USING MOLECULAR TECHNIQUES TO QUANTIFY IRON BOUND NITROGENASE IN *TRICHODESMIUM* IMS 101 AND NATURAL POPULATIONS

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ABSTRACT OF THE THESIS

Using Molecular Techniques to Quantify Iron Bound Nitrogenase

in *Trichodesmium* IMS 101 and Natural Populations

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Iron has been widely recognized as a potentially key factor in promoting nitrogen fixation by *Trichodesmium*. Data from both laboratory and field studies demonstrates that increasing the iron concentrations stimulates growth, photosynthetic rates and nitrogen fixation of both cultured and natural populations. However, quantitative studies that elucidate relationships between cellular iron quotas and physiological mechanisms have been limited. In this study, molecular techniques enabled quantification of the amount of nitrogenase expressed in iron-replete cultures of *Trichodesmium* IMS 101 over a diel cycle. A standard of the purified iron component of nitrogenase was generated from an expression and protein purification system. Using this standard and known values of intracellular carbon, the amount of nitrogenase per carbon at peak expression was measured, 0.038 mg nitrogenase: mg C. The quantity of iron bound in the nitrogenase structure was then calculated; Fe:C equal to 236.53 μmol: mol. Using estimates of *Trichodesmium* biomass from the literature, the amount of iron bound in the nitrogenase structure was calculated for various

ocean regions, resulting in 2.22 μmol m^{-3} and 0.05 μmol m^{-3} of iron bound in nitrogenase in the subtropical North Atlantic and North Pacific, respectively.

Acknowledgements

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Chapter 1

Introduction

1.1 Purpose

Trichodesmium is a filamentous, non-heterocystous marine cyanobacterium widely distributed throughout the world's tropical and subtropical oceans. It primarily inhabits stratified, oligotrophic seas characterized by warm temperatures, deep light penetration and a mixed layer depth of approximately 100m (Capone, Zehr et al. 1997). *Trichodesmium* requires a minimum water temperature of 20°C to be physiologically active (Carpenter 1983). Natural populations exhibit several morphologies, including colonies in a "puff" or "tuft" form, as well as free, individual trichomes. Unlike most diazotrophs, *Trichodesmium* is unique in that both nitrogen fixation and photosynthesis occur simultaneously during the daily light cycle (Capone, Zehr et al. 1997). Nitrogen fixation and photosynthesis persist in an inverse relationship over a diel cycle, with nitrogen fixation rates peaking around midday (Capone, Oneil et al. 1990; Paerl 1994). This mechanism of diazotrophy is enigmatic, as nitrogenase is strongly inhibited by oxygen.

Several hypotheses have been put forth to account for *Trichodesmium's* ability to fix N_2 and CO_2 simultaneously. An early study suggested that *Trichodesmium* formed differentiated cells in colony centers that lacked the capacity to photosynthesize, similar to heterocyst formation in freshwater

diazotrophs (Carpenter and Price 1976). However, later works revealed no apparent differences in the distribution of photosynthetic machinery between cells, and found only 20-40% of trichomes possessed cells containing nitrogenase, suggesting the presence of intracellular mechanisms of protection against oxygen toxicity (Carpenter, Chang et al. 1990; Bergman and Carpenter 1991). Intracellular processes demonstrated to assist in nitrogenase protection include high respiration rates, superoxide dismutase and the Mehler reaction (Kana 1993). The entire cycle appears to be under the control of a circadian rhythm, which may reset with each solar cycle (Chen, Zehr et al. 1996; Chen, Dominic et al. 1998). Most likely a combination of strategies, intracellular, spatial and temporal, create the low oxygen concentrations necessary for nitrogen fixation to occur (Capone, Zehr et al. 1997; Berman-Frank, Cullen et al. 2001).

Trichodesmium contributes up to 90% of marine nitrogen fixation (Berman et al 1997, Capone et al 1997, LaRoche and Breiberth 2005), introducing a significant amount of new nitrogen to nutrient depleted waters (Capone et al 1997, Carpenter and Romans 1991). Its potentially important role as a diazotroph was first recognized in the early 1960's when nitrogen limitation was determined to be an important factor limiting production in warm, tropical seas (Dugdale, Goering et al. 1964; Goering, Dugdale et al. 1966; Dugdale and Goering 1967). Although early reports indicated that nitrogen fixation made an insignificant contribution to primary production compared with production supported by nitrate and ammonium (Carpenter and McCarthy 1975; Capone and Carpenter 1982;

Carpenter 1983), more recent studies demonstrate much higher inputs (Carpenter and Romans 1991). Currently, nitrogen fixation rate estimates range from approximately 80-200 Tg per year (Capone, Zehr et al. 1997; Gruber and Sarmiento 1997; Karl, Michaels et al. 2002).

Inputs of new nitrogen stimulate primary production in phytoplankton communities and increase the potential for export production in pelagic waters (Dugdale and Goering 1967, Karl et al 1997, Karl and Letelier, 1996). Since biologically induced nitrogen cycling is closely linked to the carbon cycle (Gruber 2005), increasing this "biological pump" can have a significant impact on the amount of carbon dioxide in the atmosphere. Current estimates of the flux of organic carbon from the euphotic zone to the ocean interior are approximately 16 Pg C yr^{-1}, about one third of total oceanic production (Falkowski 1997). Mechanisms that are capable of increasing rates of nitrogen fixation in oligotrophic waters have the potential to stimulate sequestration of organic carbon. The relationship between export production and carbon sequestration has become progressively important to understanding climate change, as anthropogenic sources continue to cause carbon dioxide levels in the atmosphere to rise.

