REGULATION OF *TRICHODESMIUM* NITROGEN FIXATION BY COMBINED NITROGEN AND GROWTH RATE: A FIELD AND CULTURE STUDY

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REGULATION OF *TRICHODESMIUM* NITROGEN FIXATION BY COMBINED NITROGEN AND GROWTH RATE: A FIELD AND CULTURE STUDY

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March 18*th*. —We sailed from Bahia. A few days afterwards, when not far distant from the Abrolhos Islets, my attention was called to a reddish-brown appearance in the sea. The whole surface of the water, as it appeared under a weak lens, seemed as if covered by chopped bits of hay, with their ends jagged. These are minute cylindrical confervae, in bundles or rafts of from twenty to sixty in each. Mr. Berkeley informs me that they are the same species (*Trichodesmium erythraeum*) with that found over large spaces in the Red Sea, and whence its name of Red Sea is derived. Their numbers must be infinite: the ship passed through several bands of them, one of which was about ten yards wide, and, judging from the mud-like colour of the water, at least two and a half miles long. In almost every long voyage some account is given of these confervae. They appear especially common in the sea near Australia; and off Cape Leeuwin I found an allied but smaller and apparently different species. Captain Cook, in his third voyage, remarks, that the sailors gave to this appearance the name of sea-sawdust.

From the journals of Charles Darwin aboard the H. M. S. Beagle

DEDICATION

For Doug, whose remarkably indulgent nature enabled me to try something new...again. All those months apart while I was at sea are finally coming to fruition. For Margaret and Tink whose loving support helped shape a woman who knew she could do this and gave her the tenacity to try. For Nick and Lisa whose laughter got me through.

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AR	Acetylene reduction
ARA	Acetylene reduction activity
C	Carbon
C_2H_2	Acetylene
CF-IRMS	Continuous flow isotope ratio mass spectrometer
d	day
D	Dilution rate
DIN	Dissolved inorganic nitrogen
DON	Dissolved organic nitrogen
GF/C	Glass fiber filter/type C (nominal pore size 1.2 µm)
GF/F	Glass fiber filter/type F (nominal pore size 0.7 µm)
GOM	Gulf of Mexico
h	Hour
L	Liter
L:D	Light:dark
µ	Specific growth rate
µM	Micromolar (micromoles L^{-1})
µmol	Micromole
m	meter
mL	Milliliter

mM	Millimolar (millimoles L^{-1})
N	Nitrogen
N_2	Dinitrogen
NW	Northwest
NO_3^-	Nitrate
P	Phosphorous
PC	Particulate carbon
PN	Particulate nitrogen
PO_4^{3-}	Phosphate
POM	Particulate organic matter
TP	Total phosphorous
Z	Depth

SUMMARY

Trichodesmium is a globally significant marine diazotroph responsible for supplying new N to the oligotrophic regions in which it is found. Though it has been studied for decades, our understanding of the ways in which environmental factors can affect N_2-fixation rate remains limited. A continuous culture of *Trichodesmium* was established and characterized to determine the extent to which growth rate and the presence and uptake of nitrate affect N_2-fixation rates. Results indicate that N_2-fixation increases linearly with growth rate and that the uptake of nitrate inhibits N_2-fixation up to 70% and in a concentration dependent manner at initial nitrate concentrations <10μM. Results from a field study of this cyanobacterium show that areal N_2-fixation rates in the Gulf of Mexico are comparable to measurements made in other oligotrophic basins. Stable isotope evidence from this field study confirms that *Trichodesmium* is important to C cycling as well. These findings can be added to modeling efforts used to quantify *Trichodesmium* N_2-fixation on an oceanic scale. Implications for future research are discussed.

CHAPTER 1

INTRODUCTION

Trichodesmium sp. is a filamentous, non-heterocystous marine cyanobacterium important to the introduction of new nitrogen (N) to oligotrophic tropical and subtropical seas worldwide (Capone, et al. 1997). It was first reported to fix N_2 by Dugdale, et al. (1961) and since has been the focus of many research expeditions, modeling efforts, theses, and dissertations yet still there is much we do not know about its ecology and its physiology. This enigmatic bloom-forming cyanobacterium necessarily fixes N_2 concurrently with photosynthesis yet does not obviously separate these processes temporally or spatially even though the enzyme necessary to fix N_2 can be inactivated by photosynthetically produced O_2. Actively growing, diazotrophic *Trichodesmium* exist in natural populations as single filaments and as colonies, however, we are still unsure of the ultimate cause of colony formation. *Trichodesmium* have only a few known consumers, are a physical substrate for a wealth of epiflora and epifauna, and one species reputedly emits a cyanotoxin. This non-motile cyanobacterium can be found deep in the water column "mining" for phosphate (P), at the surface in dense aggregations, and are able to maintain a subsurface maximum ostensibly to avoid photoinhibition of both C and N_2-fixation. Due to the characteristic absorption and fluorescence of one of their light-harvesting pigments, phycoerythrin, and to the backscattering from their gas vacuoles, *Trichodesmium* can be remotely sensed using SeaWiFS satellite images. Extensive blooms have even been photographed from space. Despite the myriad limitations that face the oceanographers who study the large, oligotrophic, central oceanic gyres, *Trichodesmium* has been studied for decades. Though the physiology of natural

populations is still perplexing, their global significance as a supplier of N to oligotrophic gyres is indisputable (Capone, et al. 1997).

Natural History

Global distribution

Trichodesmium has been reported in nearly all tropical and subtropical waters worldwide. This cyanobacterium has been studied in the Atlantic (Goering, et al. 1966, Carpenter, et al. 1995, Carpenter, et al. 2004), Pacific (Karl, et al. 1997, Letelier, et al. 1996), and Indian Oceans (Jyothibabu, et al. 2003, Lugomela, et al. 2002) as well as in the Caribbean (Carpenter, et al. 1977), China (Saino 1977, Chen, et al. 2003), Sargasso (Orcutt, et al. 2001), and Arabian Seas (Capone, et al. 1998). Though it has been seen in all of these tropical seas, its distribution can be extremely irregular with large surface aggregations occurring only when conditions are optimal. High sea surface temperature and irradiance, low ambient nutrients, and calm, quiescent seas (Capone, et al. 1998, Eleuterius, et al. 198, Carpenter 1983) can promote dense aggregations. Under optimal conditions surface aggregations can cover hundreds of thousands of square kilometers (Capone, et al. 1998). Recent modeling efforts have confirmed that *Trichodesmium* distribution is defined by high light intensity, weak vertical mixing, and low concentrations of DIN (Hood, et al. 2004).

Janson, et al. (1999) analyzed both the *het*R gene fragment and the 16S rDNA gene sequence of natural samples collected from the Caribbean Sea, the central Atlantic, and the southern Pacific oceans. They found three distinct clades among these natural

populations. These clades are comprised of *T. hildebrandtii* and *T. thiebautii*, *T. contortum* and *T. tenue,* and *T. erythraeum* along with the two common lab strains NIBB1067 and IMS 101 (Janson, et al. 1999). Carpenter and Price report *T. thiebautii* as the most common species encountered in the Sargasso Sea, tropical North Atlantic and Caribbean Sea (Carpenter, et al. 1977) and Carpenter more recently confirmed that *T. thiebautii* is the most common species in the north Atlantic (Carpenter, et al. 2004). The distribution of colony morphology has been under investigation and the reasons for colony formation are still unclear. "Bundleness" has been inversely related to wind speed (Bryceson, et al. 1981) and directly related to nutrient limitation (Logan, et al. 1988). In the Pacific, single filaments are more common (Letelier, et al. 1996) than in the Atlantic where colonies make up as much as 89 to 92% of the *Trichodesmium* biomass (Carpenter, et al. 2004).

Vertical distribution

Trichodesmium has permanent gas vacuoles (Walsby, et al. 1978), which render this cyanobacterium positively buoyant. Like freshwater cyanobacteria, *Trichodesmium* can regulate its buoyancy such that they frequently maintain a subsurface maximum (Kromkamp, et al. 1986, Rijn, et al. 1985). Often when dense surface aggregations are encountered, the majority of the biomass sits just below a thin lens of water. In the Caribbean Sea and sub-tropical Atlantic, Carpenter and Price (1977) and Carpenter and McCarthy (1975) report subsurface maxima between 10 and 40 m and the same has been shown to be true for the southern East China Sea (Chang, et al. 2000). More recently, on three cruises in the tropical N Atlantic, the maximum was encountered at 12m in

May/June of 1994 and October 1996 and as deep as 40m in April 1996 (Carpenter, et al. 2004). 65% of total biomass was found in the upper 20m and as much as 94% of the biomass was in the upper 50m (Carpenter, et al. 2004). It is clear that the majority of the biomass is found in the upper euphotic zone however, trichomes have been found at 175m in the Western Sargasso Sea (Carpenter, et al. 1975) and as deep as 200m at station ALOHA in the North Pacific subtropical gyre (Letelier, et al. 1996). These depths are well below the nutricline and, depending on the time of day and level of cloud cover, are below the 1% light level as well.

There is no evidence for chromatic adaptation with depth (McCarthy, et al. 1979). Therefore, because this phytoplankter does not adapt its light-harvesting pigments, it cannot permanently make a living at depths at which it is unable to gather enough light to photosynthesize. Though the majority of *Trichodesmium* biomass is found above 50m, trichomes and colonies are commonly found throughout the water column. Therefore, theories have developed that revolve around the vertical migration of this cyanobacterium. A 15m subsurface maximum might indicate the avoidance of photoinhibition of both photosynthesis and N_2-fixation and may support the theory that *Trichodesmium* spp. are able to descend in the water column to acquire phosphate. It is hypothesized that carbohydrate storage at the surface temporarily counteracts the positive buoyancy of trichomes and colonies, which then allows this non-motile cyanobacterium to migrate downward to the nutricline to acquire phosphate (Karl, et al. 1992,Villareal, et al. 2003). Studies of the gas vacuoles found within *Trichodesmium* have revealed that the vacuoles are extremely strong and will not burst under the pressure encountered at the depth of the nutricline (Walsby, et al. 1978). As the stored carbohydrates are respired at

depth, the trichomes and colonies again become positively buoyant and return to the surface nutrient replete. Empirical evidence and models show that a vertical migration to at least 70m is possible (Villareal, et al. 2003) though trichomes have been found much deeper in the water column (Letelier, et al. 1996). We report herein that while *Trichodesmium* is at the nutricline to acquire phosphate, cells may take up nitrate as well, which may also help support growth upon return to the surface (Holl, et al. submitted).

It is generally accepted that *Trichodesmium* C and N_2-fixation are negatively affected by decreasing light intensity though a systematic field study of these effects has not yet been published. Carpenter et al., report a decrease in *Trichodesmium* volumetric C-fixation rate with depth to the 1% light level in the sub-tropical N Atlantic (Carpenter, et al. 2004). As part of a *Trichodesmium* N_2-fixation model, Hood, et al. report photoinhibition of N_2-fixation in 7 out of 17 incubations of samples incubated in 100% I_o in the subtropical and tropical Atlantic (Hood, et al. 2002). In the Central N Pacific, Mague et al report some evidence of photoinhibition of *Trichodesmium* photosynthesis at the highest light intensity tested and they also report that *Trichodesmium* N_2-fixation attenuates with decreasing light intensity in incubator experiments with natural populations (Mague, et al. 1977). A decrease in *Trichodesmium* N_2-fixation with depth was reported for three of five stations (Goering, et al 1966). In a study of *Trichodesmium* in the Gulf of Mexico we report an attenuation of N_2-fixation with depth as light intensity decreases from 50% incident light, where our rates exhibit a maximum, to the 1% light level. In culture under nutrient replete conditions, increasing light intensity from 10 μmol quanta m^{-2} s^{-1} to 160 μmol quanta m^{-2} s^{-1} increased N_2-fixation activity in *Trichodesmium* (Fu, et al. 2003).

Trophic Interactions

Trichodesmium colonies host a whole cadre of organisms (Sheridan, et al. 2002) including heterotrophic bacteria (Paerl, et al. 1989) and other species of filamentous non-heterocystous cyanobacteria (Siddiqui 1992b). Interestingly *T. thiebautii*, but not *T. erythraeum*, has been reported to produce a toxin (Hawser, et al. 1991, Hawser, et al. 1992) to which these associated organisms must be immune or insensitive. The toxin in approximately 50% of *T. thiebautii* culture samples caused more than 50% lethality in *Artemia salina* and calanoid and cyclopoid copepods but not harpacticoid copepods were found susceptible to the *T. thiebautii* toxin in bloom samples (Hawser, et al. 1992). Additionally, *Trichodesmium* fresh tissue, lyophilized homogenates, and chemical extracts were rejected by two of three fish species considered generalist consumers (Bullard, et al. 2002).

There are few known grazers of *Trichodesmium* (O'Neil, et al. 1992). The cyclopoid copepod, *Macrosetella gracilas* is the most well known and the distribution and life cycle of this copepod has been directly related to the distribution and abundance of *Trichodesmium* (Calef, et al. 1966, Bottger-Schnack, et al. 1989). *M. gracilas* not only ingests *Trichodesmium*, up to 21% in ^{15}N labeled feeding assays (O'Neil 1998) and from 90 to 126% of its body carbon (Roman, et al. 1978), but also uses *Trichodesmium* as a place to deposit their eggs and as a "nursery" for the development for naupliar stages of growth (O'Neil, et al. 1992).

Because there are so few known grazers it has been widely accepted that *Trichodesmium* sp. is important to oligotrophic systems primarily as a supplier of new N

by way of DON excretion (Capone, et al. 1994, Glibert, et al. 1994, Mulholland, et al. 2000, Mulholland, et al. 1999). In fact Glibert, et al., report that as much as 50% of *Trichodesmium* N_2- fixation is released as DON (Glibert, et al. 1994) and Capone et al., report that approximately 25% of the concurrent N_2-fixation in *Trichodesmium* is released as glutamate (Capone, et al. 1994). Mulholland et al., reports the direct release of NH_4^+ by *Trichodesmium* in batch culture (Mulholland, et al. 2000). We know that 10 to 15% of cells along a trichome contain active nitrogenase (Lin, et al. 1998, Bergman, et al. 1991), thus it has been hypothesized that *Trichodesmium* exudes this DON as a means of supplying N to the non-diazotrophic cells along the trichome or within a colony. Capone et al., report that the K_s for glutamate in *Trichodesmium* is high and therefore, it is possible that glutamate is the common means for N exchange in *Trichodesmium* (Capone, et al. 1994).

Newly fixed N can be traced through the organisms living in an oligotrophic marine system by determining the ratio of the natural abundance of the two stable isotopes of N. Newly fixed N has a stable isotopic signature close to that of atmospheric N, a standard of 0 ‰, and therefore isotopically light N in the biota can indicate diazotrophy at the base of the food chain (Wada, et al. 1980, Wada, et al. 1976). Isotopically light N is fixed by diazotrophs and subsequently taken up by non-diazotrophic phytoplankton. These light phytoplankton are then ingested by zooplankton thus the diazotrophic signal is propagated up the food chain. Isotopically light particulate matter and zooplankton have been identified in regions where *Trichodesmium* N_2-fixation is prevalent (Montoya, et al. 2002, Capone, et al. 1998).

The stable isotopic signature of N in the amino acids of an organism can also indicate diazotrophy in an oligotrophic system (McClelland, et al. 2003). The isotopic ratio of the essential amino acid phenylalanine does not change from food source to consumer and as such can be used as an indicator of the N source at the base of the food chain (McClelland, et al. 2002). Glutamate changes approximately 7‰ from food to consumer and therefore is a good indicator of trophic level (McClelland, et al. 2002). The stable isotopic signature of amino acids in zooplankton from a transect across the Atlantic Ocean has shown that *Trichodesmium* was a food source (or a source of DON to non-diazotrophic phytoplankton that were, in turn, ingested by these zooplankton) (McClelland, et al. 2003).

