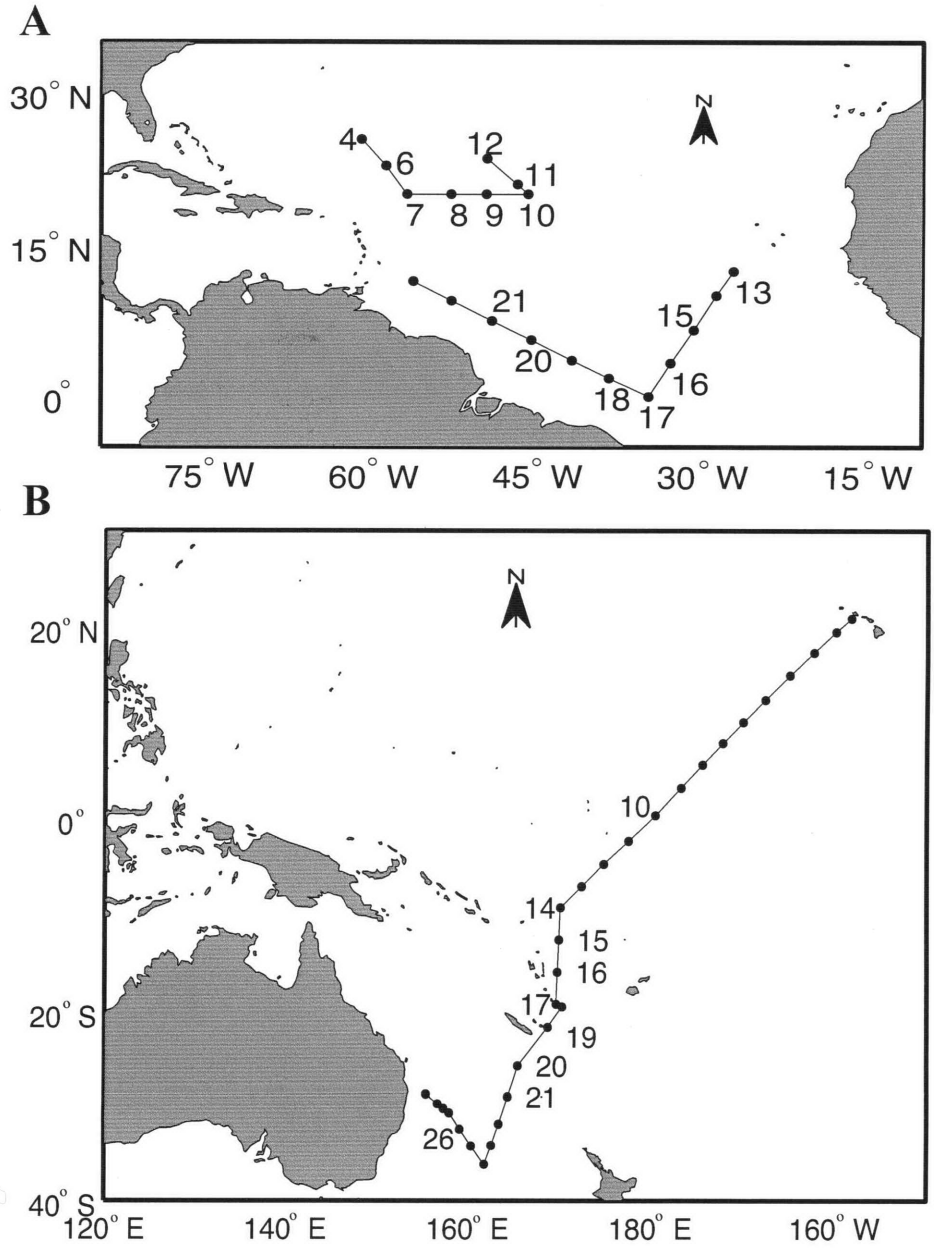

Figure 1. Map of cruise tracks. A. OC399-4 (stations 4-12) in the Sargasso Sea in March 2004. SJ0609 (stations 13-21) east-to-west transect across the equatorial Atlantic Ocean in July 2006. B. KM0701 north-to-south transect through the Western Pacific Warm Pool during January and February 2007.