Iron is known to limit primary production in ocean regions such as the high nutrient low chlorophyll (HNLC) areas of the Southern Ocean and equatorial Pacific Ocean, where high production rates would be expected from the existing environmental conditions (Martin, Gordon et al. 1991). Studies by Paerl et al

broadened the case for iron limitation to include areas of nitrogen limitation that receive the majority of new carbon and nitrogen from diazotrophic growth (1994). Iron is now widely recognized as a potentially key factor in promoting nitrogen fixation in oligotrophic waters (Capone, Zehr et al. 1997). It is an essential nutrient for many physiological systems that promote phytoplankton growth, including photosystems I & II, and the nitrogenase enzyme complex. Paradoxically, low concentrations of dissolved iron, as low as 0.2 nmol kg^{-1} and below, exist in the surface waters of much of the open ocean (Johnson, Gordon et al. 1997).

Data from both laboratory and field studies supports the importance of iron to *Trichodesmium* populations (Rueter 1988). Increasing the concentration has been demonstrated to stimulate growth, photosynthetic rates and nitrogen fixation of both cultured and natural populations (Rueter 1988; Rueter, Ohki et al. 1990; Paerl, Prufert-Bebout et al. 1994). However, quantitative studies that elucidate relationships between cellular iron quotas and essential physiological mechanisms have been limited. Research by Berman-Frank et al investigated *Trichodesmium*'s cellular iron quotas and discovered a quantitative relationship between growth rates and nitrogen fixation (2001). Modeling with the results suggested that iron availability has the potential to limit nitrogen fixation in up to 75% of the global ocean (Berman-Frank et al, 2001). Kustka et al. also examined cellular iron quotas using data derived from the iron content and reaction rates of intracellular processes, as well as diel variability in natural populations,

generating cellular Fe:C ratios of 38 μmol:mol in moderately iron-limited conditions (2003).

The purpose of this work is to provide further quantitative data directly related to the iron costs of the physiological mechanisms related to nitrogen fixation. It is broken down into three primary sections. First, molecular techniques were applied to quantify the amount of nitrogenase in *Trichodesmium* IMS 101 cultures expressed over a diel cycle. Second, the known quantities of iron in the nitrogenase structure were used in calculating the total amount of iron bound in nitrogenase in *Trichodesmium* IMS 101 cultures. Third, published biomass data of natural populations of *Trichodesmium* was used to estimate the amount of iron bound in nitrogenase in different ocean regions. From this data, and using some basic assumptions related to production in the tropical and subtropical oceans, an upper boundary for the physiological iron requirements of nitrogen fixation was defined.

1.2 Nitrogenase Function and Structure

Biological nitrogen fixation is an energetically expensive process that reduces gaseous N_2 to the biologically available ammonium (NH_4^+). The complete chemical reaction demonstrates the large quantities of ATP and reductant required to fix N_2.

$$N_2 + 8H^+ + 8e^- + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16\ Pi$$

The enzyme structure responsible for this process is the nitrogenase complex, which is composed of two components. Component I is the molybdenum-iron

containing protein (MoFe), or dinitrogenase, which houses the substrate-binding site where N_2 is reduced. The MoFe protein is encoded by the nif K and nif D genes, ranges from 200-240 kDa and contains approximately 30 Fe molecules (Burgess and Lowe 1996; Paerl and Zehr 2000). Component II is the iron containing protein (Fe), or dinitrogenase reductase, responsible for transferring electrons to the MoFe protein in a way that reduces N_2 to ammonium (Burgess and Lowe 1996). The Fe protein is encoded by the nif H gene, has a molecular weight of approximately 60 kDa and contains four Fe molecules (Burgess and Lowe 1996; Paerl and Zehr 2000). Each nitrogenase structure contains one MoFe protein with an alpha2beta2 structure and two attached iron proteins. Two major factors in regulating nitrogen synthesis include the availability of fixed inorganic nitrogen and the concentrations of cellular oxygen (Paerl and Zehr 2000).

Chapter 2

Methods of Nitrogenase Purification and Quantification

2.1 Target Gene Amplification

The nif H gene was amplified from cultured *Trichodesmium* IMS 101 with the polymerase chain reaction (PCR). Forward and reverse primers were obtained from Integrated DNA Technologies using the complete sequence of the nif H gene (NCBI U90952); forward primer: 5'-ATG CGT CAT ATG GCA TTT TAC GGA-3', reverse primer: 5'-TGT TGC TTT CAT ATG TGC ATÇ TTG-3'. PCR product was cleaned with the standard protocol from a QIAquick PCR Purification kit and quantitated using a Beckman DU640 Spectrophotomer

2.2 Cloning

The Novagen pET Vector System was utilized in cloning and expression of the target nif H gene. Specifically a pET 14b system was used, equipped with a T7 expression promoter, an N-terminal His tag and ampicillin (amp) resistance. The nif H product was cloned into a Nde 1 site. The vector was dephosphorylated with calf intestinal alkaline phosphatase (CIAP) to reduce self-ligation. The ligation reaction used T4 DNA Ligase. The recombinant nif H product was then transformed into the non-expression cloning host, NovaBlue, and grown overnight on LB agar plates supplemented with ampicillin. Eight colonies were selected and grown overnight in LB + amp. Colonies were then subjected to plasmid mini prep. The plasmid DNA from the mini prep was directionally screened for proper orientation using a vector primer and internal gene primer

through PCR; two clones demonstrated correct orientation. Fragments were sequenced with an ABI-3100 Avant Genetic Analyzer to verify the integrity and orientation of the nif H gene using a Big Dye preparation.