Trichodesmium sp. has a characteristically heavy C isotopic signature as well (Carpenter, et al. 1997) that can be used as a tracer for the direct incorporation of *Trichodesmium* sp. into the biomass of a particular region. Typical values for the particulate C in oligotrophic systems are from –22‰ to –25‰ however *Trichodesmium* values are much heavier isotopically, and range from -15.2 to -11.9‰ (Carpenter, et al. 1997). The stable isotope of carbon as an indicator of *Trichodesmium* as a food source or as a source of heavy DOC has not been used widely but our work in the Gulf of Mexico indicates that as much as 60% of the carbon found in the zooplankton of the 250 and 500μM size fractions may be coming from direct *Trichodesmium* ingestion. We believe that this may be true for other oceanic basins at times when *Trichodesmium* is a dominant member of the phytoplankton community.

Stoichiometry

There are several reports in the literature of flexible elemental stoichiometric ratios in *Trichodesmium*. Letelier and Karl (Letelier, et al. 1996) report C:N for *Trichodesmium* of 6.3 in the subtropical N Pacific. Both McCarthy et al., (1979) and Carpenter et al., (2004) report a C:N of 6.5 for *Trichodesmium* in the central N Atlantic and in the tropical N Atlantic respectively. Mague et al., report a *Trichodesmium* C:N of 4.1 in the central N Pacific (Mague, et al. 1977) and Post et al., report values of 4.1 to 4.3 in a study of a *Trichodesmium* bloom in the Gulf of Aqaba (Post, et al. 2002). We report herein a mean C:N ratio across a range of steady state growth rates in culture of 5.6 ± 0.35 (mean ± SD, N=93). All values, with the exception of those of Mague et al., and Post et al., are close to the canonical Redfield ratio of 6.6. It is not extraordinary that uni-algal cultures and natural population samples of this diazotroph might differ from the total marine phytoplankton community upon which Redfield was based (Redfield 1934, Falkowski 2000) particularly since these C:N ratios are below Redfield, which indicates this diazotroph is not N limited.

At low growth rates and under P or N limitation, phytoplankton can exhibit large variability in their stoichiometry (Goldman 1986). *Trichodesmium* is no exception, and it is in the N:P ratios of both natural and cultured populations that we begin to see the P-sparing ability of *Trichodesmium* as demonstrated by its amazingly flexible stoichiometry. Letelier and Karl report an N:P of 45 (Letelier, et al. 1996), significantly different than that of Redfield N:P of 16. In bloom conditions Karl et al., report an N:P of 125 for *Trichodesmium* sp. (Karl, et al. 1992). Sanudo-Wilhelmy, et al., (2001) report a range of *Trichodesmium* sp. N:P in the tropics of 35 to 61 and in the subtropics of 14 to

30. Mulholland et al., reports N:P ratios of 25 or higher in natural populations along the north coast of Australia and in a batch culture study of *Trichodesmium* sp. growing on DOP, report a wide range of N:P ratios from 4.4 to 156 dependent on the initial DOP concentration (Mulholland, et al. 2002). Herein we report a mean N:P ratio of 27.1 ± 4.2 (mean ± SE, N=74) across the range of steady state growth rates attained in continuous culture. Interestingly, the high variability in this ratio did not correlate with growth rate.

Physiology

Diel Nitrogenase Activity

In *Trichodesmium*, nitrogenase activity increases rapidly at the start of the light day, peaks at midday, decreases throughout the afternoon, and is completely shut down until the following morning. Nitrogenase is produced de novo every day approximately 2 hours prior to first light (Capone, et al. 1990) and there is evidence that an additional protein is translated late in the afternoon that inhibits nitrogenase synthesis thus shutting down nitrogenase activity late in the light day (Capone, et al. 1990). The nitrogenase protein appears to be completely degraded throughout the night and absent from the cells several hours before dawn. The pattern of gene expression and nitrogenase activity has been shown to be robust even in constant light and therefore is thought to be the result of an endogenous rhythm (Ohki, et al. 1992, Chen, et al. 1998). The transcription of two photosynthetic genes, *psb*A and *psa*A, are also controlled by an endogenous rhythm and transcription of these two genes follows transcription of *nif*HDK prior to a light cue at the beginning of the light period (Chen, et al. 1999).

Simultaneous N_2-fixation and Photosynthesis

A major focus of *Trichodesmium* culture and fieldwork has been to understand the way in which this diazotroph is able to fix di-nitrogen in the presence of photosynthetically produced O_2. Many cyanobacteria separate these processes either spatially with specialized sites for N_2-fixation, heterocysts, or temporally by fixing N_2 at night and C during the day. In *Trichodesmium*, both processes proceed simultaneously and obligately during the day with no obvious mechanism of separation. Initially it was suggested that only cells in the interior of colonies fixed N_2 and that these "micro-aerobic" zones within the colonies could support N_2-fixation (Fogg 1974, Paerl 1994). However, Ohki, et al. found the highest rates of N_2-fixation in a culture of strain NIBB 1067 during exponential growth when the culture consisted primarily of single trichomes (Ohki, et al. 1991). Additionally, nitrogenase exists in the active form during the day and in only 15 to 10% of the cells along a trichome (Lin, et al. 1998, Bergman, et al. 1991) and CO_2 uptake is reduced in N_2-fixing cells (Lin, et al. 1998). Thus, some form of cellular differentiation may be involved.

The photosystem I to photosystem II (PSI to PSII) ratio was determined to be approximately 25 in *Trichodesmium* (Subramaniam, et al. 1999), which is extremely high for a marine phytoplankter. Cyclic electron transport in PSI generates ATP for nitrogenase activity and Mehler reaction activity within PSI may also protect nitrogenase from the O_2 formed in PSII (Raven, et al. 1988, Kana 1993) via the formation of O_2 radicals.

The most recent work studied the combination of both temporal and spatial segregation as a means of protecting nitrogenase. In this work, the protection of

nitrogenase was shown to be via electron flow through PSII and the subsequent oxidation of the quinone pool (Berman-Frank, et al. 2001). High respiration rates early in the photoperiod further reduce the quinone pool, which sends a negative feedback to PS electron transport, down regulates PSII, and allows for high N_2-fixation rates during the photoperiod when O_2 consumption exceeds O_2 production (Berman-Frank, et al. 2001). Spatial separation is evident in the production of H_2O_2 occurring at high rates in nitrogenase containing cells (Berman-Frank, et al. 2001). The accumulation of H_2O_2 is a result of super oxide dismutase activity reducing the radicals produced during Mehler reaction activity.

Regulation of N_2-fixation

Trace Metal Limitation

Though it has been studied for decades *Trichodesmium* N_2-fixation rate measurements still vary considerably and the source of this variation remains unclear. Because nitrogenase has a large metallic component, limited access to molybdate (Howarth, et al. 1985) or iron (Rueter, et al. 1992, Berman-Frank, et al. 200) has been implicated in variability in N_2-fixation rates. As supporting evidence of iron limitation in *Trichodesmium*, a model of *Trichodesmium* distribution mimics the pattern of high dust deposition (Tyrrell, et al. 2003), which is the greatest input of Fe in the oligotrophic ocean (Duce, et al. 1991, Baker, et al. 2003). More recently another model has determined that iron concentration may limit the absolute amount of *Trichodesmium* biomass but does not control the occurrence of *Trichodesmium* (Hood, et al. 2004).

The catalytic activity of nitrogenase per mole of iron is the lowest of any iron-containing enzyme in nitrogen metabolism (Raven, et al. 1988). In fact Raven, et al. report that the iron requirement for diazotrophs is approximately 100x higher than the requirement for non-diazotrophic phytoplankton. Kustka, et al. present revised iron use efficiency data for *Trichodesmium* and report that for their predicted set of requirements (diazotrophic growth of 0.1 d^{-1}, PS1:PSII ratio from 1-4, and 48% Mehler activity) *Trichodesmium* would require only 7-11 times more iron than a non-diazotrophic (eukaryotic) phytoplankter (Kustka, et al. 2003). The available iron in the Atlantic and Caribbean Sea could support and possibly even exceed that which is necessary for the observed rates of *Trichodesmium* N_2-fixation (Kustka, et al. 2003). In the Gulf of Mexico, iron rich dust deposition has been implicated in a 100-fold increase the *Trichodesmium* biomass (Lenes, et al. 2001). Culture studies of the effects of iron limitation on N_2-fixation have determined that N_2-fixation is more sensitive to Fe stress than is cellular yield (Fu, et al. 2003) and that cellular iron quota, photochemical quantum yield, PSI:PSII ratio, as well as N_2-fixation rate all decline in iron-limited culture conditions (Berman-Frank, et al. 2001). The genetic marker, IdiA, has been used as an indicator of iron stress in *Trichodesmium* (Webb, et al. 2001). Achilles, et al. have shown that inorganic iron (III) as well as siderophore-bound iron (III) is bioavailable to *Trichodesmium* (Achilles, et al. 2003) and small, newly-formed, colloidal-complexed iron is available to *Trichodesmium* also (Wang, et al. 2003).

Though shipboard trace metal experiments are problematic, a great deal of evidence supports the theory that iron availability can affect growth rate and N_2-fixation rate in diazotrophs. However, the opposite has also been reported. In field studies in the

central Atlantic, N_2-fixation rates have been shown to be independent of both dissolved iron and *Trichodesmium* colony iron content (Sanudo-Wilhelmy, et al. 2001) or co-limited by both low iron and low phosphate availability (Mills, et al. 2004).

Phosphate Availability

Similar to trace metal limitation, it has been suggested that the lack of available phosphate in the euphotic zone of oligotrophic oceans can inhibit growth and N_2-fixation (Wu, et al. 2000, Wu, et al. 2003, Sanudo-Wilhelmy, et al. 2001). Stihl et. al, (2001) and Mulholland et al., (2002) report alkaline phosphatase activities in both phosphate deplete cultures and in natural populations and both studies report the ability of *Trichodesmium* to take up organic P in the form of glycerophosphate. Illustrating *Trichodesmium*'s remarkable P-scavenging ability under growth limiting oligotrophic conditions. An ELF labeling technique, which labels PhoA, the enzyme responsible for alkaline phosphatase activity, has been shown to detect cell-specific phosphorous stress (Dyhrman, et al. 2002). It is still unclear to what extent limited P availability affects the rate of N_2-fixation in natural populations. However, in culture, inorganic phosphorous up to 1.2 μM and organic phosphorous have been shown to stimulate N_2-fixation in *Trichodesmium* (Fu, et al. 2003).

Combined N uptake

Another potential source of variability in N_2-fixation rates is the presence and preferential uptake of combined N. In addition to its ability to fix dinitrogen, *Trichodesmium* can take up various forms of combined nitrogen (NH_4^+, NO_3^-, urea,

amino acids, and DON) from solution (Carpenter, et al. 1975, Glibert, et al. 1988, Goering, et al. 1966, Mulholland, et al. 1999, Mulholland, et al. 2001). Most studies of both natural populations and cultures show that uptake of combined N is extremely low and even undetectable. In the Sargasso Sea, Goering et al. (Goering, et al. 1966) report specific uptake rates that ranged from undetectable to 9×10^{-5} h^{-1} in non-bloom conditions to 1.75×10^{-3} h^{-1} in bloom conditions and Carpenter and McCarthy (1975) reported nitrate uptake rates at or below their limit of detection in the Sargasso Sea and reported no detectable NO_3^- uptake at substrate concentrations below 10 μg-atoms $N \cdot L^{-1}$. Mulholland et al. (Mulholland, et al. 1999, Mulholland, et al. 2001) report a stimulation of NO_3^- uptake when elevated NO_3^- (10μM) is added to batch cultures in exponential growth phase but do not report inhibited N_2-fixation at a 1μM initial NO_3^- concentration (Mulholland, et al. 2001). We report here the inhibition of N_2-fixation across the entire range of initial NO_3^- concentrations we tested in our continuous cultures (0.25μM to 20μM) and propose that *Trichodesmium* is able to overcome the inhibition of N_2-fixation by NO_3^- uptake if the initial NO_3^- concentration is above 2.5μM (Holl, et al. submitted).

Growth Rate

One cannot know or predict the growth rate or history of a population that one might encounter at sea yet N_2-fixation rate measurements necessarily are affected by growth rate. We know that N_2-fixation decreases linearly from early exponential growth to stationary phase in batch culture (Mullholland, et al. 2000, and reported herein) however to date, all N_2-fixation rate measurements and experimental manipulations have been made on batch cultures in exponential growth phase.

We have established and maintained *Trichodesmium* in continuous culture across a range of growth rates in order to establish a stable N_2-fixation rate over a period of at least five days. Our data suggest that as growth rate increases, total culture biomass decreases and N_2-fixation rates increase across the range of growth rates at which steady state growth was achievable for *Trichodesmium* IMS 101.

At the Level of the Gene

Regulation of N_2-fixation is still not well characterized in *Trichodesmium*, however the genome of *Trichodesmium* has recently been completed by the DOE joint genome project, which will undoubtedly facilitate this endeavor. In *Trichodesmium* as well as in many other cyanobacteria, the Fe protein of nitrogenase is subject to post-translational modification when under high O_2 tension or in the presence of ammonium (Bergman, et al. 1997). In *Trichodesmium*, the modified Fe protein has been detected under high O_2 tension (Zehr, et al. 1993) and in the presence of NO_3^- (Ohki, et al. 1991) and the modification is reversible when the conditions are once more favorable for N_2-fixation.

In addition to post-translational regulation, many of the genes encoding for N assimilation in cyanobacteria are repressed by ammonium and are under control of a transcription regulator that is common to the different N assimilation pathways in cyanobacteria (Herrero, et al. 2001). NtcA is a CAP family transcriptional regulator that mediates N control in cyanobacteria and appears to respond to the C:N balance of the cell (Lee, et al. 1999). NtcA is positively autoregulatory and is necessary for the expression of genes encoding for the proteins involved in the assimilation of N sources, for

heterocyst development, and for the full expression of glutamine synthetase (Herrero, et al. 2001). It is clear that NtcA is important for N control in cyanobacteria and, in combination with the circadian rhythm (Ohki, et al. 1992), is likely responsible for control of the N assimilation pathways in *Trichodesmium* as well.

Modeling Efforts

Because of the difficulties inherent in tracking *Trichodesmium* biomass in situ, a number of models of *Trichodesmium* distribution and their potential to supply new N to oligotrophic systems have been published of late. One of these is a model by Fennel et al., (2002) of the N_2-fixation dynamics of *Trichodesmium* at station ALOHA that takes into account the physical forcing that may affect N_2-fixation in this diazotroph (Fennel, et al. 2002). This model accounts for temperature, irradiance, and wind speed, allows for fluctuating N:P ratios in the inorganic and organic pools, and ultimately captures the interannual variation in diazotrophic biomass in the subtropical North Pacific (Fennel, et al. 2002). Hood, et al (2001) model N_2-fixation by *Trichodesmium* in the North Atlantic and implications of this N_2-fixation upon the drawdown of DIC as well as export flux (Hood, et al. 2001). Their results point to significant interannual variation in N_2-fixation rates as a result of decadal-scale climate fluctuations. In a model designed to estimate *Trichodesmium* N_2-fixation via remote sensing, Hood, et al. (2002) employs the use of empirical measurements of N_2-fixation with respect to light intensity from shipboard incubator experiments as well as enumerated vertical trichome profiles in conjunction with SeaWiFS-derived *Trichodesmium* chlorophyll concentration.

CHAPTER 2

ESTABLISHMENT AND CHARACTERIZATION OF A CONTINUOUS CULTURE OF *TRICHODESMIUM* IMS 101

Introduction

Trichodesmium is an important diazotroph broadly distributed in subtropical and tropical oligotrophic oceans (Capone, et al. 1997, Tyrrell, et al. 2003). Its ability to fix dinitrogen and its cosmopolitan distribution make it an important contributor to the pool of new nitrogen in the oligotrophic ocean (Carpenter, et al. 1991, Capone, et al. 1997). Natural populations have been studied for decades, yielding wide variation in reported rates of N_2-fixation (Capone, et al. 1997, Mulholland, et al. 1999, Letelier, et al. 1996). Seasonality, availability of dissolved species including combined N (Holl, et al. submitted, Mulholland, et al. 2001), phosphorous (Sanudo-Wilhelmy, et al. 2001,Wu, et al. 2003, Mulholland, et al. 2002), trace metals (Berman-Frank, et al. 2001, Kustka, et al. 2003) or combinations of these factors (Mills, et al. 2004) may all contribute to variation in the N_2-fixation rate measurements observed in the field.