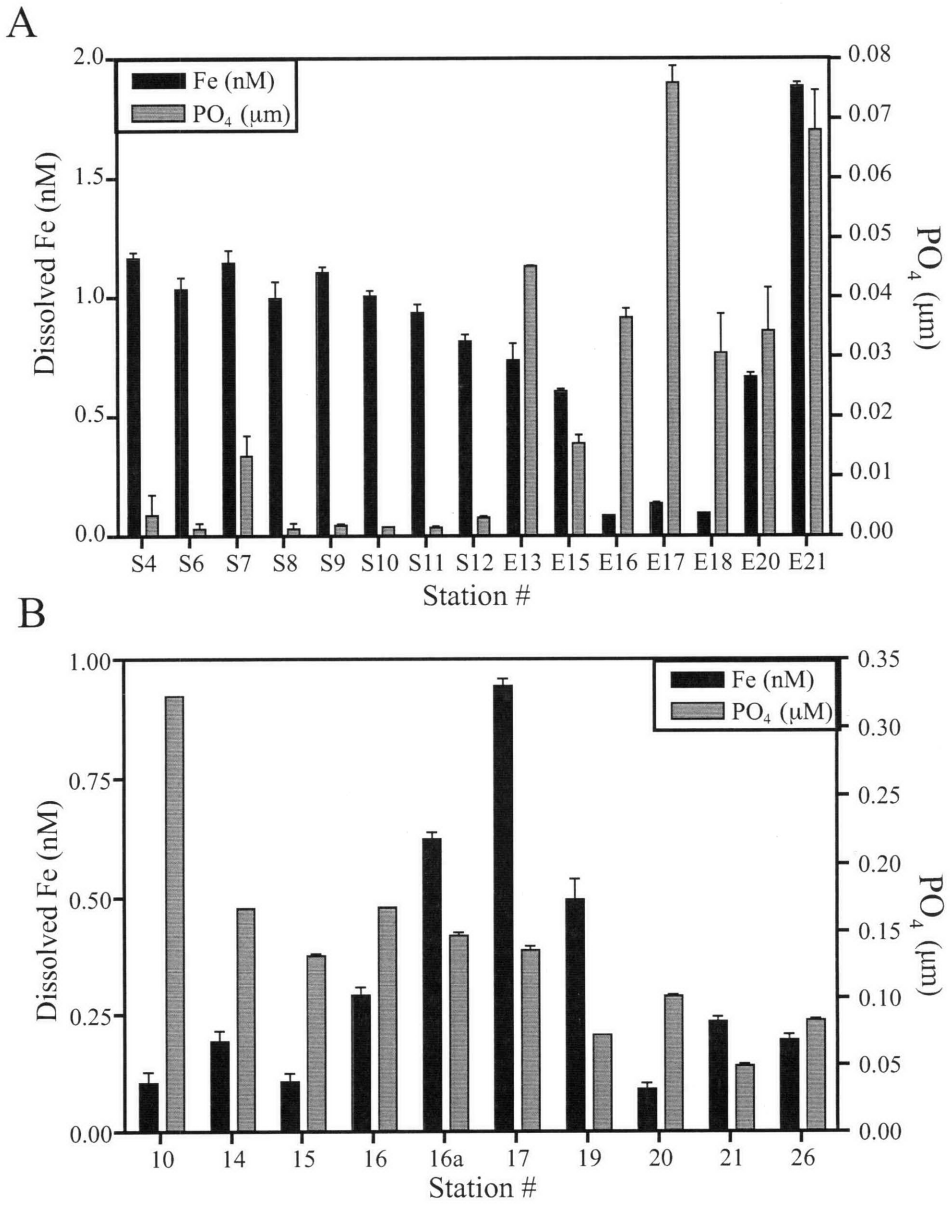

Figure 2. Dissolved (< 0.4 μm filtered) Fe and PO_4 measured in surface seawater samples from the Atlantic Ocean (A) and Pacific Ocean (B). Station numbers correspond to stations locations listed in Table 2 and plotted in Figure 1. Error bars represent the standard deviation of duplicate (PO_4) and triplicate (Fe) samples.