2.3 Transformation into Expression Host and Induction

Transformation and induction of the nif H protein was accomplished with protocols from the pET System Manual. The pET-14b expression vector with the correctly oriented and sequenced nif H fragment was transformed into a BL21(DE3)pLysS expression host containing a lacUV5 promoter and the gene for T7 RNA polymerase. The transformation reaction was plated on two agar plates infused with both amp and chloramphenicol. One colony was selected and grown in 10ml of LB + amp + chloramphenicol overnight and transferred to 1L of LB. Expression of the nitrogenase protein was then induced with the addition of 4 ml isopropyl-B-D-thiogalactopyranoside (IPTG). Cultures were grown to an optical density (OD) of 0.4, harvested at 250 ml volumes, centrifuged into pellets and stored at -80^{o}C. Induction of the target protein was confirmed on 10% polyacrylamide gels with sodium-dodecyl-sulfate (SDS-PAGE) and staining with Gel Code, followed by Western blotting. The target protein was extracted using BugBuster Protein Extraction Reagent and Benzonase Nuclease on both a small and large scale. Each pellet was subjected to a series of steps alternating incubation of the pellet in BugBuster and Benzonase Nuclease with centrifugation at 14,000 x g until all inclusion bodies were separated from the soluble pool.

Finally, the inclusion bodies were solubilized in a NaH_2PO_4/Tris/Urea buffer, filtered and collected as denatured clear lysate.

2.4 Protein Purification

Purification of the nitrogenase protein was achieved using Qiagen's QIA*express* System, which purifies the target protein through nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography compatible with 6xHis tagged proteins. First, the denatured lysate was quantified using a BCA Assay Kit; total protein was 42 mg with a concentration of 11.2 mg/ml. The column was carefully packed with 50% Ni-NTA superflow slurry and primed with a buffer composed of NaH_2PO_4/Tric-Cl/urea. Using High Performance Liquid Chromatography (HPLC), the proteins were washed and eluted using a series of NaH_2PO_4/Tric-Cl/urea buffers, each with decreasing pH. Purification involved four steps; two washes through the column with a buffer at a pH of 6.3 followed by two elution cycles through the column with a buffer at a pH of 5.9 and 4.3, respectively. Results were visualized with SDS- PAGE and staining with Gel Code.

2.5 Standard Derivation with Quantitative Western Immunoblotting

A standard derived from dilutions of the purified nitrogenase protein was created using quantitative western immunoblotting. A universal antibody against dinitrogenase reductase from *A. vinelandii and R. rubrum* was obtained by the generous donation of Professor Paul Ludden. The nif H protein was run on SDS-PAGE in amounts of 1140ng, 380ng, 114ng & 38ng for each successive lane and transferred to a polyvinylidene difluoride membrane (PVDF) membrane. The

membrane was probed with the nif H antibody at a dilution of 1:30,000. The secondary antibody (anti-rabbit), equipped with an HRP-conjugate, was also probed at a dilution of 1:30,000. Visualization was accomplished with SuperSignal chemiluminescence. The resulting bands were quantified by densitometry with Image J software obtained from the NIH website.

2.6 Growth of Trichodesmium Cultures

Two *Trichodesmium* IMS 101 cultures, denoted as experiment 1 and experiment 2, were grown in a 12:12 hour Light/Dark (L/D) cycle at 26°C under 80 μmol quanta m^{-2} s^{-1} of light. Cultures were grown in 10 & 5 Liters of YBCII media as defined by Chen et al (1996) under constant bubbling. Cells were harvested on 5μ pore size polycarbonate filters during exponential growth phase. Growth rates for experiment 2 were determined utilizing carbon specific measurements.

2.7 Sampling over a Diel Cycle

Once *Trichodesmium* IMS 101 cultures reached exponential growth, a series of samples were collected over one diel cycle. Beginning at a time point before the beginning of the light phase (7am), 15ml, 10ml and 5ml samples were filtered onto 5μ pore size polycarbonate filters and pre-combusted GF/F filters. Sampling continued every three hours until one hour past the end of the 12-hour light cycle (10am, 1pm, 4pm, 7pm, 10pm). All filters were stored at -80°C for further processing; including quantitative western blotting, measurements of total

carbon and nitrogen, and measurements of photochemical quantum yields (fv/fm).

2.8 *Trichodesmium* IMS 101 Protein Quantification

Total protein was extracted from 15 ml of filtered *Trichodesmium* IMS 101 in buffer containing 4% SDS, 15% Glycerol, 0.05% Bromothymol blue / 4% SDS, 0.1M Sodium Carbonate, PMSF and DTT. Total proteins were quantified with standard BCA and measured on a Molecular Devices Emax precision microplate reader. Proteins were subjected to a similar series of steps as the original standard. First, the total proteins were separated using SDS-PAGE polyacrylamide gel electrophoresis on 10% gels, and then transferred to the PVDF. The total protein in each lane from experiment one was 17.25 μg. The total protein in each lane from experiment two was 18 μg. As previously described, proteins were probed on the same western blot as the standard, i.e. using the same antibody (dinitrogenase reductase derived from *A. vinelandii* and *R. rubrum*) at the same dilution of 1:30,000. Immunoblot quantification was once again completed using densitometry with the Image J software obtained from NIH.