Trichodesmium has now been cultured and studied under controlled laboratory conditions for more than a decade (Mulholland, et al. 1999, Mulholland, et al. 1999, Ohki, et al. 1986, Chen, et al. 1996, Saino, et al. 1978, Fu, et al. 2003) yet much of its physiology remains poorly understood. Typically, metabolic and physiologic work has been carried out on batch cultures in exponential growth phase. However, N_2-fixation rates do not remain constant during exponential growth (Mulholland, et al. 1999)

potentially leading to significant variation in rates measured at different time points. The highest rates of N_2-fixation are typically observed in early phase cultures (Mulholland, et al. 1999), which also release significant quantities of combined N that is then available for reuptake (Mulholland, et al. 1999, Glibert, et al. 1994). The decline in N_2-fixation rate has been attributed to the uptake of combined N released by the growing culture in the form of NH_4^+ (Mulholland, et al. 1999, Mulholland, et al. 1999) as well as dissolved amino acids such as glutamine and glutamate (Capone, et al. 1994, Carpenter, et al. 1992, Mulholland, et al. 1999). The switch from a culture acquiring N solely from N_2-fixation to one in which recycling and combined N uptake is dominant occurs during mid exponential growth (Mulholland, et al. 2000).

Growth rate is intimately related to N_2-fixation rate in diazotrophs but this relationship has not been studied extensively in *Trichodesmium*, in part because of the challenges associated with developing a continuous culture of this organism. A continuous culture approach allows maintenance of a constant physiological state under which controlled rate measurements and experimental manipulations can be carried out. We established a continuous culture of *Trichodesmium* in which we could study the interactions among growth rate, N_2-fixation rate, and biomass under steady state growth conditions. To our knowledge, this is the first successful continuous culture of *Trichodesmium*, and the first study to yield insight into the steady state dynamics of N_2-fixation and biomass production in this globally significant diazotroph.

Materials and Methods

Culture conditions

Batch cultures – In three trials, triplicate unialgal cultures of *Trichodesmium erythraeum* (IMS-101) were grown at 26°C with a 12:12 light:dark cycle and a daytime photon flux of approximately 170 μE m^{-2} s^{-1}. We used an artificial seawater medium with trace metal and vitamin concentrations as described by (Chen, et al. 1996). This medium contains no added N and an initial phosphate concentration of 5 M. Each culture was grown in a 2.5 L Nalgene 3-port polycarbonate magnetic culture vessel fitted with a tetra-fluoroethylene stir bar and polypropylene/polyvinylidene fluoride stirring assembly in a volume of 1.5 L.

Continuous cultures – Two replicate unialgal cultures of *Trichodesmium erythraeum* (IMS-101) were grown as continuous cultures under the same temperature and light cycle conditions as our batch cultures. We used the same N-free artificial seawater medium with trace metal and vitamin concentrations as described by (Chen, et al. 1996) and an initial phosphate concentration of 5 M. Both cultures were grown in the same vessels as described above with a working volume of 1.7 L. New medium was introduced continuously with a Manostat peristaltic pump at dilution rates ranging from 0.27 d^{-1} to 0.67 d^{-1}. The culture vessel was continuously mixed by suspended magnetic stir bar. A stream of 0.2 m filtered air maintained a slight positive pressure in the headspace to facilitate overflow of the culture through a riser tube and helped minimize airborne contamination. These cultures were not axenic but aseptic techniques were used to minimize contamination. Cultures were considered to be at steady state when in-vivo

chlorophyll fluorescence in the culture overflow was constant for five or more consecutive days.

Analyses and Calculations

At steady state, the continuous culture growth rate (μ) was equal to the medium dilution rate (D), which in turn was controlled by the speed of the peristaltic pump. We measured the volume of the culture overflow daily to monitor dilution rate.
In-vivo fluorescence was measured on a subsample of each culture at the start of the light day (0900 local time) using a Turner model 112 fluorometer at a door setting of 10x. Nutrient samples were immediately filtered through a pre-combusted (450˚C for 2 to 4 h) 25 mm Whatman GF/F filter and stored frozen until analysis of nitrate, phosphate and ammonia with a Lachat QuikChem FIA 8000 nutrient analyzer. The filters were dried at 60° C and then packed in tin capsules for analysis of elemental and stable isotope composition by continuous-flow isotope ratio mass spectrometry (CF-IRMS) using a Carlo Erba NC 2500 elemental analyzer interfaced to a Micromass Optima mass spectrometer.

N_2-fixation was measured using the acetylene reduction assay (ARA) carried out as described in Capone and Montoya (2001). In brief, incubations were carried out in 30 mL Nalgene vials fitted with Teflon-lined septum caps with a 3 mL headspace to which we added 3 mL of acetylene. Incubations were conducted under the same light and temperature conditions as the continuous cultures. The ethylene concentration in the headspace was measured in triplicate by gas chromatography using a SRI model 8610c gas chromatograph fitted with a 2m Hayesep A column and a flame ionization detector.

Acetylene reduction rates were calculated for the three-hour (0900 to 1200 local time) experimental incubation using the average of three headspace measurements at the final time point. We used a reduction ratio (C_2H_2:N_2) of 4:1 to convert acetylene reduction to N_2-fixation rates (Capone, et al. 2001). The rates for the replicate vials were then averaged and normalized to the particulate N concentration measured at the start of the incubation and to the total time of the incubation to obtain an hourly N_2-fixation rate normalized to biomass. Hourly rates were extrapolated to daily rates based on the daily time course of N_2-fixation described by Mulholland and Capone (2001).

Total phosphorous (TP) was measured on a fraction of the particulate filter by persulfate oxidation (Raimbault, et al. 1999). Oxidizing agent was made fresh daily and oxidized samples were filtered through a Millex-GV 0.22 μm filter unit before analysis of total nitrate and phosphate with a Lachat QuikChem FIA 8000 nutrient analyzer.

Results

Batch cultures

N_2-fixation decreased as biomass increased from the first day of exponential growth in each of the three trials (Figure 1 panels A – C) and did not stabilize at any time during exponential growth. The hourly N_2-fixation rate in all three trials ranged from 0.5 x 10^{-2} h^{-1} to 0.3 x 10^{-1} h^{-1} with a mean of 0.2 ± 0.07 x 10^{-1} h^{-1} (mean ± SD, N = 19). Mean daily N_2-fixation rates within each trial were 0.5 ± 0.2 d^{-1} (mean ± SD, N = 6), 0.3 ± 0.06 d^{-1} (mean ± SD, N = 7), and 0.3 ± 0.1 d^{-1} (mean ± SD, N = 6), respectively (Figure 1 panels A – C). The slope of the linear portion of the curve of PN vs. time was used to calculate specific growth rates that ranged from 0.39 d^{-1} to 0.45 d^{-1} with a mean of 0.42 ± 0.03 d^{-1} (mean ± SD, N=9) during the exponential growth phase.

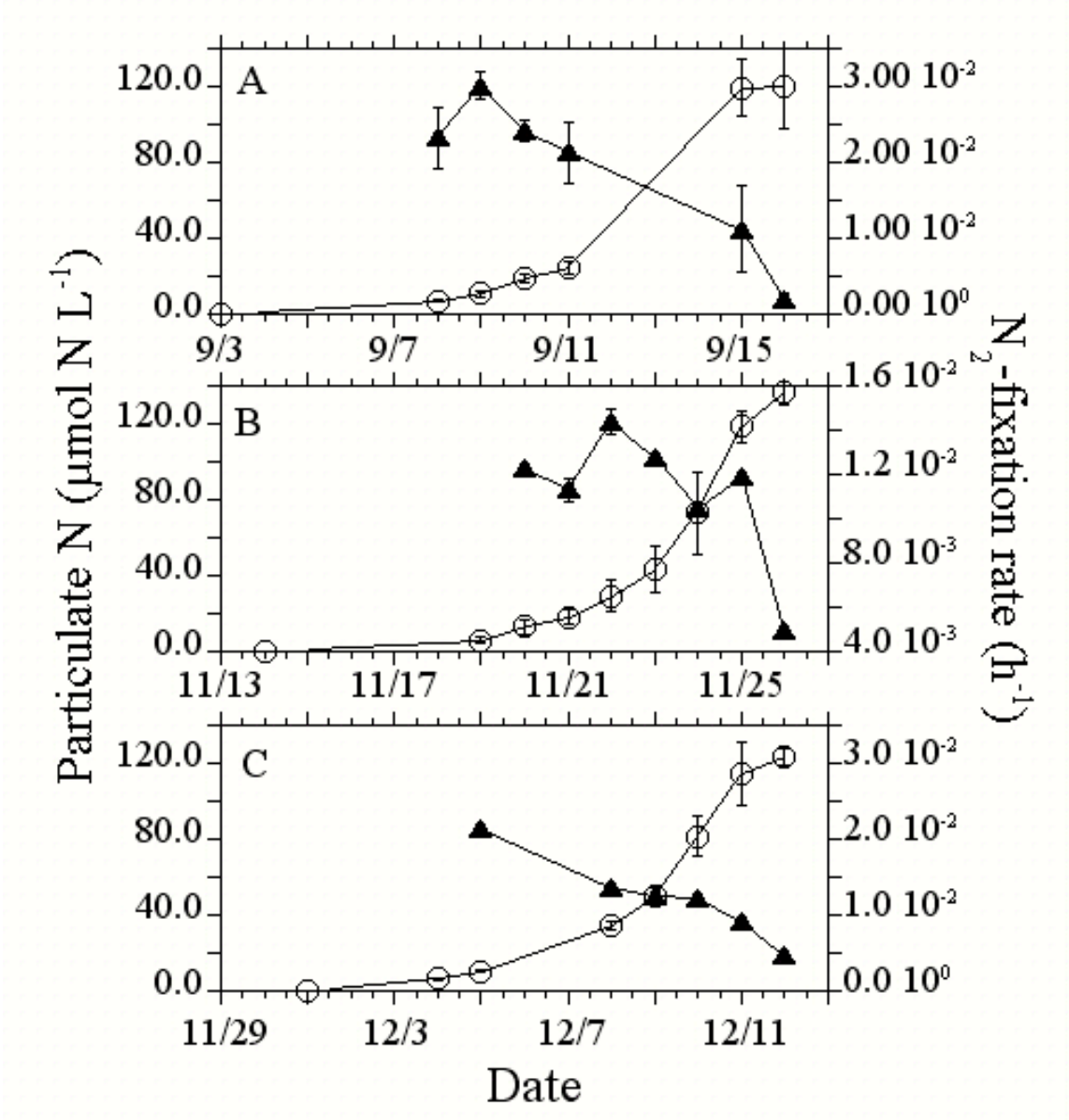

Figure 1 – Time course of PN accumulation (open circles) and specific N_2-fixation (triangles) in Batch Culture

In our batch cultures, phosphate concentrations declined from an initial value of 5μM to below the limit of detection during the middle to late exponential growth phase. Ammonium concentration increased in two of the three trials from 0.5 μM at the start of exponential growth to 0.8 μM at the end of exponential growth (nutrient data not shown) and did not change during exponential growth in the third set of batch cultures. Nitrate was not measurable at any time in these batch cultures.

Continuous cultures

In our continuous cultures, N_2-fixation rate and biomass (PN) are inversely related, reflecting the normal relationship between growth rate and biomass in a continuous culture. Particulate N concentration decreased linearly as growth rate increased from 0.27 to 0.67 d^{-1} (Figure 2). With each adjustment of the dilution rate we measured in-vivo chlorophyll fluorescence, PN, and ARA daily. Steady state was defined as less than 5% variation in in-vivo chlorophyll fluorescence for 5 or more consecutive days. We determined the maximum growth rate of *Trichodesmium* under these culture conditions, $\mu_{max} = 0.67\ d^{-1}$, by increasing the dilution rate until we observed washout of biomass.

Biomass specific N_2-fixation rates, normalized to PN concentration, increased with growth rate to a maximum of 0.77 d^{-1} at a growth rate of 0.51 d^{-1} (Figure 2). The increase was linear (N_2-fixation rate = 1.63 x μ (d^{-1}) – 0.24, $R^2 = 0.76$, N=7 $p<0.05$) with growth rate from $\mu = 0.27$ to 0.51 d^{-1} (Table 1). Interestingly, the two steady state experiments with $\mu > 0.57\ d^{-1}$ showed reduced N_2-fixation rates. Our biomass specific N_2-fixation rates are equal to instantaneous N-specific growth rates, which are linearly

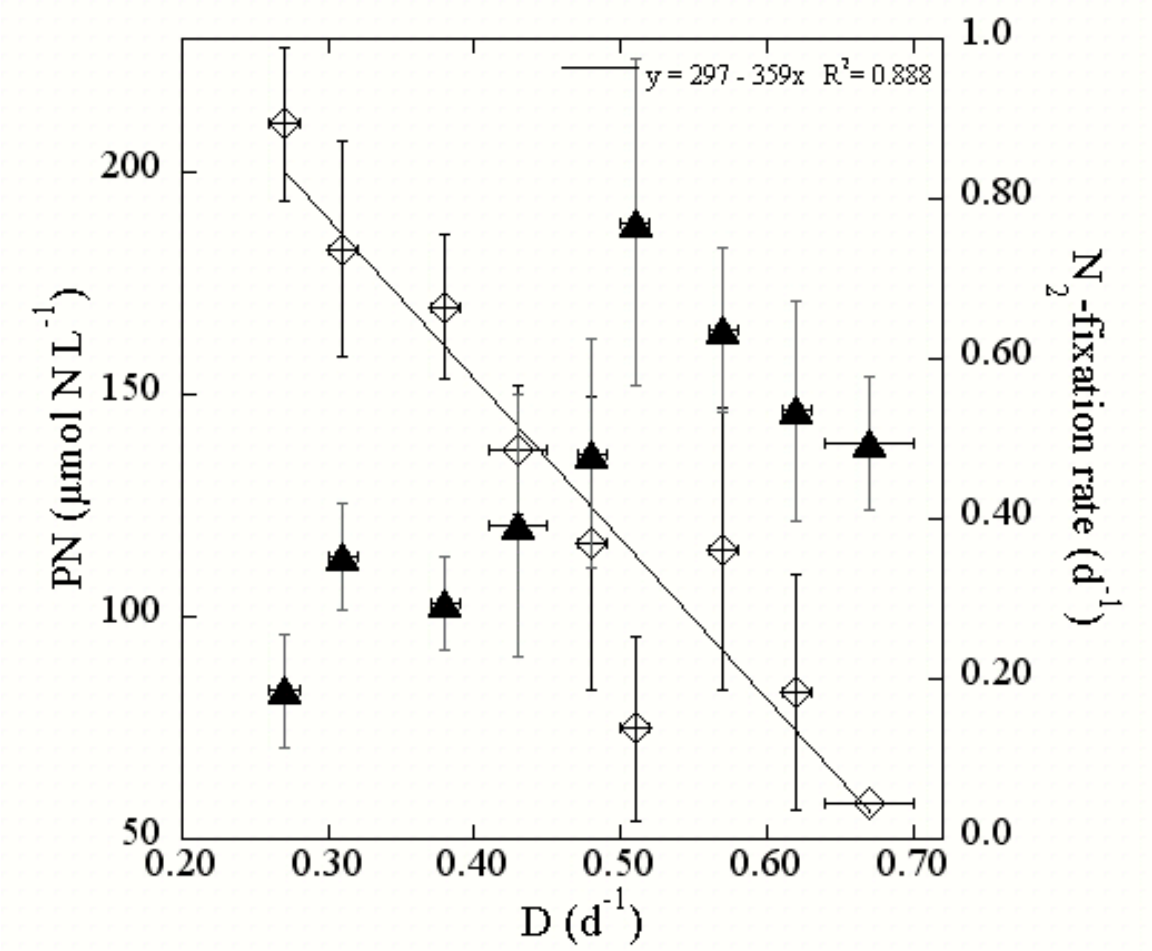

Figure 2 – Particulate N (open diamonds) and N_2-fixation (triangles) as a function of specific growth rate.