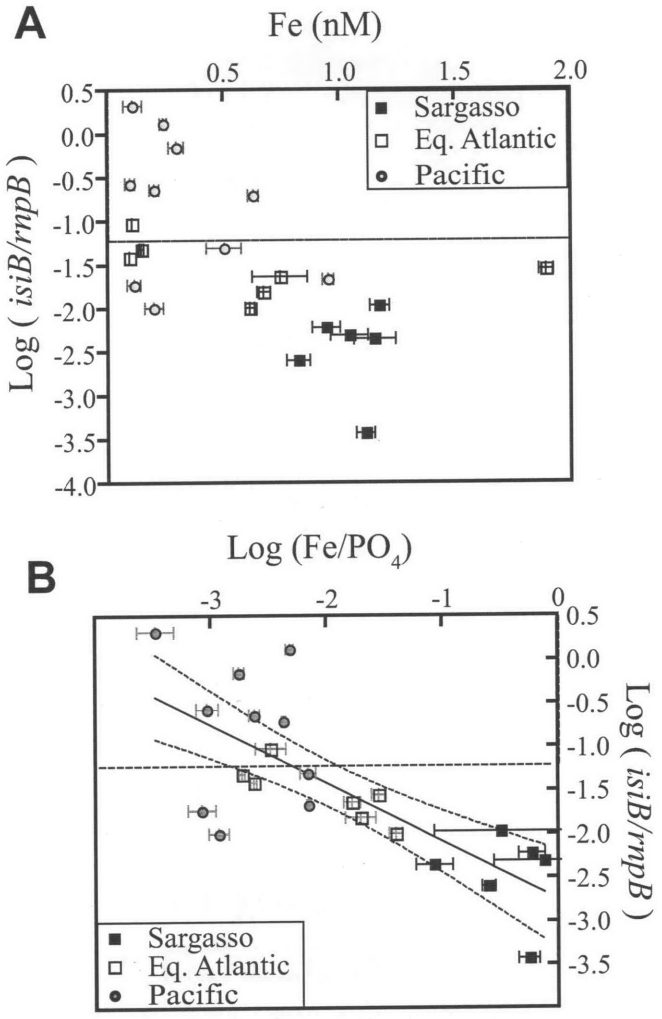

Figure 3. Relationships between log(*isiB*/*rnpB*) and both dissolved [Fe] (A) and log([Fe]/[PO_4]) (B). A dashed line is placed on each plot at the value log(*isiB*/*rnpB*) = -1.2, which is the value associated with a 50% reduction in N_2 fixation in Trichodesmium from previous work (Chappell and Webb submitted). The solid line in (B) represents the linear regression of log([Fe]/[PO_4]) verses log(*isiB*/*rnpB*) with the dashed curves on either side representing the 95% confidence intervals. In both plots, error bars represent the standard deviation of triplicate analyses.

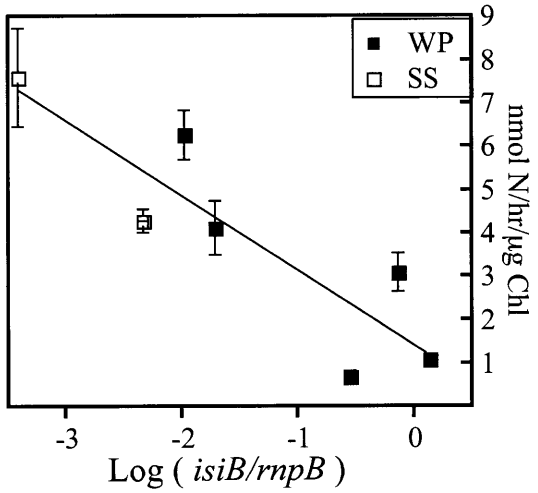

Figure 4. Relationship between log (*isiB/rnpB*) and N_2 fixation rates measured at two stations from the Sargasso Sea (SS7, SS10) and five stations from the Western Pacific (14, 15, 16, 20, 21). The linear relationship between log(*isiB/rnpB*) and nmol N/hr/µg Chl for just the Western Pacific Ocean samples is plotted as a solid line. The same relationship including the data from Sargasso Sea is plotted as a dashed line.