2. 9 Photochemical Quantum Yields (Fv/Fm)

Measurements of the photosynthetic quantum yield (Fv/Fm) were immediately made at each time point over the diel cycle with a fast repetition rate fluorometer (FRRF). Fv/Fm was determined from two parameters: the initial

dark-adapted fluorescence Fo, and the maximum fluorescence, Fm with the equation Fv/Fm = (Fm-Fo)/Fm.

2.10 C:N Analysis

Measurements of elemental carbon and nitrogen in *Trichodesmium* IMS 101 samples were completed with the Carlo Erba NA1500 Series 2 Elemental Analyzer. 15ml samples were collected on pre-combusted GF/F filters and stored at -80^{o}C. Samples were placed in tin foil capsules and processed through the analyzer where they were combusted and measured with a mass spectrophotometer; carbon and nitrogen quantities were processed with Eager 200 software.

Chapter 3

Quantifying Nitrogenase and the Iron Bound in Nitrogenase

3.1 Protein Expression and Induction

In these experiments, the level of nitrogenase expressed in *Trichodesmium* IMS 101 cultures was quantitated over a diel cycle. To accomplish this, a standard of purified recombinant nitrogenase protein was generated via an *E. coli* expression system. The Nif H gene was first amplified using standard PCR, which resulted in 35.32 ng/μl of the nif H product. The nif H fragment was ligated into a pET 14b expression vector. This vector comes equipped with a T7 expression region induced by T7 RNA polymerase, as well as an N-terminal His tag. These modifications are designed to facilitate the purification of the nitrogenase protein. The vector with the nif H gene was then successfully transformed into NovaBlue, and selected colonies were screened. Two colonies demonstrated the appropriate band size (37 kb) and orientation. The ligated gene (pET 14b + nif H) was then successfully transformed into a BL21(DE3)pLysS expression host. This host contains the necessary gene for T7 RNA polymerase and the lacUV5 promoter region, as well as, a small amount of T7 lysozyme, an inhibitor of T7 RNA polymerase that provides an extra level of stability to the target gene. Once induced with the IPTG, the majority of the protein was found to be located in inclusion bodies, which were solubilized for further quantification.

3.2 Nitrogenase Purification

Nitrogenase tagged with 6xHis was purified with HPLC using a Ni-NTA superflow column. A distinct band is clearly shown at the predicted size of 37kDa. The four lanes present in Figure 1 reflect the different stages of purification. With each rinse, a buffer at a successively lower pH was responsible for rinsing away contaminating proteins. The multiple bands in lane one represent total protein in the denatured lysate after one wash through the column; lane two represents the second wash fraction through the column. Lane three is eluted protein at a pH of 5.9. Lane four is the purified nitrogenase eluted at a pH of 4.5. At this pH, a large concentration of the protein was effectively purified and collected.

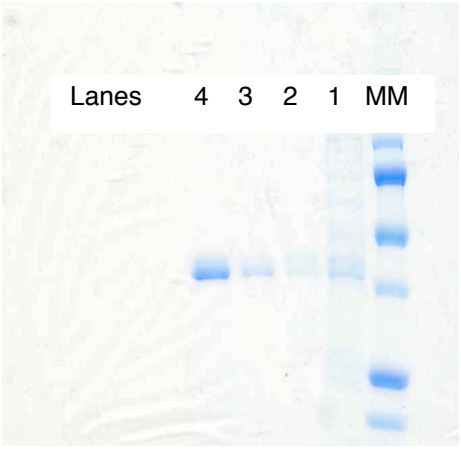

Figure 1 – SDS-Page of Purified Nitrogenase from Trichodesmium IMS 101 - Lane 1 is the total protein of denatured lysate. Lane 2 is the second wash fraction. Lane 3 is eluted protein using elution buffer at a pH of 5.9. Lane 4 is the final purified protein eluted at pH of 4.5

3.3 Quantification of Purified Nitrogenase

The purified iron component of the nitrogenase protein was quantified and used to set up a standard curve with western blot analysis. The final concentration of the purified nitrogenase was determined to be 0.19 μg/μl.

Dilutions of the purified protein were then run on a western blot. The membrane was probed with the dinitrogenase reductase antibody**.** Figure 2a shows the relative expression of serial dilutions of the purified nitrogenase in nanograms; 38ng, 114ng, 380ng and 1140ng. The dilutions were linear over the range tested. Figure 2b shows quantification of the nitrogenase concentration (ng) versus pixel density.

Figure 2a

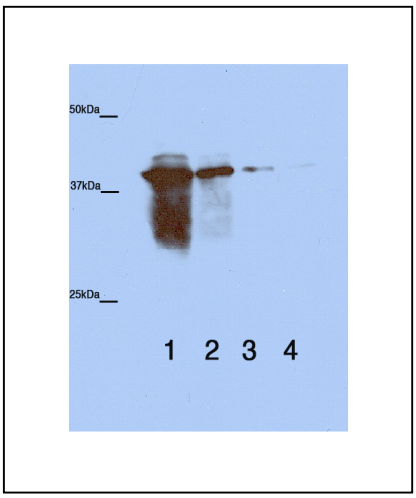

Figure 2b

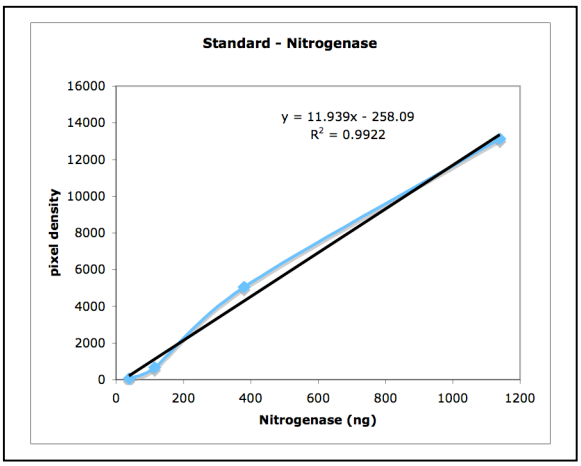

Figure 2 – Standard Curve of Purified Nitrogenase, a) Serial dilutions of the purified nitrogenase protein; concentration of protein in lane 1 is 1140ng, lane 2 is 380ng, lane 3 is 114ng & lane 4 is 38ng. b) Linear regression of nitrogenase concentration (ng)