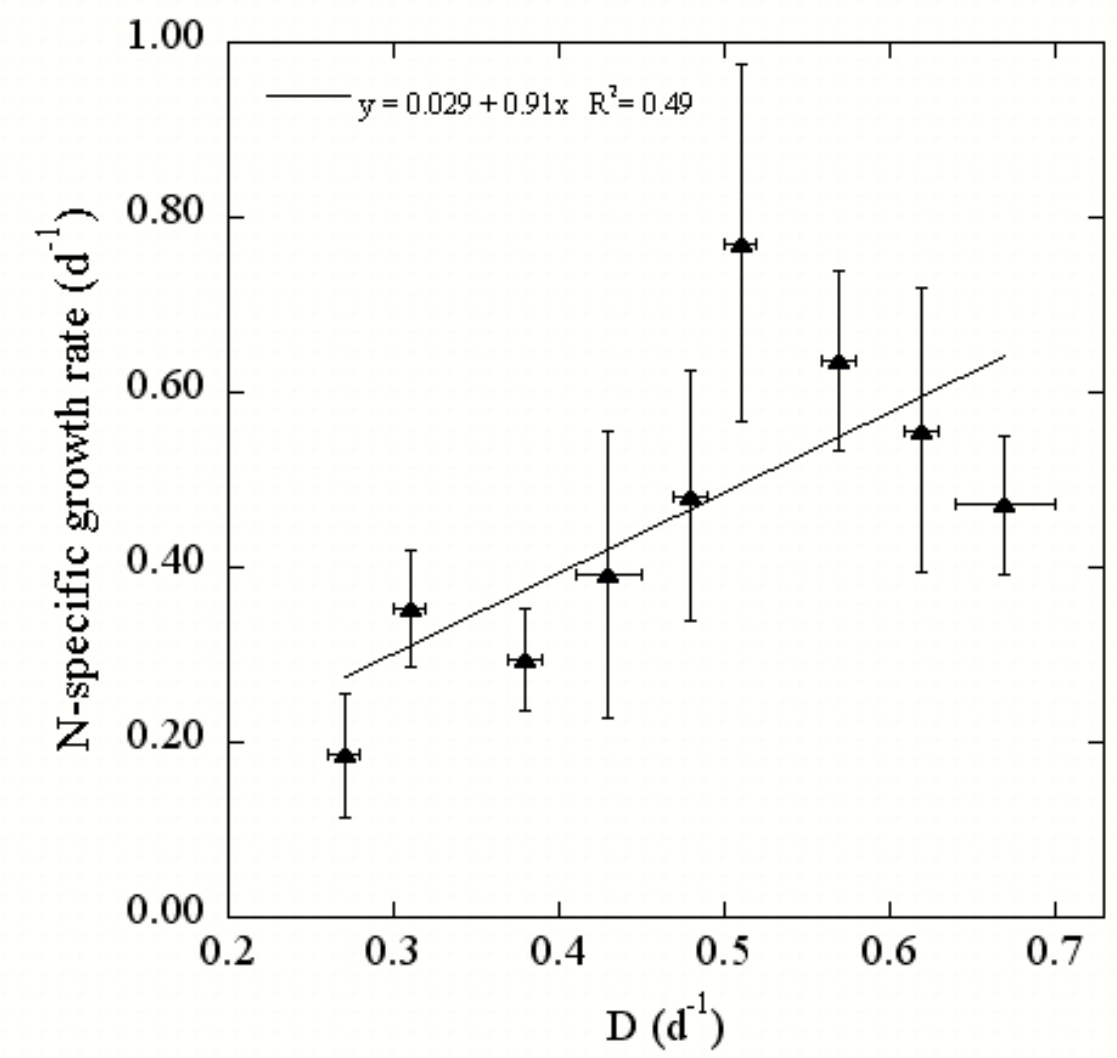

Figure 3 – N-specific growth rate as a function of dilution rate

related to dilution rate (D) across the entire range of growth rates with a slope of 0.91, R^2 = 0.49 (Figure 3).

Neither elemental (C:N, N:P) nor fluorescence:PN ratios varied significantly with growth rate. C:N ratios varied from 4.58 to 6.56 with a mean of 5.63 ± 0.35 (mean ± SD, N= 93). N:P ratios showed considerably greater variation, with a range of 8 to 54 and a mean of 27.14 ± 7.42 (mean ± SD, N=74), but there was no correlation with growth rate. The fluorescence:PN ratio, which we used as a proxy for biomass specific fluorescent pigment concentration, did not change across our range of growth rates (Table 1).

Discussion

Our batch culture study showed a clear decrease in N_2-fixation rate during the exponential growth phase. This decrease could reflect increasing phosphate limitation (Wu, et al. 2000) and/or re-uptake of newly fixed and exuded N as the culture matures (Mulholland, et al. 1999, Glibert, et al. 1994). N_2-fixation rates in early exponential phase are roughly double those measured in late exponential phase (Figure 1 panels A-C). The constantly changing growth conditions in a batch culture can cause variation in N_2-fixation rate and may help explain much of the variation found in field populations. The pattern of decreasing N_2-fixation rates during exponential growth in batch culture necessitates the establishment of a continuous culture of *Trichodesmium* in which one can maintain a steady state growth rate, and therefore N_2-fixation rate. This physiological steady state provides a standard or baseline, which can then be experimentally manipulated.

Table 1 – Dilution rate, PN, N-specific growth rate, and fluorescence:PN ratio for the range of growth rates in continuous culture.

D (d^{-1})	PN ($\mu mol\ N\ L^{-1}$)	N Specific growth rate (d^{-1})	SD	N	Fluorescence: PN ratio
0.27	210.82	0.19	0.07	12	0.13
0.31	182.66	0.35	0.07	10	0.14
0.38	169.64	0.29	0.06	10	0.13
0.43	137.57	0.39	0.16	10	0.15
0.48	116.53	0.48	0.14	14	0.15
0.51	74.79	0.77	0.20	12	0.20
0.57	115.24	0.64	0.10	10	0.12
0.62	83.06	0.53	0.14	12	0.13
0.67	57.95	0.49	0.08	10	0.15

In continuous culture, decreasing biomass with increasing growth or dilution rate is the normal pattern for nutrient or light limited chemo- or cyclostats. This pattern has been shown for natural phytoplankton assemblages from an oligotrophic lake (Suttle, et al. 1986), the marine microalga, *Isochrysis galbana* (Grima, et al. 1997), the diazotrophic unicellular cyanobacterium *Gloeothece sp.* (Ortega-Calvo, et al. 1991), the freshwater alga *Scenedesmus sp.* (Rhee, et al. 1981), the coccolithophorid *Emiliania huxleyi* (Riegman, et al. 2000), the marine diatom *Thalassiosirea fluviatilis* (Laws, et al. 1980), and for the freshwater cyanobacteria *Anabaena sp.* and *Aphanizomenon flos-aquae* (Nobel, et al. 1997). Likewise, in our continuous cultures, as dilution rate increased PN concentration decreased linearly (Figure 2) (R^2=0.88) and we were able to maintain a steady state biomass concentration at each of the growth rates across the range we tested for 5 or more days.

The lowest steady state we attempted to maintain was $\mu = 0.27\ d^{-1}$. At low growth rates, the culture was fairly dense, potentially leading to self-shading and light-limitation of growth. However, the ratio of fluorescence to PN concentration did not change across our range of growth rates (Table 1), suggesting that self-shading and photoadaptation at low growth rates was not significant. To determine if *Trichodesmium* could maintain a steady state lower that $0.27\ d^{-1}$ would have required further manipulation of the culture conditions to alleviate density dependent effects.

C:N ratios remained constant at all growth rates, which reflects close coupling of carbon and N_2-fixation across the range of growth rates we investigated. N:P ratios also remained stable across the growth rates investigated and stable macronutrient ratios suggest we had achieved balanced growth in our continuous cultures. A C:N:P ratio of

152:27:1 and no measurable residual P in the overflow, at even high growth rates, suggests P limitation at all growth rates. This limitation may have been overcome by APA activity (Stihl, et al. 2001, Dyhrman, et al. 2002, Mulholland, et al. 2002) since N:P ratio did not deviate significantly across the range of growth rates we investigated.

It is difficult to compare continuous culture studies as the culture conditions often differ depending on the physiological processes under investigation. An even greater distinction is that, with the exception of *Gloeothece sp., Anabaena sp.,* and *Aphanizomenon flos-aquae,* the organisms studied to date are not diazotrophs and were supplied with a source of combined N, as opposed to our obligately diazotrophic culture conditions. Among the diazotrophic species studied in continuous culture, the freshwater cyanobacteria *Anabaena sp*. and *Aphanizomenon flos-aquae* are the most closely related to *Trichodesmium*. However, both of these cyanobacteria are heterocystous and thus spatially separate O_2 production and N_2 fixation, in contrast to the nonheterocystous *Trichodesmium* (Berman-Frank, et al. 2001). This fundamental physiological difference may distinguish them from *Trichodesmium* in terms of growth rate and N_2-fixation rate.

Therefore, though it is unicellular, the marine cyanobacterium *Gloeothece sp.* may be a better comparison to our work. In continuous culture, *Gloeothece sp.* fixes dinitrogen concomitantly with photosynthesis and during light phase regardless of the imposed light-dark cycle (Ortega-Calvo, et al. 1991). N_2-fixation in *Gloeothece sp.* increased with increasing growth rate until the culture began to washout. The nitrogen content of the diazotrophic *Gloeothece sp.* culture decreased with increasing growth rate as did protein and chlorophyll a (Ortega-Calvo, et al. 1991). N_2-fixation rates of *Gloeothece sp.* measured at three dilution rates (Ortega-Calvo, et al. 1991) increased as

dilution rate increased from 0.12 to 0.36 d^{-1}. As seen in *Gloeothece sp.* cultures, *Trichodesmium* N_2-fixation rate increased with increasing dilution rate and N content of the culture decreased linearly with increasing dilution rate (Figure 2). Though there was some variation in N_2-fixation rate at each growth rate (Figure 2), the increase in specific N_2-fixation rate was linear from a growth rate of 0.27 d^{-1} to 0.51 d^{-1} ($R^2 = 0.76$). At the two highest growth rates tested, N_2-fixation decreased, but only the highest growth rate tested yielded a specific N2-fixation rate markedly lower than the dilution rate (Figure 3).

Trichodesmium may excrete DON (Capone, et al. 1994, Glibert, et al. 1994, Mulholland, et al. 1999), which then becomes a potential source of combined N to individual cells that do not possess actively fixing nitrogenase (Berman-Frank, et al. 2001, Lin, et al. 1998). We did not measure DON concentration directly in this study but our data allow us to constrain the magnitude of excretion and re-uptake under steady state conditions. ARA measures nitrogenase activity rather than the incorporation of N_2 into protein. As a result, ARA will reflect the production and accumulation of DON in the medium as well as the production of new biomass, yielding N-specific activity rates greater than the continuous culture dilution rate when DON production is important. Accumulation of significant quantities of DON in the growth medium can occur only if the N-specific rate of N_2-fixation measured by ARA is greater than the culture dilution rate.

In our continuous cultures, *Trichodesmium* fixed N_2 at a specific rate indistinguishable from D at all except the highest dilution rate tested (Figure 3). Our data imply that little or no DON accumulated in solution, therefore either DON excretion was unimportant, or rapid uptake prevented accumulation of DON following excretion. Our

data do not allow us to distinguish between these two possibilities, though they show a closed N budget across a range of steady state growth rates for this important cyanobacterium.

A consistent challenge in field studies of *Trichodesmium* is assessing the growth status and history of a natural population of *Trichodesmium* at the time of sampling. Some of the variation in N_2-fixation rates measured on field populations may reflect differences in the growth phase of the population at the time of sampling, as well as the potentially inhibitory effects of trace metal limitation, phosphate limitation, non-optimal temperature and light regimes, as well as the presence of combined N. Our work clearly shows that N_2-fixation rate is intimately related to growth rate. Growth rate and the rate at which a bloom may be progressing need to be included in models of *Trichodesmium* N_2-fixation in order to accurately assess the impact of new nitrogen supplied by this important diazotroph to the oligotrophic waters in which it thrives.

CHAPTER 3

INTERACTION BETWEEN N_2-FIXATION AND NITRATE UPTAKE IN CONTINUOUS CULTURES OF *TRICHODESMIUM* IMS 101

Introduction

Trichodesmium is a non-heterocystous, diazotrophic marine cyanobacterium found in oligotrophic tropical and sub-tropical seas worldwide (Capone, et al. 1997). It can form extensive blooms and appears to supply a significant quantity of newly fixed nitrogen to the oligotrophic ocean (Capone, et al. 1997, Carpenter, et al. 1991). Much effort has gone into quantifying the rate of N_2-fixation in natural populations (Carpenter, et al. 1975, Mulholland, et al. 1999, Saino, et al. 1978) and in lab cultures of *Trichodesmium* (Mulholland, et al. 1999, Mulholland, et al. 1999) and in extending such measurements to estimate the impact of *Trichodesmium* N_2-fixation on the regional and basin scale (Hood 2002). In addition to its ability to fix dinitrogen, *Trichodesmium* can take up various forms of combined nitrogen (NH_4^+, NO_3^-, urea, amino acids, and DON) from solution (Carpenter, et al. 1975, Glibert, et al. 1988, Goering, et al. 1966, Mulholland, et al. 1999, Mulholland, et al. 2001). Studies of natural populations (compiled in Mulholland, et al. 1999) have shown that *Trichodesmium* has a high capacity for NH_4^+ uptake and that amino acid and urea uptake rates are typically low and variable but detectable. In contrast, NO_3^- uptake rates were either very low or undetectable in most studies to date. In the Sargasso Sea, Goering, et al. (1966) used a tracer addition of 2 □g-atoms $^{15}NO_3^-{\cdot}L^{-1}$ to measure nitrate uptake and reported specific uptake rates that ranged from undetectable to 9 □ 10^{-5} h^{-1} in non-bloom conditions to 1.75

$\sim 10^{-3}$ h^{-1} in bloom conditions. Carpenter and McCarthy (1975) reported nitrate uptake rates at or below their limit of detection in the Sargasso Sea and reported no detectable NO_3^- uptake at substrate concentrations below 10 μg-atoms $N \cdot L^{-1}$.

In batch cultures growing exponentially, Mulholland (Mulholland, et al. 2001) has shown that NO_3^- uptake is stimulated by increased concentrations of NO_3^- (1 μM and 10 μM additions). However, short-term rates of N_2 fixation were not affected by additions of 1μM NO_3^- to cultures or to natural populations of *Trichodesmium* (Mulholland, et al. 2001).

Although *Trichodesmium* is generally thought to obtain the great majority of its N ration via N_2-fixation, rates measured in the field show wide temporal and spatial variation (Mulholland, et al. 1999). The causes of this variability remain unclear, but environmental factors including the availability of combined nitrogen may contribute to the observed variation in N_2-fixation rates. Relatively little effort to date has been devoted to studying the utilization of combined nitrogen sources by *Trichodesmium*, or the interactions among potential nitrogen sources. While it is clear that *Trichodesmium* can in fact take up at least some forms of combined N, the effects of combined N uptake on the rate of N_2-fixation have not been systematically studied. Here we report on the effects of NO_3^- exposure on both N_2-fixation and NO_3^- uptake by *Trichodesmium* (IMS-101) maintained in continuous culture on a nitrogen-free medium. In this study, we focused on quantifying the physiological interactions between N_2-fixation and uptake of environmentally relevant concentrations of NO_3^-.

Materials and Methods

Continuous Culture Conditions

A unialgal culture of *Trichodesmium erythraeum* (IMS-101) was grown in continuous culture at 26° C with a 12:12 light:dark cycle and a daytime photon flux of approximately 128 μmol $m^{-2} \cdot s^{-1}$. This culture was not axenic but every effort was made to keep contamination to a minimum. We used an artificial seawater medium with trace metal and vitamin concentrations as described by Chen, et al. (1996). This medium had no added N and a phosphate concentration of 10 μM. Each culture was grown in a 1 L Nalgene® 3-port polycarbonate magnetic culture vessel fitted with a tetra-fluoroethylene stir bar and a polypropylene/polyvinylidene fluoride stirring assembly. The volume of the culture was maintained at 1.5 L by passive overflow, and new medium was introduced continuously at a dilution rate of 0.3 d^{-1}. The culture vessel was gently mixed by a suspended magnetic stir bar and a stream of 0.2 μm filtered air maintained a slight positive pressure in the headspace to facilitate overflow of the culture through a riser tube as well as to minimize airborne contamination. Cultures were considered to be at steady state when in-vivo chlorophyll fluorescence in the culture overflow was constant for three or more consecutive days.