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CHAPTER 5. SUMMARY

5.1 SUMMARY

This thesis sheds light on the role that iron (Fe) plays in controlling nitrogen (N) fixation in *Trichodesmium* as well as providing data to increase our understanding of Fe chemistry in the understudied region of the southwestern Pacific Ocean. It is the first demonstration using a calibrated molecular method to show Fe limitation of *Trichodesmium* in the field. As is generally the case with a study of this kind, in addition to answering questions, the data points to new questions that need to be answered.

The data presented in Chapter 2 is a contribution to the growing dataset of dissolved Fe and Fe speciation in the surface ocean. It covers a region where there is limited data on trace metal concentrations, which receives very low dust deposition (Duce and Tindale, 1991; Jickells, 1999; Wagener et al., 2008). The profiles of dissolved Fe ([Fe]) are well in line with previous work showing low Fe (~0.2 nM) in the surface ocean, a small subsurface maximum in Fe (~0.4 nM) (Bruland et al., 1994; Wu et al., 2001; Boyle et al., 2005) and an increase in Fe below the euphotic zone to values that can range between 0.4 nM and 1 nM (Bruland et al., 1994; Johnson et al., 1997; Wu et al., 2001; Boyle et al., 2005; Johnson et al., 2007). Values for both the total amount of ligand present ([L] = 0.44 – 2.2) and the conditional binding constant ($K_{Fe',L} = 10^{11.7} – 10^{12.9}$) are within the range of reported values from other open ocean studies of [L] ranging from 0.33 – 2.5 nM and $K_{Fe',L}$ ranging from $10^{10.6}$ - $10^{13.9}$ (Rue and Bruland, 1995; van den Berg, 1995; Rue and Bruland, 1997; Boye et al., 2001; Powell and Donat, 2001; Boye et al., 2003; Croot et al., 2004; Boye et al., 2005; Cullen et al., 2006; van den Berg, 2006; Buck and Bruland, 2007; Kondo et al., 2007; Kondo et al., 2008; Rijkenberg et al., 2008). While calculations of [L] appear to be associated with a small range of values, calculations of $K_{Fe',L}$ appear to be much more variable as is evident from the wide range of values reported in the literature. It is unclear whether these differences are a result of true variability of the stability constants of ligands present in given samples or have to do with analytical or mathematical differences in how this value is determined. Given the importance of this parameter to determining the value for Fe', the inorganically bound

fraction of dissolved Fe that is though to be an important variable in determining Fe bioavailability, accurate measurements of $K_{Fe',L}$ are critical. A complicating factor in comparing Fe speciation data from different studies is that there are many competitive ligands used to generate the data and forms of mathematical analysis that people use to interpret their data and there has not been significant effort made to standardize these measurements. The intercalibration of Fe speciation methods, which is proposed as part of GEOTRACES, should help alleviate any concerns about the ability to compare results between groups.

That caveat aside, by comparing the multitude of Fe speciation studies that have been done throughout the world's oceans, it is becoming apparent that there are specific relationships governing ligand concentration in the surface ocean, which appear to be related to the amount of total dissolved Fe ([Fe]) present in a given sample and unrelated to biological parameters (Buck and Bruland, 2007; Chapter 2). This relationship suggests that at higher values of [Fe], biological factors are less involved in controlling [L] than their role as siderophores might imply. This does not mean that Fe binding ligands are not siderophores; it could have to do with different factors such as association with colloidal Fe and/or the type of organic ligand present resulting in a protection from UV degradation of ligands. More data on the composition of ligands in the field such as the recent study in the North Atlantic looking at ferrioxiamines (Mawji et al., 2008) in addition to other ligand classes could help answer if ligand composition is the predominant factor governing this relationship. In addition, studies looking at UV degradation of ligands in the field focusing on the colloidal verses soluble fraction of the Fe-ligand pool could also help answer the question of what is driving the relationship between [Fe] and [L] in samples with higher Fe.