3.4 Growth

In order to examine the expression of nitrogenase in vivo in *Trichodesmium* IMS 101, two cultures were established under a diel cycle, experiment 1 and experiment 2. Cells were harvested during exponential growth. Data collected from experiment 1 was incomplete and a growth curve was not able to be determined. Figure 3 depicts the growth rate from experiment 2. Based

on total carbon (mg/μl), growth from days 1-8 was calculated as 0.158 cells per day, with a doubling time of 3.796 days.

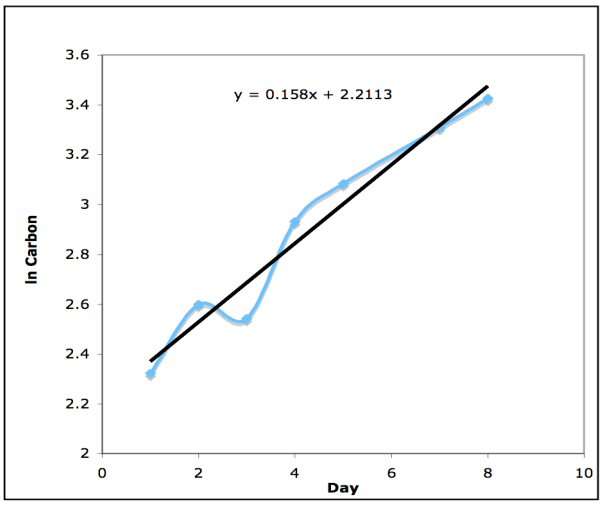

Figure 3 – Growth curve – Linear regression of growth rate of Trichodesmium in experiment 2.

3.5 Quantitative Western Blotting

Figure 4 presents the western blot analysis from which measurements of total nitrogenase protein extracted from *Trichodesmium* IMS 101 were taken. The blot was probed with the same antibody as described previously. Every sample reveals a major band at approximately 37kD, confirming the presence of the nitrogenase protein. The bands visualized in each lane were utilized in quantifying the total amount of nitrogenase protein expressed in each sample. In experiment 1, samples were collected over a diel cycle at 10am, 1pm, 4pm, 7pm, 10pm, 4am and 7am. In experiment 2, samples were collected at 7am, 10am, 1pm, 4pm, 7pm and 10pm. Although there is variability between the two experiments, the blot clearly demonstrates that both cultures exhibited similar

patterns of nitrogenase expression over a diel cycle. One possible explanation of the variability is that sampling was completed at two different stages of the growth cycle, i.e. experiment 1 samples were taken during the initial phases of exponential growth and experiment 2 samples at a later stage of exponential growth. This could potentially affect extrapolations to natural populations. However, no specific conclusions are possible because of the lack of carbon data in experiment 1 cultures.

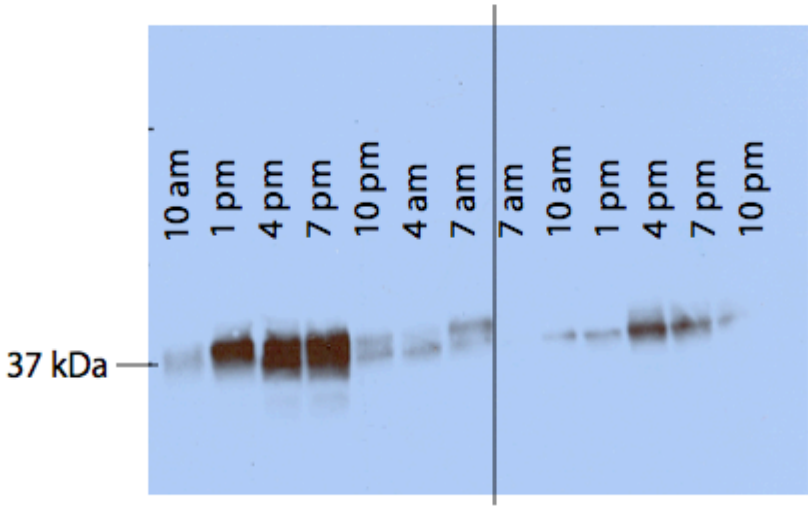

Figure 4 – Western immunoblot with standard derived from the purified iron component of the nitrogenase protein over a diel cycle during exponential growth. The left side represents expression from experiment 1, the right side represents expression in experiment 2.

3.6 Total Nitrogenase Proteins from *Trichodesmium* Cultures

To evaluate levels of nitrogenase in whole extracts of cultured *Trichodesmium* IMS 101, we used the standard of purified nitrogenase from Figure 2. Table 1 depicts the absolute quantification of expressed nitrogenase proteins from experiments 1 and 2. This data corroborates the western blot analysis demonstrated above. In each experiment, the expressed protein has a

range of one order of magnitude between peak and minimum expression times. Peak expression in both experiments occurred between 4-7pm.