Experimental Manipulations

Just before the start of the light day (08:00 local time) the medium pump was stopped, the culture was split and half was transferred to either an additional acid-washed culture vessel or an acid-washed 500 mL Nalgene polycarbonate bottle. One vessel was then amended with NO_3^- while the other was left unamended as a control. Immediately following the NO_3^- addition, and at hourly intervals throughout the day, samples were

collected from the control and experimental (NO_3^- amended) vessels for measurement of nutrient and particulate nitrogen (PN) concentrations. Additional samples were collected from each vessel for duplicate acetylene reduction assays every two hours beginning immediately after the NO_3^- addition to the experimental vessel and continuing through the light day.

Acetylene reduction assays were carried out as described in Capone and Montoya (2001). In brief, incubations were carried out in 30 mL Nalgene vials fitted with Teflon-lined septum caps with a 3 mL headspace to which we added 3 mL of acetylene. Each assay vial was incubated for three hours under the same light and temperature conditions as the original continuous culture. Upon termination of each incubation, the ethylene concentration in the headspace was measured in triplicate by gas chromatography using an SRI 8610c gas chromatograph fitted with a 2m Hayesep A column and a flame ionization detector.

Analyses and Calculations

Fluorescence was measured on an aliquot of each culture at the start of the light day using a Turner fluorometer model 112 at a door setting of 10x. Nutrient samples were filtered through a 25 mm Whatman GF/F filter immediately after collection and stored frozen until analysis with a Lachat QuikChem FIA 8000 nutrient analyzer. The filters were dried at 60° C, then packed in tin capsules for analysis of carbon, nitrogen and stable isotope composition by continuous-flow isotope ratio mass spectrometry (CF-IRMS) using a Carlo Erba NC 2500 elemental analyzer interfaced to a Micromass Optima mass spectrometer.

Acetylene reduction rates were calculated for each three-hour experimental incubation using the average of three terminal headspace measurements. We used a reduction ratio (C_2H_2:N_2) of 4:1 to convert acetylene reduction to N_2-fixation rates (Capone, et al. 2001). The rates for the replicate vials were then averaged and normalized to the particulate nitrogen concentration. The total N_2-fixation during the light day was computed by integrating the rates of the six sequential acetylene reduction incubations started at two-hour intervals spanning the entire light day.

NO_3^- uptake rates were calculated from the rate of disappearance of nitrate from solution, and then normalized to particulate nitrogen concentration. Both the acetylene reduction and the nitrate uptake rates were normalized to the particulate nitrogen concentration measured at 11:00 (local time) to produce biomass-specific rates for comparison across experiments.

Results

NO_3^- uptake and inhibition of N_2-fixation

We observed measurable inhibition of N_2-fixation even at the lower end of the range of NO_3^- additions tested (0.5 – 20 μM) (Figure 4), and the inhibition persisted through much or all of the light day in most cases. In experiments with small nitrate additions (< 5 □M) we observed some recovery of N_2-fixation activity approximately 8 hours after the NO_3^- addition (Figure 4B). The recovery of N_2-fixation activity appeared to coincide with a reduction of ambient NO_3^- concentrations below about 0.5 μM. In experiments with large initial NO_3^- additions the ambient concentration of NO_3^- did not decrease to such low levels over the course of the experiment, and no recovery of N_2-fixation activity was observed (Figure 4A). The inhibition effect showed apparent

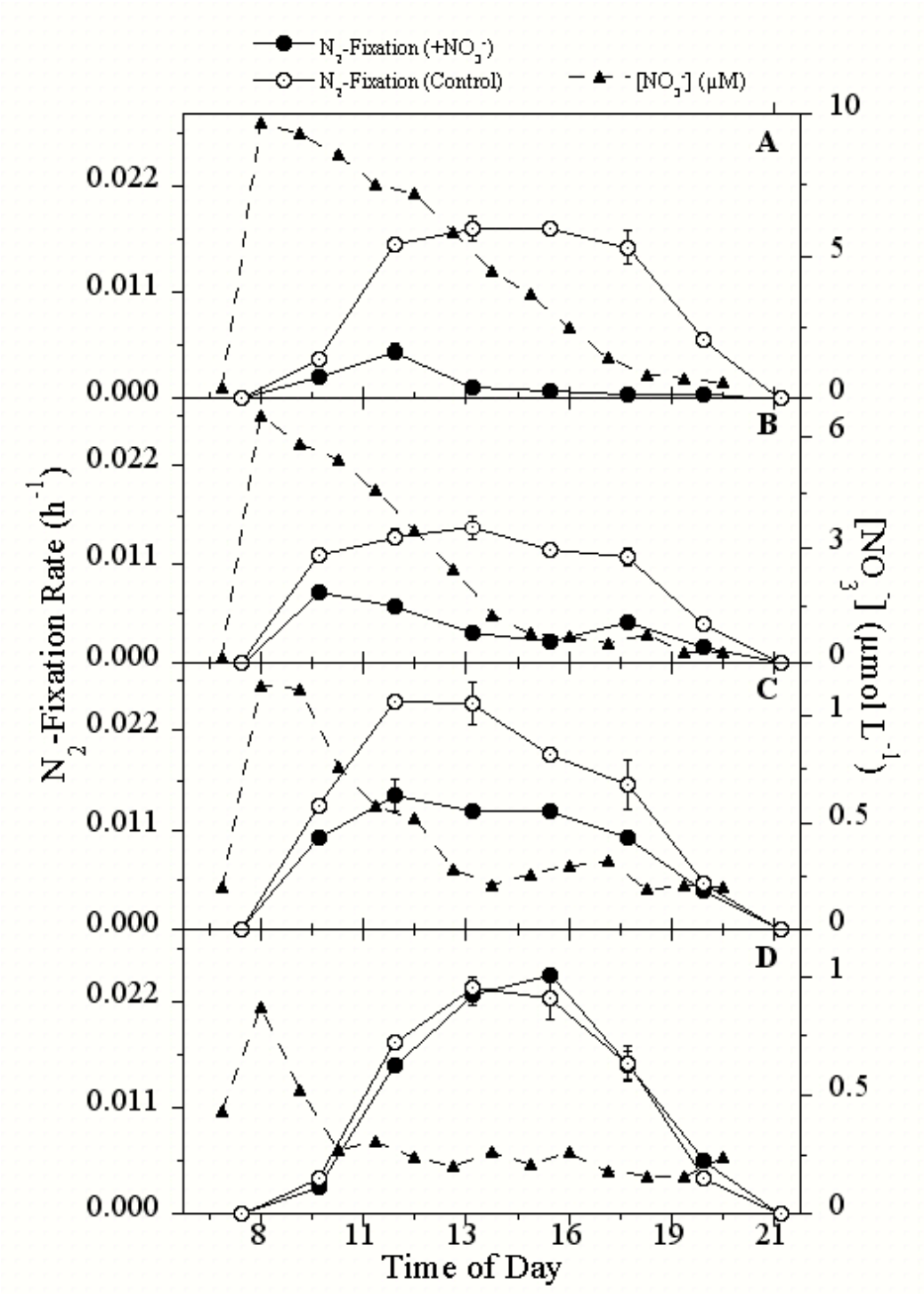

Figure 4 – Time course of N_2-fixation in Nitrate Amended Cultures

saturation kinetics, reaching an asymptotic value of about 70% inhibition at an initial NO_3^- concentration of 10 μM (Figure 5).

We did not find complete inhibition of N_2-fixation at even the highest NO_3^- concentrations used in this study. For example, a 20 μM NO_3^- addition suppressed N_2-fixation by 66%, yet N2-fixation still accounted for 8% of the total N assimilation by *Trichodesmium* in the experimental vessel (Figure 6) despite the presence of abundant NO_3^- throughout the day.

NO_3^- Uptake Kinetics

Our data clearly show immediate and continuing uptake of added NO_3^- during the "daytime" portion of the 12:12 L:D cycle. Nitrate uptake rates (V_{NO3-}) increased with increasing initial NO_3^- concentration ($V_{NO3-} = 8.7 \times 10^{-4} + 1.4 \times 10^{-3}[NO_3^-]$, $R^2 = 0.85$). Nitrate uptake rates during the 14 hour incubation ranged from 1.5 □ 10^{-3} h^{-1} at the lowest initial NO_3^- addition, 0.5μM, to 2.4×10^{-2} h^{-1} at the highest NO_3^- addition, 20 μM. Nitrate consumption was negligible during the dark portion of the 12:12 L:D cycle. The instantaneous N-based specific growth rate (V_{total}) in the treatments was computed using total N uptake (NO_3^- uptake plus N_2-fixation) over the 14 hour experimental time period and PN measurements made at the end of the experimental time period. V_{total} varied between 0.12 d^{-1} and 0.61 d^{-1} as a linear function of initial NO_3^- concentration ($V_{total} = 0.1 + 2.4 \times 10^{-2}$ $[NO_3^-]$, $R^2 = 0.83$).

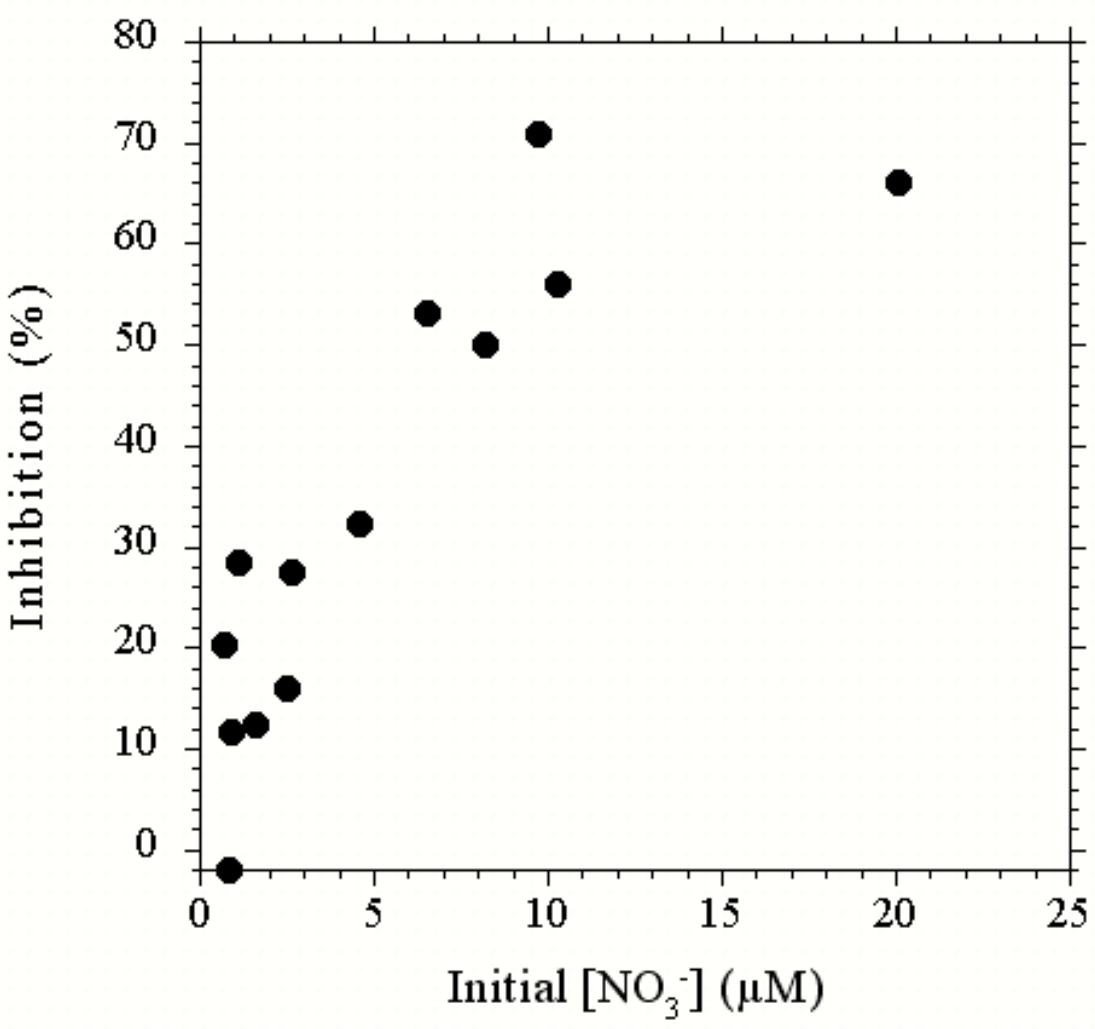

Figure 5 – Relative inhibition of N_2-fixation as a function of initial nitrate concentration

Only those treatments with the highest initial NO_3^- concentrations (7 μM and 20μM) had N-based instantaneous growth rates comparable to the steady state growth rate of these cultures before the start of the experiment, 0.3 d^{-1} (i.e., a doubling time of 2.3 d). An initial NO_3^- concentration of 20 μM produced a doubling time of 1.14 d, while the lowest initial NO_3^- addition tested (0.5 μM) produced a doubling time of 5.85 d in these experiments. ΔV_{total}, defined as the difference between V_{total} in the treatment and control incubations, increased with increasing initial NO_3^- concentration ($\Delta V_{total} = -6.7 \times 10^{-2} + 1.8 \times 10^{-2}$ [NO3-], R^2=0.72). By definition, ΔV_{total} is therefore negative when control V_{total} is higher than treatment V_{total} and this pattern was observed in four of the five treatments amended with <2.5μM NO_3^-.

Effect of NO_3^- uptake on total N assimilation

The impact of NO_3^- on the total rate of N uptake by *Trichodesmium* varied across the range of NO_3^- concentrations investigated. At high concentrations (>2.5 μM) the uptake of NO_3^- more than compensated for the inhibition of N_2-fixation. This resulted in greater total N consumption in the treatments amended with >2.5μM NO_3^- than in the corresponding controls (Figure 7).

Small additions of NO_3^- (< 2.5 μM) led to significant inhibition of N_2-fixation by *Trichodesmium* (Figures 4C and 4D). However, at the lower initial NO_3^- concentrations (<2.5 μM) most treatments were just able make up for the inhibition of N_2-fixation by taking up NO_3^-. Therefore, total N consumption in the treatments was not significantly different than total N consumption in the corresponding controls (Figure 7).