Another factor that is apparent from the data presented in Chapter 2, is that the inorganically bound fraction of Fe ([Fe']) predicted based on these data is vanishingly small (<1 pM). It is much lower than the value of [Fe'] associated with Fe limitation in many species of phytoplankton (Brand, 1991; Chapter 3). In light of recent work suggesting that many organisms are capable of taking up Fe from organic ligands

(Hutchins et al., 1999; Maldonado and Price, 2001; Soria-Dengg et al., 2001; Achilles et al., 2003; Shaked et al., 2005), the usefulness of determining bulk Fe speciation in field samples to approximate bioavailable Fe is called into question. It may be more useful to improve methods to identify and measure the concentration of specific ligands, similarly to what has recently been done with ferrioxiamines (Mawji et al., 2008) and test the bioavailability of Fe bound to them to various phytoplankton groups, although that is a very labor-intensive proposition.

Limited information regarding the bioavailability of different forms of Fe was one of the driving reasons behind the goal of developing a molecular method to assess Fe limitation of *Trichodesmium* in the field. Because of research suggesting that there were differences in N_2 fixation rates between different species (Carpenter et al., 1993) and a growing body of work separating the cultured representatives of the *Trichodesmium* genus into two distinct clades (Orcutt et al., 2002; Annette Hynes, personal communication), merely looking at Fe limitation in *Trichodesmium erythraeum* seemed unlikely to ensure that the method would prove useful in the field. The work presented in Chapter 3 of this thesis shows clade-specific responses to growth under different Fe and temperature conditions. In light of results from Chapter 4 that indicate that representatives of the *Trichodesmium tenue* (Ten) clade are the most abundant or at least the most active in the open ocean, it is important that future work to evaluate how different physical and chemical factors affect *Trichodesmium* focus on representatives of the Ten clade in addition to the *T. erythraeum* (Tery) clade.

In addition to evaluating the differential responses to Fe and temperature between the two phylogenetic clades of *Trichodesmium*, Chapter 3 included the development of a calibrated molecular method to assess clade-specific Fe limitation of N_2 fixation. This is the first calibrated molecular method for Fe limitation and the first method to evaluate Fe limitation that enables for the distinction between the two clades. In addition to the gene predicted to encode for flavodoxin, *isiB* (Leonhardt and Straus, 1992), which is the gene used in the molecular assay, two other genes that could potentially be of interest in future molecular assays were identified. One, *idiA*, is predicted to encode for a protein involved

in a high-affinity ATP-driven Fe (III) uptake system (Michel et al., 1996), and the other, *feoB*, is predicted to encode for a protein involved in Fe (II) uptake (Kammler et al., 1993). The data in Chapter 3 showed that these two genes were also expressed during Fe limitation of axenic *T. erythraeum* (IMS101) and well conserved across the *Trichodesmium* genus. Developing a similar qPCR method for one or both of these genes could provide additional information about the timing and control of transcription of the Fe stress response. For example, one gene might be turned on earlier than the others with the onset of Fe limitation or turned off with a different response time following the alleviation of Fe limitation. An experiment looking at how the expression of one or all of the genes responds to long-term Fe deprivation, beyond what is necessary to draw down Fe that has been stored within the cell, could provide useful information about how *Trichodesmium* adapts to growth in low Fe environments. While these additional experiments could provide useful information, they were unnecessary to accomplish the goal of this work, which was to design a clade-specific RNA normalized assay for Fe limitation of *Trichodesmium* N_2 fixation and use it to assess Fe limitation of *Trichodesmium* in the field.