Table 1: Total Nitrogenase from Whole Extracts of *Trichodesmium*

Diel Cycle	Time	pixels (y)	Nitrogenase (ng)
Experiment 1	10am	1569	153.03
	1pm	11071	948.91
	4pm	13955	**1190.47**
	7pm	15907	**1353.97**
	10pm	2357	219.04
	4am	1588	154.62
	7am	3024	274.90
Experiment 2	7am	91	29.24
	10am	531	66.09
	1pm	982	103.87
	4pm	5981	**522.58**
	7pm	4174	**371.23**
	10pm	652	76.23

3.7 Fe:C Quotas in Nitrogenase

Calculations of Fe:C were achieved using the quantitative nitrogenase and carbon data from the above experiment with known iron requirements for the nitrogenase structure (Fe & MoFe), (Table 2). Calculations were performed based on the assumption that the iron requirements of the nitrogenase (Fe + MoFe) structure are two Fe proteins for every one MoFe protein, which gives a total of 2 Fe proteins to 38 total proteins in the enzyme structure.

To do the calculations, the amount of nitrogenase per carbon was determined in each 15 ml sample collected over the diel cycle (7am, 10am, 1pm, 4pm, 7pm, 10pm). Using the 2:1 relationship of the Fe:MoFe proteins and the molecular weight of the Fe protein (other ratios are possible), we were able to

convert the nitrogenase per carbon (mg/mg) to Fe:C (μmol/mol) with the following equation:

> **Fe:C (μmol:mol)** = Nitrogenase x Carbon (mg)/ Carbon (mol) x (1/molecular weight of MoFe) x ratio of nitrogenase complex to Fe protein x total Fe per complex

In experiment 2, measurements of the Fe:C demonstrated a range of values over the diel cycle; as expected the lowest values resulted before the light cycle started and after it finished when no nitrogen fixation took place; the highest values resulted during peak hours of nitrogenase expression (Table 2). The Fe:C data indicates the amount of iron bound in the nitrogenase complex at each time point in iron-replete cultures.

Table 2 – Iron bound in the Nitrogenase Structure

Time	Nitrogenase[a] (mg)	Carbon[b] (mg)	Nitrogenase/Carbon[c] (mg/mg)	Fe/C[d] (μmol/mol)
7am	0.00087	0.47569	0.0018	11.32
10am	0.00209	0.43516	0.0048	29.64
1pm	0.00359	0.54963	0.0065	40.32
4pm	**0.01873**	**0.48943**	**0.0383**	**236.53**
7pm	0.01411	0.61241	0.0231	142.46
10pm	0.00251	0.46699	0.0054	33.20

a. nitrogenase in 15ml of filtered *Trichodesmium* collected at each time point of the diel cycle
b. carbon in 15ml of filtered *Trichodesmium* at each time point
c. calculated nitrogenase/carbon
d. convert to iron/carbon using the MW of the Fe protein & ratio of 2:1 Fe proteins to MoFe protein

3.8 Biomass Data

By using Fe:C data from Table 2 and estimates of *Trichodesmium* biomass from the literature, the amount of iron bound in the nitrogenase structure was calculated for various ocean regions. As previously mentioned, calculations

were based on peak nitrogenase expression measured in an iron-replete culture. The biomass data in Table 3 comes from published estimates for three key ocean regions, the Subtropical North Pacific, Tropical North Atlantic and Arabian Sea. The value of 50 ng C/trichome is the quantity of particulate carbon (PC) measured in single filaments collected at the ALOHA Station in the subtropical North Pacific (Letelier and Karl 1996). The lowest value of nitrogenase per cubic meter was 0.11 mg nitrogenase/m^3 at the ALOHA station in the subtropical N. Pacific; the highest value reflected a bloom in the Arabian Sea, 19 mg nitrogenase/m^3. The tropical North Atlantic samples fell between the two points but were also very high compared to samples from the ALOHA Station, at 4.28 mg nitrogenase/m3. Iron values ranged from 0.05 μmol Fe-nitrogenase/m^3 for the subtropical North Pacific to 9.86 μmol Fe-nitrogenase/m^3 in a concentrated *Trichodesmium* bloom. The key result of these calculations is that each value represents an estimate of the potential upper limit of iron bound in the nitrogenase structure in each ocean region.

Table 3 – Iron bound in *Trichodesmium* biomass, based on published biomass estimates from 1991

Location	Trichomes/m^3	Carbon ng /Trichome	Carbon mg/m^3	Carbon mol/m^3	Nitro-genase mg/m^3	μmol Fe in Nitro-genase/ m^3
Sub-tropical N.Pacific[a]	56700	50	2.835	0.00024	0.108	0.056
Arabian Sea Bloom[b]	10^7	50	500	0.0417	19.00	9.855
Trop. NA[c]	2250000	50	112.5	0.094	4.275	2.217

a. Letelier & Karl, 1996
b. Capone et al., 1998
c. Carpenter et al., 2004 - Based on data from May-June 1994 Cruise

3.9 C:N Analysis

Total carbon ranged from 0.029 mg/μl to a peak value of 0.040 mg/μl at the point of highest nitrogen fixation; nitrogen ranged from 0.0063 mg/μl to 0.0086 mg/μl (Table 4). C:N values were similar at all time points and reflect a culture that is actively fixing nitrogen. The average C:N over the diel cycle was 4.7.