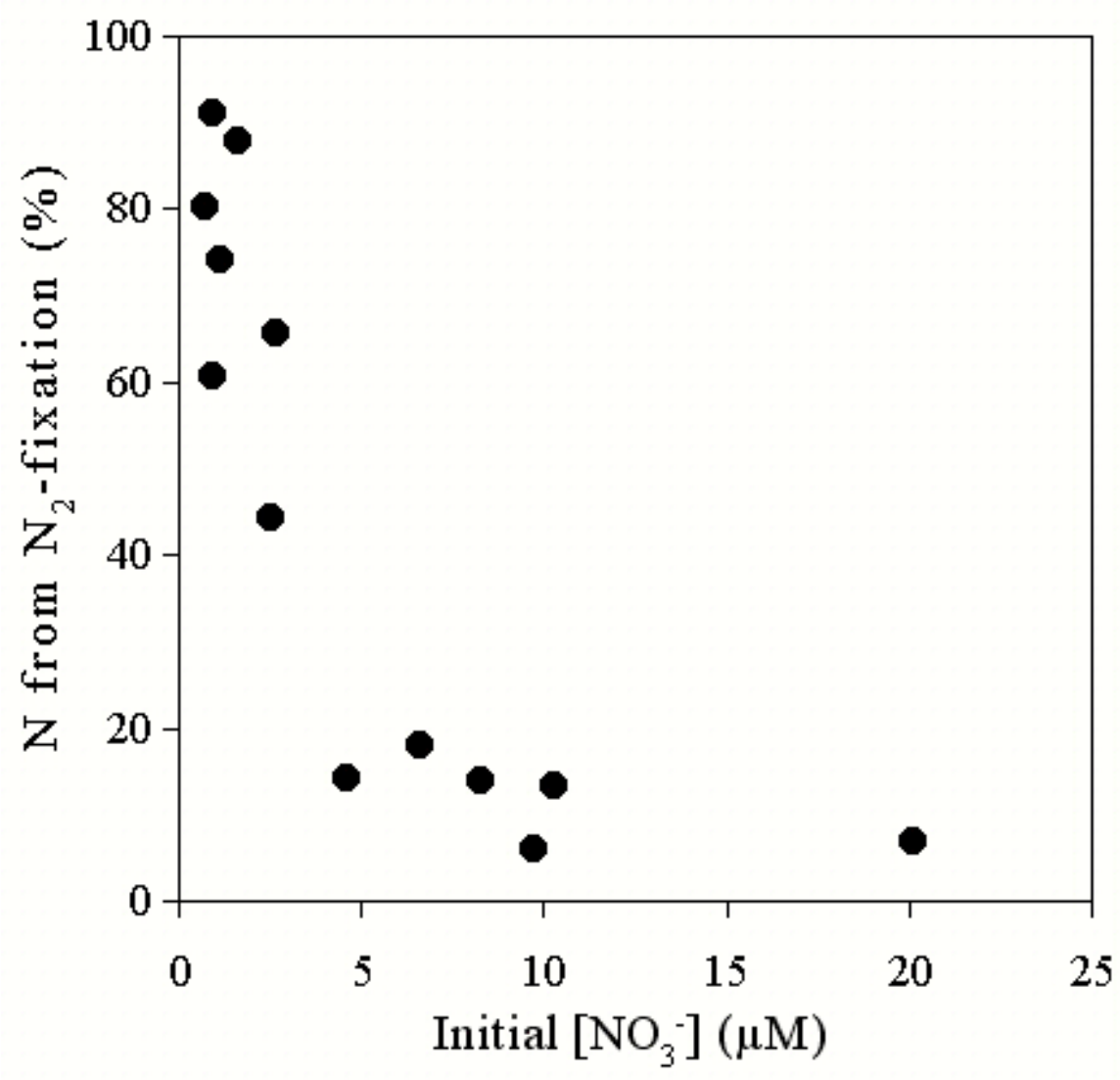

Figure 6 – Contribution of N_2-fixation to total N uptake as a function of initial nitrate concentration.

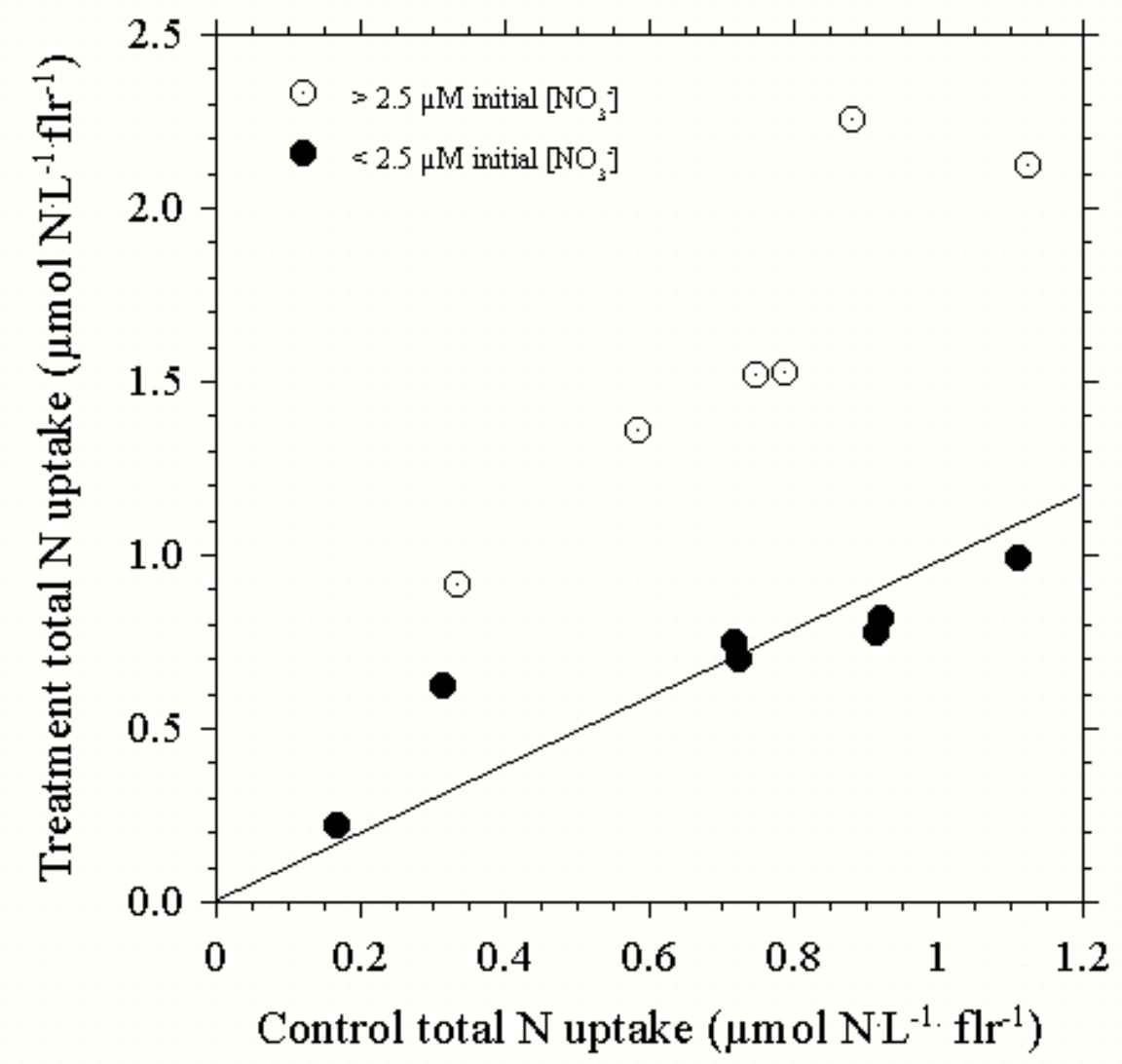

Figure 7 – Total N uptake in nitrate amended treatments as compared to total N uptake in corresponding unamended controls.

Discussion

The ability to fix dinitrogen potentially gives *Trichodesmium* a strong competitive advantage in oligotrophic waters where the availability of combined nitrogen may severely limit the growth of non-diazotrophs. Although appreciable NO_3^- concentrations are uncommon at the surface in regions where *Trichodesmium* is typically found, mixing events may inject NO_3^- into the surface layer and NO_3^- is always available near the base of the mixed layer where *Trichodesmium* colonies and free trichomes can be found. As a result, *Trichodesmium* in the field may encounter measurable concentrations of NO_3^- on a regular basis. However, relatively little is known about the effects of combined nitrogen on the N acquisition strategy of *Trichodesmium.*

N_2-fixation by *Trichodesmium* in culture and natural populations exhibits a reproducible diurnal pattern when incubated under simulated in situ conditions: N_2-fixation rates increase rapidly at the start of the light day, peak at midday, and decrease in mid-afternoon when nitrogenase is inactivated and subsequently degraded (Capone, et al. 1990). At night, N_2-fixation is completely shut down until the following morning. Previous studies have shown that nitrogenase is produced *de novo* each day approximately 2 h prior to first light (Capone, et al. 1990) and that transcription of nitrogenase genes as well as the activity of the protein itself is regulated by an endogenous clock (Chen, et al. 1998).

NO_3^- Inhibition of N_2-Fixation

The inhibition of N_2-fixation followed a reproducible temporal pattern in our experiments. NO_3^- was added to the culture flask immediately preceding the daily

dark/light transition. In the presence of NO_3^-, NO_3^- uptake and N_2-fixation occurred simultaneously and N_2-fixation was typically inhibited as early as our first time point. Even a 0.5 μM initial NO_3^- concentration induced immediate consumption of NO_3^- by *Trichodesmium*. These treatments show a clear increase in NO_3^- uptake rate as well as an increase in the inhibition of N_2-fixation as the initial NO_3^- concentration is increased. In experiments with an initial NO_3^- amendment greater than 5 μM, the ambient NO_3^- concentration and uptake rate remained high throughout the afternoon, and we found no recovery of N_2-fixation activity during the remainder of the light day (Figure 4A). In treatments amended with less than 5 μM NO_3^- we observed some recovery of N_2-fixation late in the afternoon when NO_3^- concentrations fell below 0.3 – 0.4 μM (Figure 4). Treatments with low initial concentrations of NO_3^- (<2.5 μM) typically resulted in the ambient concentration decreasing to 0.1 - 0.3 μM by midday, after which the rate of N_2-fixation in the experimental vessel increased and approached that of the control.

In all of our experiments, NO_3^- uptake by *Trichodesmium* rapidly lowered the ambient concentration in the experimental vessel, but higher initial concentrations naturally led to a longer exposure of the experimental culture to elevated NO_3^- concentrations. The persistence of significant ambient NO_3^- concentrations through the early afternoon when nitrogenase activity is normally decreasing may be critical in maintaining a high degree of inhibition of N_2-fixation through the end of the light day (Figure 4).

In exponentially growing batch cultures, Mulholland et al. (2001) found inhibition of N_2-fixation after a 10 μM NO_3^- amendment at the start of the light day. However, they did not report significant inhibition of N_2-fixation relative to the corresponding control

for a 1 μM NO_3^- amendment. The physiology of cells growing at steady state in a continuous culture clearly differs from that of cells in exponential growth in batch culture, which may explain the difference between our results and those of Mulholland et al. (2001). It is possible that the uptake we observed is a result of "luxury consumption", a strategy that may not be available to exponentially growing batch cultures. We have not yet completely characterized the growth kinetics of our continuous cultures, but our results clearly show that *Trichodesmium* at steady state can take up NO_3^- from solution at significant rates, and that NO_3^- uptake has a strong inhibitory effect on N_2-fixation over the range of concentrations tested. Our results also demonstrate that *Trichodesmium* can meet essentially all of its instantaneous N demand via NO_3^- assimilation when concentrations are high enough.

Kinetics of NO_3^- Uptake and Inhibition of N_2-fixation

Our results clearly show immediate NO_3^- uptake when *Trichodesmium* is exposed to NO_3^- at the start of the light day. In the presence of NO_3^-, the rate of N_2-fixation is reduced by as much as 70% relative to unamended controls (Figure 5). The inhibition of N_2-fixation increases with NO_3^- concentration and reaches values between 50 and 60% at initial nitrate concentrations above roughly 10 μM (Figure 5). Nitrate uptake rates increase with increasing initial concentration and these addition experiments do not appear to exhibit saturation kinetics at concentrations < 20 μM NO_3^-. Previous studies have shown that exposure to NO_3^- leads to a post-translational modification that inactivates the Fe-protein of the nitrogenase complex (Ohki, et al. 1991), the enzyme complex responsible for the reduction of dinitrogen. However, this work involved

cultures that had grown for 15 generations in the presence of 2 mM nitrate (Ohki, et al. 1991) and our amendment experiments show significant inhibition on an hourly timescale and at much lower (μM) NO_3^- concentrations. Should this post-translational modification occur rapidly, our data suggest that this response mechanism may become at least temporarily saturated at NO_3^- concentrations of 10 μM or greater.

The interaction between N_2-fixation and NO_3^- uptake may reflect the competition between these two processes for energy and reductant. Both NO_3^- uptake and N_2-fixation occur during the day in *Trichodesmium* and it is likely that they compete for photosynthetic energy and reductant, particularly in experiments like ours where NO_3^- uptake and N_2-fixation occur simultaneously. Reduction of either NO_3^- or N_2 to NH_3 or N_2 to NH_3 requires a transfer of six electrons to N, but NO_3^- reduction and N_2-fixation involve very different energetic pathways. Ferredoxin and NAD(P)H are required by nitrate reductase and nitrogenase requires ferredoxin and ATP for N_2-fixation. Indirect repression of nitrogenase by NO_3^- and the immediate uptake of NO_3^- may reflect a preference for a particular N source and/or the redox or energetic state of the cells. It is also clear from our results that if NtcA, the transcriptional regulator of N uptake and assimilation (Herrero, et al. 2001), is active in *Trichodesmium* as it is in many other cyanobacteria then a hierarchy of N preference is established at the gene level. Repression of N_2-fixation in our experiments may be caused by the intracellular accumulation of ammonium from NO_3^- uptake and reduction.

Effects of NO_3^- on Total N Uptake

The effect of NO_3^- exposure and uptake on the total rate of N uptake by *Trichodesmium* varied across the range of NO_3^- concentrations we investigated. Small

additions of NO_3^- (<2.5 μM) led to significant inhibition of N_2-fixation by *Trichodesmium.* However, over the 14 h time-course of our experiments, these treatments were just able to make up for the inhibition of N_2-fixation by uptake of available NO_3^-. Therefore, total N consumption in these treatments (<2.5 μM) was not significantly different than total N consumption in the controls. In treatments with initial NO_3^- concentrations greater than 2.5μM, the uptake of NO_3^- more than compensated for the observed inhibition of N_2-fixation, resulting in greater total uptake of N in the amended treatments than in the controls.

Our data show that N_2-fixation and NO_3^- uptake interact strongly and that the presence of NO_3^- at low concentrations similar to those found at the base of the nutricline can inhibit total N uptake in *Trichodesmium* at least on short time scales. Over longer time scales, *Trichodesmium*, strain NIBB1067, exhibits similar growth rates on 2 mM NO_3^- and under N-free conditions (Ohki, et al. 1991). In contrast to our results, acetylene reduction by strain NIBB1067 was not inhibited in cells grown in N-free medium and subsequently incubated for 7 hours in the presence of 2 mM nitrate (Ohki, et al. 1991). The point during the light:dark cycle at which these cells were initially exposed to combined nitrogen was not specified but may be an important factor contributing to the differences between their results (Ohki, et al. 1991) and ours.

N specific Growth Rate

Our results show that the instantaneous N-based growth rate (V_{total}) in our experimental incubations varied with the size of the NO_3^- amendment. Cultures exposed to high initial NO_3^- concentrations (>2.5μM) showed higher total N consumption over the 14 h experimental day and therefore higher V_{total} than cultures exposed to low initial NO_3^-

concentrations (<2.5μM). Control V_{total} ranged from 0.03 d^{-1} to 0.34 d^{-1} with a mean of 0.20 ± 0.09 d^{-1} (mean ± SD, N = 12). Thus, the instantaneous growth rate in the presence of 20 μM NO_3^- (0.61 d^{-1}) represents a substantial increase in V_{total} relative to the V_{total} of the corresponding controls. There is clearly a trend for higher growth rates in treatments with high initial NO_3^- concentrations, which may reflect a preference for NO_3^- as an N source.

Immediately preceding the NO_3^- amendment in our experiments, the cultures were at a steady state growth rate of 0.3 d^{-1}. Interestingly, only the treatment bottles given high initial NO_3^- amendments (7 to 20 μM) were able to take up N at a specific rate equal to or higher than that at which the experiment was started. At lower initial concentrations or under control conditions, the treatment bottles do not take up N at the same rate at which they did at steady state under constant growth conditions.

Ecological Implications

Trichodesmium is unlikely to encounter NO_3^- concentrations in excess of 1 μM near the surface in oligotrophic waters. However, our data provide insights into the potential interaction between NO_3^- uptake and N_2-fixation deeper in the water column or at the surface immediately following a mixing event. Exposure to low concentrations of NO_3^- (< 2.5 μM) inhibited N_2-fixation and lowered instantaneous N-based growth rates within a 14 hour time period. Therefore, because *Trichodesmium* is frequently observed tens of meters below the surface, both as colonies and as free filaments, NO_3^- exposure may play a significant role in the overall nitrogen budget of *Trichodesmium*. Exposure

to, and consumption of, NO_3^- clearly affects both N_2-fixation activity and the total rate of N uptake in this cyanobacterium.

Trichodesmium abundance measurements are frequently expressed as areal standing stocks in order to compute areal N_2-fixation rates. Therefore, published data on the vertical distribution of *Trichodesmium* are limited. However, at several stations along a subtropical north Atlantic cruise track in April of 1996 *Trichodesmium* filaments were found at depths where NO_3^- concentrations were between 1.8 μM and 12.2 μM (Carpenter, et al. 2004). Our experiments indicate that NO_3^- at these concentrations would inhibit N_2-fixation as well as alter total N uptake in *Trichodesmium*. The available data from the Pacific suggest that surface aggregations of *Trichodesmium* will rarely encounter NO_3^- concentrations high enough to inhibit N_2-fixation. However, Letelier and Karl (1996) found free trichomes at depths as great as 200 m at Station ALOHA between October 1989 and December 1992. At ALOHA, during this time period, the nutricline typically started at about 100 m depth (http://hahana.soest.hawaii.edu), so trichomes or colonies at or below this depth would encounter enough NO_3^- to inhibit N_2-fixation and potentially affect total N uptake as well as growth rate.