The ability to distinguish between the two clades, enabled not only the quantification of the levels of Fe limitation associated with N_2 fixation in the field, but also the determination of the relative contribution of the two clades to the active population of *Trichodesmium*. The field data indicates that the Ten clade is the dominant active form of *Trichodesmium* in the field. One important caveat to this conclusion is that the samples focused on *Trichodesmium* colonies, which were collected in a manner that selected against the collection of individual trichomes. It may be that looking at the cDNA extracted from a specific volume of water and collected using a filter that would catch free trichomes in addition to colonies would indicate that the Tery clade is quantitatively important. If nothing else, the data suggests that the colonial forms of *Trichodesmium* in the open ocean areas sampled in this study are from the Ten clade. In the future, it might also prove useful to compare the relative messenger RNA (mRNA) data that the assay provides with a quantitative measurement of DNA from these stations.

This could be used to determine if Tery clade representatives are present in a dormant form or at very low levels compared to the Ten clade in most of the open ocean, which may enable the Tery clade to become a more significant component of the *Trichodesmium* population when conditions change. Comparing that information with auxiliary data such as nutrient and Fe concentrations could help determine which factors are controlling niche differentiation between the clades.

The data in Chapter 4 represents the first survey quantifying Fe limitation of N_2 fixation of *Trichodesmium* in open ocean gyres. The data supports model predictions that there is widespread Fe limitation of *Trichodesmium* N_2 fixation in the Pacific Ocean (Moore et al., 2004). In comparing results of the [Fe] value associated with Fe limitation in the field with the [Fe'] value that found to be limiting in the lab, it appears that all the dissolved [Fe] may be available to *Trichodesmium*, including the Fe that is bound to organic ligands. In addition, by evaluating *isiB* expression in regions of varying Fe and phosphorus (P) concentrations and not just focusing on areas believed to be Fe stressed, it appears that there is a relationship between *isiB* expression and Fe:P. This relationship allowed for the determination of a critical Fe:P value that defines where Fe limitation of *Trichodesmium* commences. This value is in good agreement with the value that was used to predict that Fe was the controlling factor in the Pacific Ocean (Moore et al., 2004). In light of this data suggesting that Fe/P is what drives the transition to Fe limitation, a corresponding method looking at P limitation would help determine if there are areas where co-limitation of *Trichodesmium* populations is occurring and what the boundaries of Fe/P are that are associated with Fe-P co-limitation.

In conclusion, this thesis has provided data on Fe chemistry for a region where there are few measurements. It has confirmed that Fe ligands are prevalent even in low dust regions, though the data in Chapter 4 suggests that these measurements may prove unnecessary to attempts to predict Fe limitation of *Trichodesmium*. Determining that there are differences in the way that representatives from the two phylogenetic clades respond to chemical and physical factors and that representatives of the Ten clade are the predominant active *Trichodesmium* spp. in the open ocean are discoveries that will need

to be taken into account in future studies. The development of a method that can be used to assess Fe limitation of *Trichodesmium* in the field in a quantitative manner has provided empirical data that support model predictions of Fe limitation of *Trichodesmium*. Combining this method with a similar method designed to look at P limitation would be the best way to evaluate where these two chemical factors are controlling *Trichodesmium* N_2 fixation and to answer questions regarding Fe-P co-limitation. Developing similar methods for other important groups of phytoplankton could help answer questions about where Fe limitation is truly important in the ocean without having to deal with complications associated with what form of Fe is bioavailable to a given species.