Table 4 - Measurements of Total Carbon to Nitrogen

Time	Carbon (mg/μl)	Nitrogen (mg/μl)	C:N
7am	0.0317	0.0067	4.7
10am	0.0290	0.0063	4.6
1pm	0.0366	0.0076	4.8
4pm	0.0326	0.0071	4.6
7pm	0.0408	0.0086	4.7
10pm	0.0311	0.0064	4.9

3.10 Photochemical Quantum Yields (Fv/Fm)

Measurements of photochemical quantum yields are reflected in Table 4. Over the diel cycle, they are relatively consistent but do decrease somewhat during peak expression around 4-7pm. This general trend has been demonstrated previously (Berman-Frank, Lundgren et al. 2001).

Table 5 – Photochemical Quantum Yields

Date	**Time**	**Fv/Fm**
Experiment 2	7am	0.438
	10am	0.418
	1pm	0.41
	4pm	0.386
	7pm	0.335
	10pm	0.4

Chapter 4

Discussion and Conclusion

Diazotrophy is known to require a significant amount of iron for growth and maintenance. Previous work demonstrated that growth based on nitrogen fixation may require up to 5 times more intracellular iron than growth based on ammonium (Kustka, Sanudo-Wilhelmy et al. 2003). This increased demand is likely due to the high iron requirements of the nitrogenase complex. However, little work has been done to elucidate the total cellular iron required for diazotrophy. In this study, molecular techniques enabled quantification of the amount of nitrogenase expressed in iron-replete cultures of *Trichodesmium* IMS 101 over a diel cycle. A standard of the purified iron component of nitrogenase was generated from an expression and protein purification system (Figure 2b). Using this standard, nitrogenase expression in cultured populations was measured and normalized to carbon. The quantity of iron bound in the nitrogenase structure, derived from the known iron content of the nitrogenase complex, ranged two orders of magnitude. To our knowledge, this is the first study to directly quantitate Fe:C within the nitrogenase structure.

Nitrogenase expression in cultures of *Trichodesmium* IMS 101 from two independent experiments followed a diel cycle (Figure 4, Table 1,2) consistent with other studies (Chen, Zehr et al. 1996). In both experiments, populations exhibited peak expression between 4-7pm. Data from experiment one was incomplete so the following discussion primarily focuses on data derived from

experiment two, unless otherwise noted. Protein expression began before the onset of the light cycle (7am) at two orders of magnitude less than maximum. Following completion of the light cycle (8pm), nitrogenase quickly degraded. C:N averaged approximately 4.7 over the entire day, indicating the presence of nitrogen fixation. C:N values measured during the diel cycle and throughout exponential growth are within the range of published data for cultured *Trichodesmium* populations (Mulholland and Capone 2000). Data from most assays was collected for each time point in the cycle (every three hours), however, the amount of nitrogenase expressed at the peak of the cycle was considered the most important for the purpose of this study. Therefore, unless otherwise specified, the following discussion focuses on data generated from the maximal protein expression at 4 p.m.

Studies focused on iron quotas for *Trichodesmium* are limited, but at least two are known which calculated cellular iron quotas and growth (Berman-Frank, Cullen et al. 2001; Kustka, Sanudo-Wilhelmy et al. 2003). Unlike these studies, measurements of Fe:C in this work strictly focus on the amount of iron bound in the structural component of the nitrogenase complex. The results from this report were generally higher than previous estimates of total intracellular Fe:C, and related more closely to data derived from natural populations, although at least one experiment with an iron-replete culture demonstrates compatible values (Kustka, Sanudo-Wilhelmy et al. 2003). Direct comparisons cannot be drawn as neither of the previous studies specifically measured Fe:C in the nitrogenase

complex, and much of the previous data was generated from cultured populations under iron limitation. However, both estimated the partitioning of cellular iron between photosynthesis and nitrogen fixation, providing important insight to the parameters of this data.

Berman-Frank et al. used mass spectroscopy and ^{59}Fe uptake to quantify the total iron quotas in cultures grown across a gradient of inorganic iron, with results of 7.1- 214 μmol Fe mol^{-1} C (2001). Intracellular Fe:C was found to be approximately 60-70% lower and ranged from 3 – 69 μmol Fe mol^{-1} C (Berman-Frank, Cullen et al. 2001), compared with our result of 236 μmol Fe mol-1 C in the nitrogenase structure alone. Field measurements of Fe:C were much closer to the total iron estimated at 450 +/- 242 μmol Fe mol^{-1} C; which translated to an intracellular iron of approximately 292 μmol Fe mol^{-1} C. Iron budgets generated from their data indicated approximately 60% of the intracellular iron was allocated to photosynthesis (Berman-Frank, Cullen et al. 2001), a value of 175 μmol Fe mol^{-1} C in natural populations. This leaves approximately 117 μmol Fe mol^{-1} C available to nitrogenase, assuming all iron is partitioned to either CO_2 or N_2 fixation.