Though not well documented in *Trichodesmium*, the frequent occurrence of *Trichodesmium* deep in the water column may well reflect a migratory strategy for obtaining PO_4^{3-} from the nutricline (Karl, et al. 1991) and recent work suggests that migration of at least 70 m is possible for *Trichodesmium* (Villareal, et al. 2003). Any *Trichodesmium* at the nutricline will be exposed to NO_3^- as well as PO_4^{3-}. Our results show unequivocally that *Trichodesmium* can take up NO_3^- from solution even at low concentrations and that this uptake immediately inhibits N_2-fixation. Furthermore,

uptake of NO_3^- at depth may help explain why N:P ratios of sinking and floating colonies are not significantly different in the Pacific (Villareal, et al. 2003). To the extent that *Trichodesmium* does migrate vertically through the water column, its uptake of NO_3^- at the nutricline represents a second mechanism, in addition to N_2-fixation, that can inject new nitrogen into the upper water column.

To date, most studies of *Trichodesmium* have focused on the large populations occurring in surface aggregations where NO_3^- concentrations are typically undetectable, but our results clearly demonstrate the potential for NO_3^- at low ambient concentrations to have a significant effect on the N budget of this organism. Further studies of the vertical distribution of *Trichodesmium* with concurrent measurements of dissolved nutrient concentrations, along with focused efforts to measure the rates of consumption of inorganic nutrients by natural populations are clearly needed.

CHAPTER 4

CHARACTERIZATION OF A BLOOM OF THE DIAZOTROPHIC MARINE CYANOBACTERIUM, *TRICHODESMIUM*: IMPLICATIONS FOR N CYCLING IN THE GULF OF MEXICO

Introduction

Both geochemical estimates and empirical measurements show that N_2-fixation is a major source of new nitrogen supporting primary production in the oligotrophic ocean. *Trichodesmium* sp., one of the first known and best studied colonial cyanobacteria (Capone, et al. 1997), is considered an important source of new nitrogen to the oligotrophic ocean and studies of this cosmopolitan cyanobacterium are still necessary as there is much we do not know about its physiology. *Trichodesmium* sp. is frequently found in the oligotrophic gyres of the world ocean. Though common in tropical and sub-tropical seas, its distribution can be irregular, yet spatially extensive when present. Dense blooms can form when the conditions are right; high sea surface temperature, high light intensity, low nutrients, and quiescent seas (Capone, et al. 1998, Eleuterius, et al. 1981). Measurements of N_2-fixation rates of this diazotroph in both bloom and non-bloom abundances have been made in the Atlantic (Goering, et al. 1966), Pacific (Karl, et al. 1997, Letelier, et al. 1996), and Indian Oceans (Jyothibabu, et al. 2003, Lugomela, et al. 2002) as well as in the Caribbean (Carpenter, et al. 1977), China (Saino, et al. 1976, Chen, et al. 2003), Sargasso (Orcutt, et al. 2001), and Arabian Seas (Capone, et al. 1998). These areal rate measurements have determined that when it is present, *Trichodesmium* is an important source of new nitrogen to nutrient-poor regions of the global ocean. This

new nitrogen may significantly amplify primary production and the consequent export of carbon to the deep ocean.

Trichodesmium sp. has been studied for decades both in natural populations and more recently in laboratory cultures (Chen, et al. 1996, Mulholland, et al. 1999, Mulholland, et al. 2001, Ohki, et al. 1986, Holl, et al. submitted) and though the importance of new nitrogen input by *Trichodesmium* to many sub-tropical and tropical gyres has been established, few studies of this cyanobacterium have been conducted in the Gulf of Mexico. To our knowledge no studies have reported N_2-fixation rates by *Trichodesmium* in the Gulf, though *Trichodesmium* is frequently present (Biddanda, et al. 1995, Eleuterius, et al. 1981).

Of the work that has been undertaken in the Gulf of Mexico, one locus of study has been the West Florida shelf where *Trichodesmium* biomass increased 100-fold due to wet deposition of nutrients from Saharan dust (Lenes, et al. 2001). The subsequent increase in DON following a West Florida shelf *Trichodesmium* bloom has been shown to be the stimulus for harmful algal blooms of the dinoflagellate, *Karenia brevis* (Walsh, et al. 2001). In the Mississippi Sound in the northeastern Gulf of Mexico, the causes of an extensive bloom of *Trichodesmium* were determined to be the combination of high sea surface temperature, high light intensity and increased salinity as well as low nutrients and low wind activity (Eleuterius, et al. 1981). Summer *Trichodesmium* blooms and the resultant increased nutrient load have been implicated in the enhancement of heteroptrophic bacterial respiration rates below the euphotic zone (Biddanda, et al. 1997). These studies have shown that *Trichodesmium* is important to the nutrient cycling in the

coastal Gulf of Mexico however, we do not know the extent to which *Trichodesmium* can supply new N to this oligotrophic region.

Here we describe an extensive bloom of *Trichodesmium* in the western Gulf of Mexico in July 2000 (Figure 8). We measured N_2-fixation rates and pigment concentrations within the bloom as well as the stable isotopic signature of the organic and inorganic nitrogen to assess the impact of this new nitrogen on the planktonic food web. Our results show that N_2-fixation by *Trichodesmium* in the Gulf of Mexico is equal to and in many cases even exceeds N_2-fixation measured in other oligotrophic gyres (Table 3).

Materials and Methods

We collected samples and carried out experiments in the northwestern Gulf of Mexico during a cruise on the R/V Longhorn in July 2000 (Figure 8). Water samples were collected for isolation of particles and for nutrient analysis with a CTD-rosette system. Zooplankton were collected in diagonal tows through the upper 100 m of the water column using a 1 m diameter net with a 220 μm mesh size. *Trichodesmium* colonies were collected at the surface by hand-casting a 30 cm diameter net with a 64 μm mesh size while the ship was adrift.

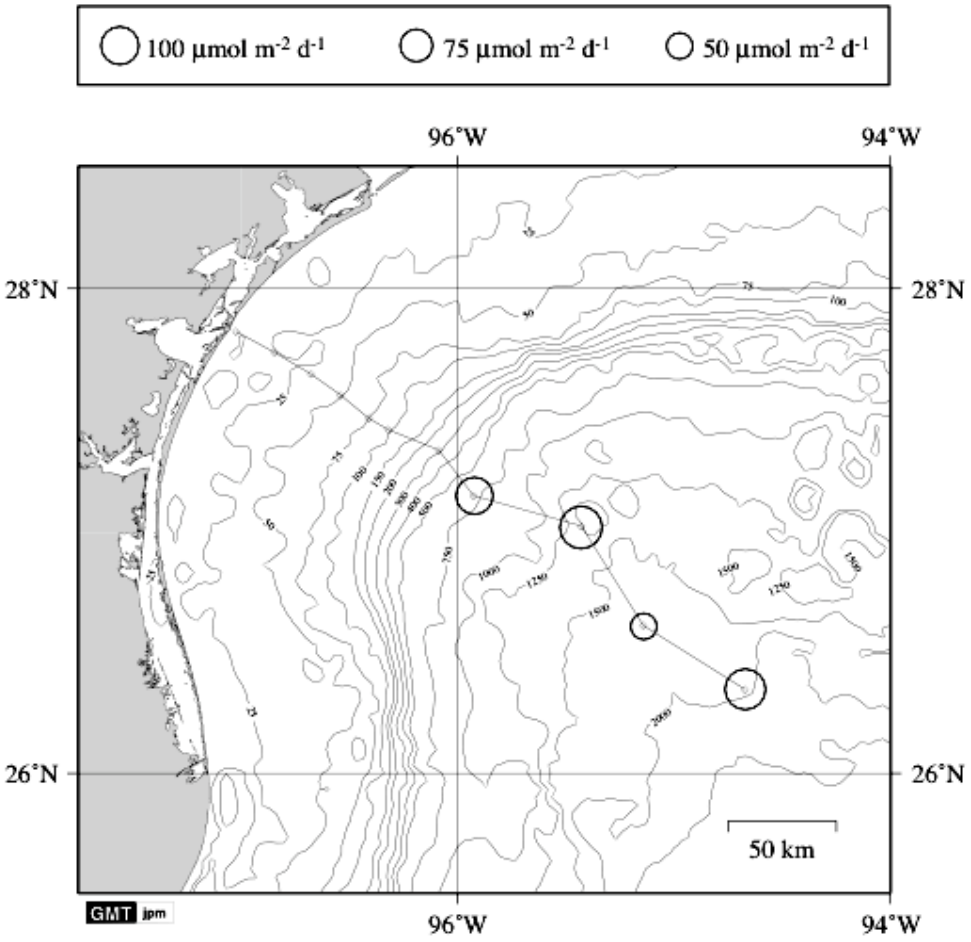

Figure 8 – Gulf of Mexico Cruise track, July 2000.

Hydrography

The CTD-rosette system was equipped with a Sea-Bird 911-Plus temperature and conductivity sensor as well as a SeaTec FL0500 fluorometer and a Datasonics atltimeter. This equipment was used to produce vertical profiles of temperature, salinity and chlorophyll fluorescence at each of the ten stations along the cruise track.

Nutrients, Chlorophyll, and Trichodesmium Abundance

Samples for measurement of dissolved nutrients and bulk chlorophyll were taken from the CTD-rosette immediately after recovery. Nutrient samples were immediately frozen for later analysis ashore. Concentrations of nitrate, phosphate, and silicate were measured with a Lachat QuikChem FIA 8000 nutrient analyzer.

Bulk chlorophyll was measured fluorometrically using a non-acidification technique (Welschmeyer, et al. 1994) after extraction in 100% methanol.

Trichodesmium abundance was determined by 3 methods: 1. For the vertical profile at station 4, the contents of a 12 L niskin bottle were filtered through a 10 μm 47 mm polycarbonate filter. The filter was then examined in a stereoscope at sea and *Trichodesmium* colonies counted. Using this method, free trichomes could be seen but not enumerated. 2. At other stations, *Trichodesmium* colony abundance was determined at the surface where bucket samples were collected, volume determined with a graduated cylinder, and colonies counted visually. 3. Discrete depth water samples (250 ml) were collected and preserved in acid Lugol's iodine (Throndsen, et al. 1978). 100 ml was settled and counted on an inverted microscope (Hasle 1978).

Stable Isotope Abundances

Suspended particles were collected for stable isotope analysis by gentle pressure filtration ($\Delta P < 7$ psi) of at least 10L of seawater through a pre-combusted 47mm Whatman GF/F filter. The filters were stored frozen for stable isotope analysis ashore. In the lab, filters were dried at 60˚C and acid-fumed to remove carbonates. A portion of each filter was then packed into a tin capsule and pelletized for elemental and isotopic analysis by continuous-flow isotope ratio mass spectrometry (CF-IRMS) using a Carlo Erba NC 2500 elemental analyzer interfaced to a Micromass Optima mass spectrometer. At selected depths, an aliquot of filtrate from the suspended particle sampling was preserved by acidification (pH 2 - 3) for isotopic analysis of nitrate ashore. Nitrate was reduced with Devarda's alloy and isolated for isotopic analysis by diffusion (Sigman, et al. 1997).

Zooplankton samples were separated into discrete size fractions by passage through a series of Nitex sieves (4000, 2000, 1000, 500, and 202µm), thoroughly rinsed with surface seawater to remove any *Trichodesmium* caught in the sieves, and frozen for later isotopic analysis ashore. In the laboratory, the samples were dried at 60°C and homogenized by grinding with a mortar and pestle. After grinding, a subsample (ca. 400 µg) was weighed and packed into a tin capsule for elemental and isotopic analysis.

Nitrogen and Carbon Fixation

Trichodesmium colonies were isolated using a plastic inoculating loop and transferred into filtered surface seawater for distribution into 250mL polycarbonate bottles fitted with silicone septum caps. The bottles were completely filled with filtered

surface seawater to exclude bubbles before being sealed. Trace additions of ^{13}C-bicarbonate and $^{15}N_2$ gas were made to each bottle using a gas-tight syringe and the samples were incubated either under simulated in situ conditions in deck incubators, or under in situ conditions on a drifting array. Shipboard incubations lasted between 6 and 8 hours and in situ incubations spanned an entire light day.

Incubations were terminated by gentle vacuum filtration through pre-combusted 25mm GF/C filters, which were stored frozen. In the lab, filters were dried at 60˚C and acid-fumed to remove carbonates before being packed into tin capsules for elemental and isotopic analysis CF-IRMS using a Carlo Erba NC 2500 elemental analyzer interfaced to a Micromass Optima mass spectrometer. N_2-fixation rates were calculated by isotope mass balance as described in Montoya et al. (Montoya, et al. 1996). To calculate CO_2-fixation rates, we estimated the concentration of dissolved inorganic carbon based on temperature and salinity (Parsons, et al. 1984) and applied the same isotope mass balance approach.

Results

Hydrography

There is considerable variation in the vertical temperature profiles along the offshore transect however, surface temperatures show little variation across the transect with one exception (station 1). The surface temperature at stations 2 through 9 is approximately 29° C and at station 1 is 27° C. The mixed layer depth is the shallowest at stations 1 and 9, at approximately 10m. At stations 2 through 4 the mixed layer depth is 30m and at stations 5 through 8 the mixed layer depth is 40m. With the exception of station 1 where the temperature at 50m is 22° C, the temperature of all other stations at

50 m converges on 27° C. It is at 50m that the temperature profiles diverge such that the temperature at stations 2 through 4 remains relatively elevated at 25° C at 100m while the temperature at the remainder of the stations drops off quickly from 50 to 100m and converges on 18° C.

Nutrient and Chlorophyll Concentrations

Nitrate concentrations ranged from below our limit of detection at the surface at all stations to 30 μM at 500m at station 1 and to 25 μM at 500m for stations 2 and 4. The start of the nitricline was considerably deeper at those stations furthest from shore. At stations 2, 3, and 4 the nitracline started at approximately 125m and at stations 1 and 5 the nitracline started at approximately 60m. We did not find measurable NO_3^- shallower than 55 m at stations 1 and 5, shallower than 103 m at stations 4, or shallower than 125m at stations 2 and 3. The phosphocline started only slightly shallower in the water column than the nitricline; 50m at stations 1 and 5 and 90 to 95m at stations 2, 3, and 4.

Bulk water chlorophyll measurements were made at all stations along the cruise tract. Chlorophyll concentrations ranged from a minimum of 0.06 μg L^{-1} at 250m at station 3 to 1.4 μg L^{-1} at 22m at station 10. Bulk chlorophyll maxima ranged from 0.3 μg L^{-1} at 119m at station 4 to 1.4 μg L^{-1} at 22m at station 10. The chlorophyll maximum increased in depth from approximately 65 meters at those stations closer to shore (1, 5, and 8) to approximately 125m at stations 2, 3 and 4 (Figure 9).

Trichodesmium Abundance

Trichodesmium was present at nine of ten stations (except Station 8) along our cruise track, and surface slicks were evident at stations 1 through 4. We used three

different approaches to quantifying *Trichodesmium* abundance. Enumerating the trichomes present in discrete 100 ml samples from the CTD-rosette and in bucket-collected samples showed the highest trichome abundance at the surface, with values ranging as high as 10^4 trichomes·L^{-1} with a mean of $1.5 \times 10^3 \pm 1.1 \times 10^3$ trichomes·L^{-1} (mean ± SE, N=10). The highest *Trichodesmium* surface abundance occurred at station 2 and was 100-fold higher than at any other station. Removing this large outlier yields a mean *Trichodesmium* surface abundance of 360 ± 159 trichomes·L^{-1} (mean ± SE, N=9). Three of the five stations sampled had a second, subsurface maximum in *Trichodesmium* abundance between 10 and 15 m depth (Table 2). Trichome abundance at these depths were as high as 440 trichomes·L^{-1} with a mean of 82 ± 41 trichomes·L^{-1} (mean ± SE, N=10) The highest subsurface peak occurred at station 2, the furthest offshore of our transect (Table 2).