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APPENDIX A: TABLE OF FE SPECIATION RAW DATA

Table A1. Selected raw data from Chapter 2 titrations

Station 11 15m

Fe_{total} (nM)	Current (nA)	Fe'	FeL	FeL/Fe'	Fe'/FeL
0.11	0.7	0.0004	0.05	125.5	0.0080
0.61	2.0	0.0011	0.43	409.4	0.0024
0.86	5.1	0.0027	0.41	152.0	0.0066
1.61	11.0	0.0058	0.64	110.1	0.0091
3.11	27.0	0.0142	0.73	51.2	0.0195
5.11	49.0	0.0258	0.79	30.6	0.0326
8.11	84.0	0.0443	0.71	16.0	0.0625

Station 13 15m

Fe_{total} (nM)	Current (nA)	Fe'	FeL	FeL/Fe'	Fe'/FeL
0.32	1.5	0.0013	0.09	66.8	0.0150
0.57	1.5	0.0013	0.34	252.3	0.0040
0.82	3.5	0.0031	0.29	92.2	0.0108
1.07	4.2	0.0038	0.43	115.3	0.0087
1.82	9.8	0.0088	0.34	39.2	0.0255
3.32	19.0	0.0171	0.46	27.2	0.0367
5.32	33.0	0.0296	0.36	12.3	0.0814

Station 14 15 m

Fe_{total} (nM)	Current (nA)	Fe'	FeL	FeL/Fe'	Fe'/FeL
0.20	0.4	0.0002	0.16	696.1	0.0014
0.45	1.1	0.0006	0.34	547.2	0.0018
0.70	1.6	0.0009	0.54	599.2	0.0017
0.95	2.4	0.0014	0.72	527.3	0.0019
1.20	3.4	0.0019	0.87	452.6	0.0022
2.20	8.3	0.0047	1.41	299.0	0.0033
3.20	19.0	0.0108	1.39	129.3	0.0077
8.20	72.0	0.0409	1.37	33.5	0.0298

Table A1. Continued

Station 16a 7m

Fe_{total} (nM)	Current (nA)	Fe'	FeL	FeL/Fe'	Fe'/FeL
0.61	0.6	0.0004	0.54	1364.5	0.0007
0.86	1.5	0.0010	0.69	696.2	0.0014
1.11	3.1	0.0021	0.77	372.0	0.0027
1.36	3.6	0.0024	0.96	401.6	0.0025
1.61	6.0	0.0040	0.94	236.8	0.0042
2.61	8.9	0.0059	1.62	274.2	0.0036
3.61	17.0	0.0113	1.72	152.4	0.0066
5.61	35.0	0.0233	1.72	74.1	0.0135
8.61	63.0	0.0419	1.62	38.6	0.0259

Station 19 15m

Fe_{total} (nM)	Current (nA)	Fe'	FeL	FeL/Fe'	Fe'/FeL
0.50	0.8	0.0004	0.43	1122.0	0.0009
0.75	1.5	0.0007	0.63	864.9	0.0012
1.00	1.0	0.0005	0.92	1897.4	0.0005
1.25	4.4	0.0021	0.89	419.6	0.0024
2.00	12.0	0.0058	1.03	177.2	0.0056
5.50	54.0	0.0261	1.14	43.4	0.0230
8.50	93.0	0.0450	0.98	21.9	0.0458

Station 26 15m

Fe_{total} (nM)	Current (nA)	Fe'	FeL	FeL/Fe'	Fe'/FeL
0.26	0.2	0.0001	0.24	2324.2	0.0004
0.51	0.5	0.0003	0.47	1784.0	0.0006
0.76	1.5	0.0008	0.63	801.5	0.0012
1.01	2.9	0.0015	0.76	498.5	0.0020
1.26	5.5	0.0029	0.78	270.7	0.0037
1.76	10.0	0.0052	0.89	169.2	0.0059
2.26	17.0	0.0089	0.77	86.9	0.0115

APPENDIX B: DEPTH PROFILES FROM SJ0609 (CHAPTER 4)

Table 1. Dissolved (<0.4 μm filtered) Fe for Station 16 (Latitude: 3.30 Longitude: -32.90), Station 17 (Latitude:0.01 Longitude: -34.90) and Station 18 (Latitude: 1.80 Longitude: -38.50).