Kustka et al. calculated cellular iron quotas and iron use efficiencies - the minimum amount of iron required for minimum growth at a growth rate of 0.1/day - considering the iron content and reaction rates of the nitrogenase complex, PSI:PSII ratios, the Mehler reaction and superoxide dismutase (Kustka, Sanudo-Wilhelmy et al. 2003). Their primary data demonstrated that iron limited growth in

cultured populations was supported by a total cellular Fe:C quota of 38 μmol Fe mol^{-1} C. However, in iron-replete cultures the Fe:C reached levels as high as 500 μmol Fe mol^{-1} C. In natural populations from the North Atlantic and Caribbean Sea, Fe:C ranged from 20-500 μmol:mol (Kustka, Carpenter et al. 2002). Unlike Berman-Frank et al., Kustka et al. predicted the majority of iron would be bound in the nitrogenase complex; 19 to 53% (2003). All Kustka et al. calculations were based on an assumption of a 5:1 Fe:MoFe ratio, which has been shown to produce maximal N_2 fixation reaction rates in some organisms (2003). An iron-replete culture with a Fe:C ratio of 500 μmol:mol Fe:C allocates between 95-265 Fe:C to the nitrogenase complex. Depending on the ratio of Fe:MoFe (2:1 or 5:1), our data suggests 124-236 *μ*mol of nitrogenase bound iron per mol C. In either case, both studies show compatible results. The high values of Fe:C are not surprising based on *Trichodesmium's* capacity for luxury iron uptake, demonstrated to be 13-fold times greater than required for moderately Fe-limited growth (Kustka, Sanudo-Wilhelmy et al. 2003). *Trichodesmium* does not cease the uptake of iron even at levels that exceed the concentration at which oxyhydroxides precipitate (Kustka, Sanudo-Wilhelmy et al. 2003). The specifics of how and where luxury iron is stored in *Trichodesmium* is currently unknown.

The concentration of iron bound in nitrogenase in *Trichodesmium* over various ocean regions, calculated from data in Table 2, spanned a range of 0.108 – 9.856 μmol nitrogenase m^{-3} (Table 3). Lowest values were found in the subtropical North Pacific, while highest values were derived from a bloom located

in the Arabian Sea (Carpenter, Subramaniam et al. 2004). In the North Atlantic, nitrogenase was 4.28 mg m^{-3} and the iron bound in nitrogenase was 2.22 μmol m^{-3}. This data focuses the difference in biomass between the three regions, highlighting the increased iron quantities that are bound in the nitrogenase structure in the subtropical North Atlantic versus the North Pacific. It also points to the importance of blooms with values more than quadruple those from the North Atlantic. Historically, it has been speculated that estimated rates of fixed nitrogen would significantly increase if *Trichodesmium* blooms were included in global nitrogen fixation estimates (Carpenter 1983; Capone, Zehr et al. 1997).

The above calculations are based on areas of concentrated *Trichodesmium* populations. To broaden this perspective to an entire ocean basin, the question of iron limitation in the North Atlantic is explored using this data and some basic assumptions. Nitrogen fixation has been studied extensively in the tropical and subtropical North Atlantic because of the region's prime environmental conditions and geographic location. This area receives ample quantities of iron through dust deposition derived from African deserts; which are considered the major source of dissolved iron into the photic zone (Duce and Tinsdale; 1991 Gao, Kaufman et al. 2001). Because of its small size and proximity to land, some researchers hypothesize a factor other than iron, such as phosphorous, may be limiting *Trichodesmium* biomass in this region (Brand 1991; Wu, Sunda et al. 2000; Sanudo-Wilhelmy, Kustka et al. 2001).

An area of the North Atlantic Ocean was outlined with a spring global sea surface temperature map generated from the NOAA Satellites and Information website. The sole requirement was sea surface temperatures 20°C and above, the lower limit for physiologically active *Trichodesmium*. This region spanned the equator to approximately 35°N, measuring 2 x 10^7 km^2 or 2 x $10^{13}m^2$. Assuming 100m as the depth of the euphotic zone gives a total volume of 2 x $10^{15}m^3$. The choice of 100m is bolstered by a recent study that measured significant *Trichodesmium* biomass to 130 m using a video plankton recorder (Davis and McGillicuddy Jr. 2006). Assuming the average chlorophyll concentration in this region to be 0.2 mg/m^3 (Falkowski, Barber et al. 1998), total chlorophyll in the basin equaled 0.4 x 10^{15} mg. If 1% of this biomass is considered *Trichodesmium* and a C:Chl ratio of 100 is used, the result is 400 x 10^12 mg C in *Trichodesmium*. Applying the ratio of nitrogenase to carbon measured in this study (0.038 mg nitrogenase:mg C), results in a total of 15.2 x 10^9 g nitrogenase or 1.52 Gg of nitrogenase in the region. Using the calculations described previously, this translates to 7.8 x 10^6 mol Fe, or 3.9 pM of iron bound in nitrogenase in *Trichodesmium*. To answer the original question of whether or not *Trichodesmium* appears to be limited in the tropical North Atlantic depends on the concentration of dissolved iron available. Choosing a reasonable concentration of 1 nM of dissolved Fe in the euphotic zone, establishes that *Trichodesmium* is not iron-limited in the tropical North Atlantic Ocean.

This data provides an important examination of nitrogenase quantification in *Trichodesmium* from a physiological perspective. Using molecular techniques, this study estimated the potential upper limit of iron bound in nitrogenase in various ocean regions. Understanding the amount of iron that is bound in the nitrogenase structure provides relevant parameters with which to explore nitrogen and iron budgets. To provide more environmentally compatible data, this study could be expanded to include cultures under varying factors such as different light regimes, iron and phosphorous limitation, as well as comparisons to nitrogen fixation rates. These methods could then be applied to natural populations for comparison of the physiological quotas between cultured and in situ populations. As the above thought experiment demonstrates, these techniques are useful in addressing questions of iron use over large-scale ocean regions. Including this data in models with measurements of dust deposition and soluble iron concentrations in the subtropical North Pacific and North Atlantic Oceans has the potential to help resolve outstanding questions of nutrient limitation. Only through a solid understanding of the physiological requirements and capabilities of *Trichodesmium* will a global picture of the relationship between iron and nitrogen fixation emerge.

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