Station	Depth (m)	Fe (nM)	Stdev
16	15	0.09	0.00
16	30	0.16	0.00
16	60	0.17	0.00
16	90	0.22	0.01
16	120	0.72	0.00
16	150	0.86	0.00
16	220	0.70	0.00
16	250	0.77	0.02
17	12	0.14	0.00
17	30	0.08	0.01
17	60	0.18	0.09
17	90	0.49	0.02
17	150	0.56	0.01
17	290	0.99	0.01
18	15	0.10	0.00
18	30	0.24	0.00
18	60	0.23	0.01
18	90	0.32	0.02
18	120	0.67	0.00
18	150	0.55	0.02
18	220	0.54	0.01
18	290	0.49	0.00

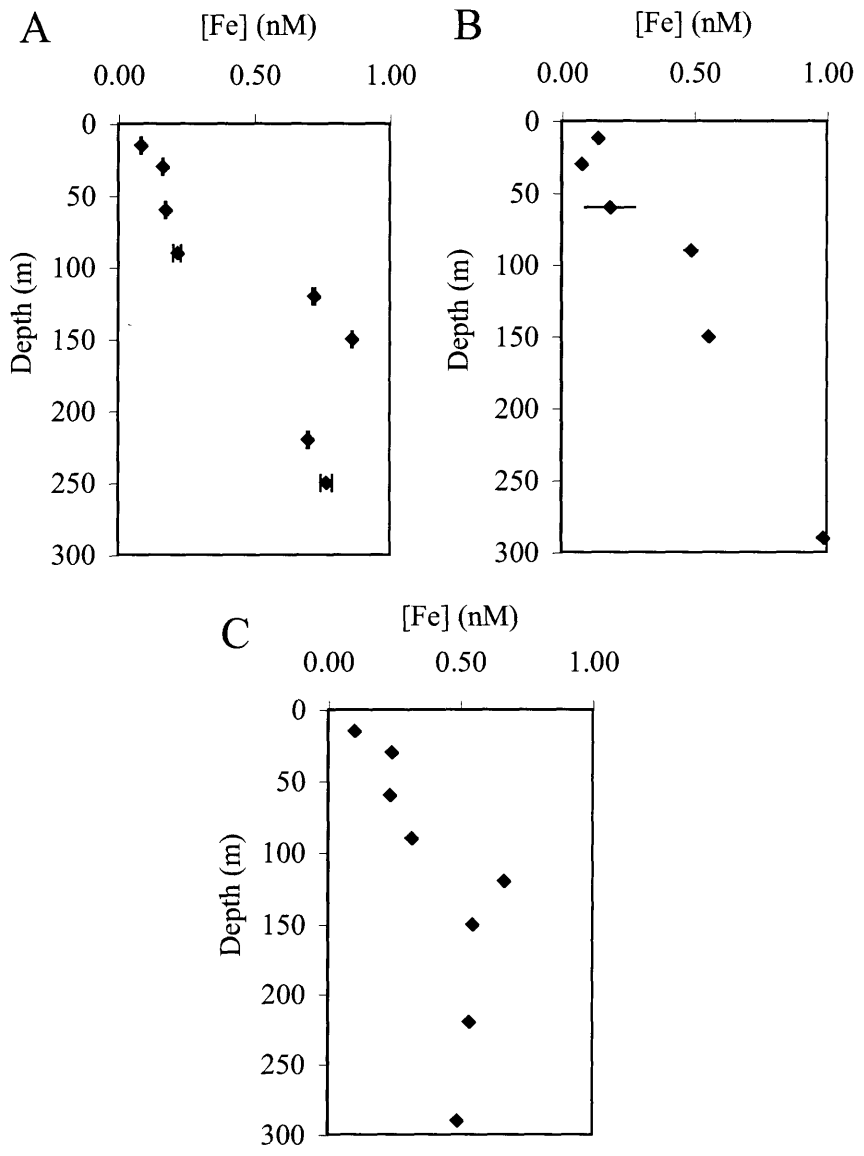

Figure 1. Dissolved Fe (< 0.4 μm filtered) depth profiles from three stations from cruise SJ0609. (A) Station 16. (B) Station 17. (C) Station 18. Error bars are standard deviations of triplicate analyses.