Table 2 - *Trichodesmium* biomass in the NW Gulf of Mexico in July 2000. Areal 100mL sample trichome counts are integrated over the upper 50m. * Denotes areal trichome counts that include surface bucket counts as well as the 100mL discrete depth counts.

Station	Date	Trichomes L^{-1} at surface	Trichomes L^{-1} at 10-15m	Trichomes L^{-1} at maximum	Depth of trichome maximum (m)	Trichomes m^{-2} to 50 m
1	7/24/2000	1060	300	1060	0	$1.3 \times 10^{8*}$
2	7/25/2000	10,200	440	10,200	0	$5.4 \times 10^{8*}$
3	7/26/2000	1000	20	1000	0	$5.5 \times 10^{7*}$
4 (100ml)	7/27/2000	400	110	400	0	$3.4 \times 10^{7*}$
4 (12L)	7/27/2000	400	640	640	10	$1.0 \times 10^{8*}$
5	7/28/2000	400	10	400	0	$2.4 \times 10^{7*}$
6	7/28/2000	0	10	30	35	2.0×10^{6}
7	7/28/2000	10	10	20	30	2.5×10^{6}
8	7/28/2000	0	0	0	--	0
9	7/29/2000	10	10	140	20	6.8×10^{6}
10	7/29/2000	0	120	120	10	2.7×10^{6}

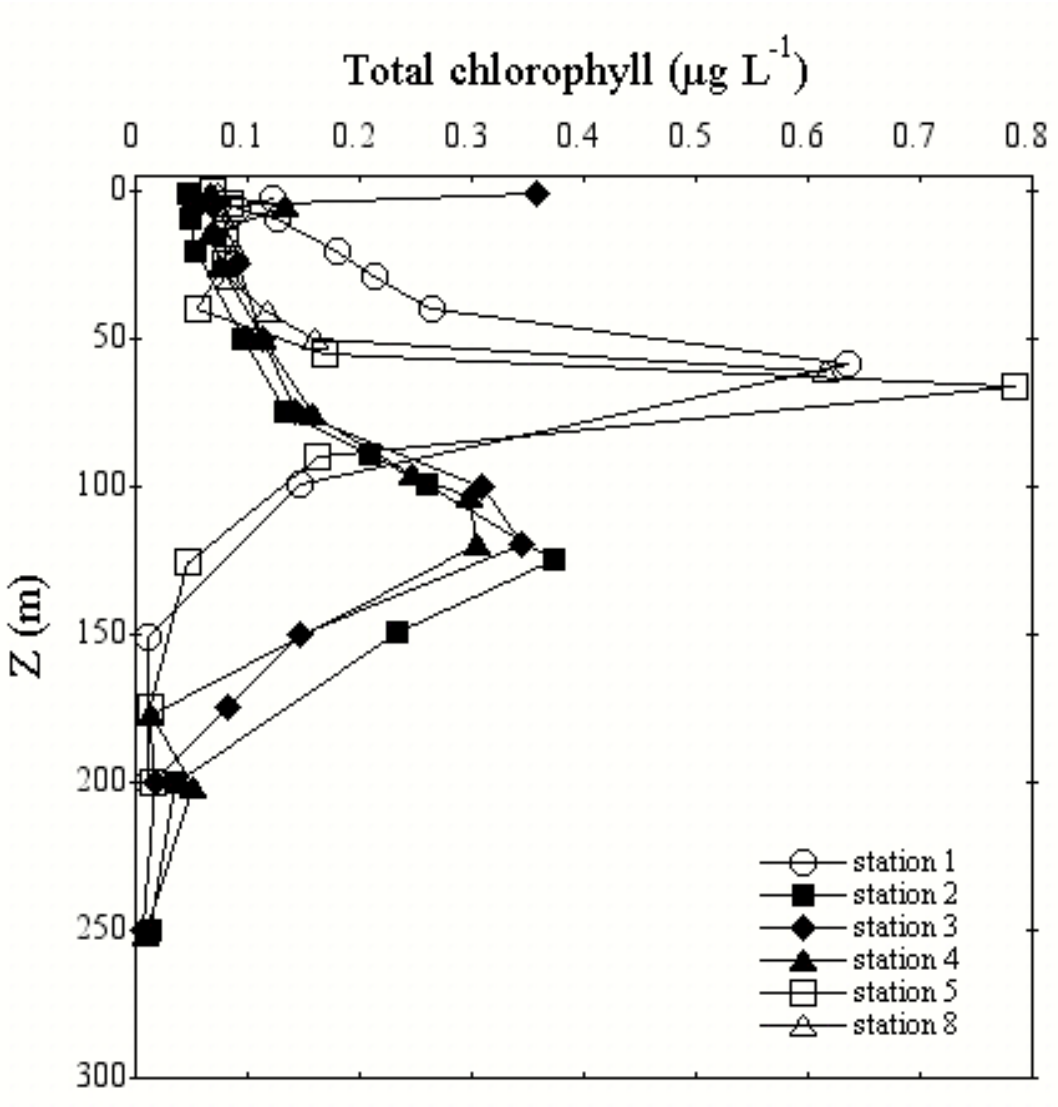

Figure 9 – Depth profiles of bulk chlorophyll concentration.

A larger discrete volume (12 L) from the CTD-rosette was sampled for *Trichodesmium* abundance at station 4, where the large volume sampling produced estimates of *Trichodesmium* abundance considerably higher than estimates based on 100 mL samples (Table 2). Thus our trichome abundance estimates based on the 100 mL and bucket samples are underestimates. Despite the difference in absolute abundance, the general shape of the profile is similar with a distinct surface maximum and the bulk of the trichomes found above 50 m. This vertical profile also shows that *Trichodesmium* trichomes were found throughout the upper water column down to the upper boundary of the nutricline and a significant number of colonies were found as deep as 175 m at this station.

Stable Isotope Abundance

$\delta^{15}N$ of Dissolved Inorganic Nitrogen

Extraction and stable isotopic analysis of the DIN was completed on stations 2 and 4. The $\delta^{15}N$ of the dissolved inorganic nitrogen at these two stations was 2.00 ‰ ± 0.45 (mean ± SD, N=3) at 200 m depth, and 4.05 ‰ ± 0.12 (mean ± SD, N=2) at 900m.

Particulate Organic Matter $\delta^{15}N$ and $\delta^{13}C$

The $\delta^{15}N$ of the particulates (>0.7µm) in surface samples decreased with distance from the shore ($\delta^{15}N = 3.0 - 0.01$ x kilometers from shore, $R^2 = 0.72$). The same trend is seen in the $\delta^{15}N$ of the particulates at 20m ($\delta^{15}N = 4.2 – 0.03$ x kilometers from shore, $R^2 = 0.87$). Vertical profiles at stations 2, 3, and 4 show a $\delta^{15}N$ minimum (-4 to -2 ‰) at approximately 20m (Figure 10), just at or below the subsurface maximum in

Trichodesmium abundance (Figure 10). Stations 1 through 4 have similar $\delta^{13}C$ profiles (Figure 11). At station 5 the $\delta^{13}C$ of POC is markedly heavier, though there is no clear trend in $\delta^{13}C$ of suspended particles along our onshore to offshore transect.

Zooplankton $\delta^{15}N$ and $\delta^{13}C$

The $\delta^{15}N$ of the zooplankton varied across the transect with the largest change in $\delta^{15}N$ in the smallest (250-500μm) size fraction (Figure 12). At stations 1, 5 and 8 the $\delta^{15}N$ of the 250-500 μm size fraction varied from 3.7 ‰ to 4.5 ‰ with a mean of 3.9 ± 0.4 ‰ (mean ± SE, N=3). The 250-500μm size fraction at stations 2, 3 and 4 varied from 1.6 ‰ to 2.2 ‰ with a mean of 2.0 ± 0.2 ‰ (mean ± SE, N=3, $p< 0.05$). The larger size fractions of zooplankton (500-1000 and 1000-2000 μm) had higher $\delta^{15}N$ relative to the 250-500μm size fraction and exhibited the same spatial trends along our transect. There was little difference between the $\delta^{13}C$ of the 250-500μm and 500-1000μm size fractions, which ranged from –18.83 ‰ to –18.15 ‰ and from –19.7 ‰ to –18.5 ‰, respectively.

A cross-plot of zooplankton $\delta^{15}N$ as a function of $\delta^{13}C$ shows two clear groups of different zooplankton $\delta^{15}N$. Zooplankton in the 250-500μm and 500-1000μm size fractions from stations 1, 5, and 8 have a higher $\delta^{15}N$ than the same zooplankton size fractions from stations 2, 3, and 4 (Figure 14). $\delta^{13}C$ is essentially unchanged across zooplankton size fractions and stations. A weighted average calculation between the two sources of C available to these zooplankton, *Trichodesmium* POC and particulate POC,

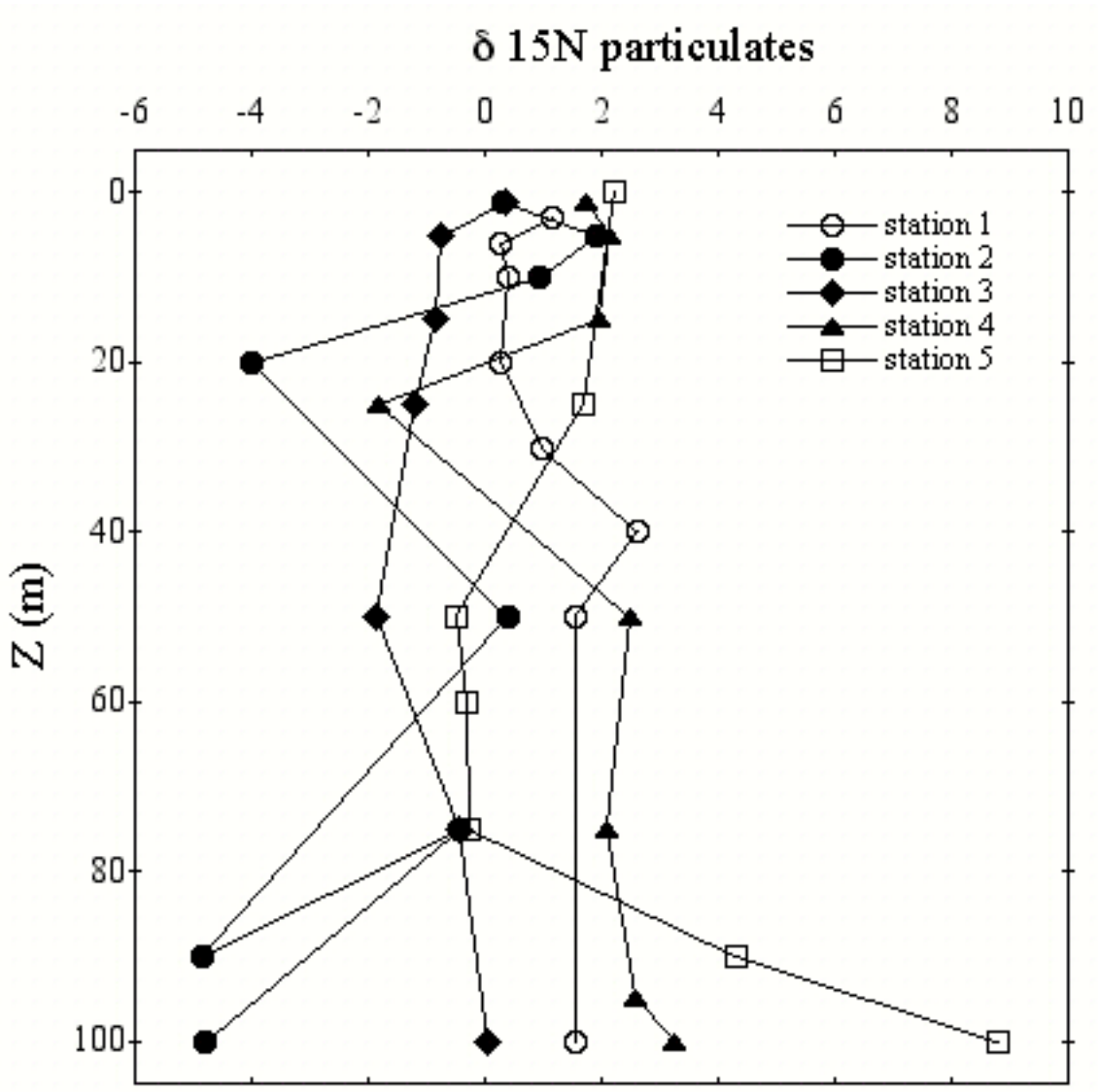

Figure 10 - $\delta^{15}N$ of the Particulate organic matter.

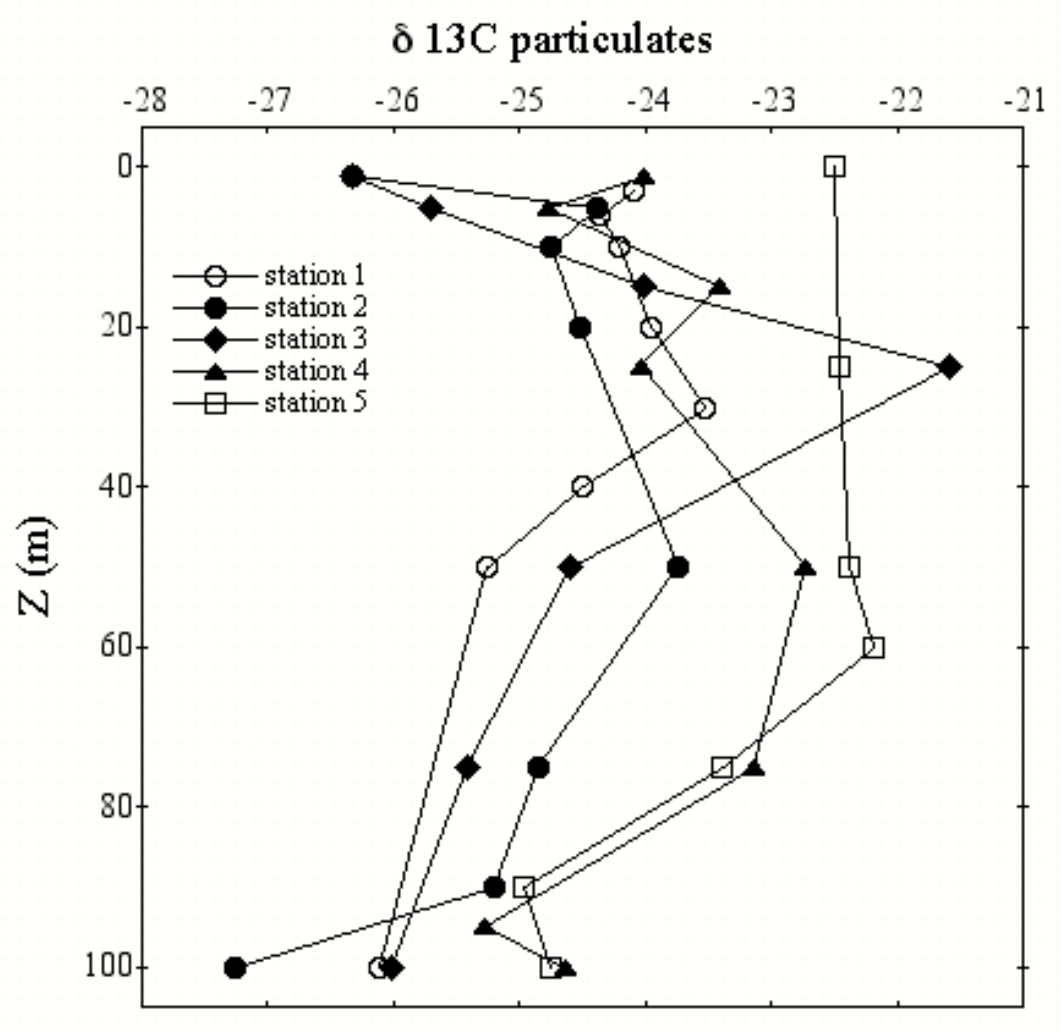

Figure 11 - $\delta^{13}C$ of the particulate organic matter.

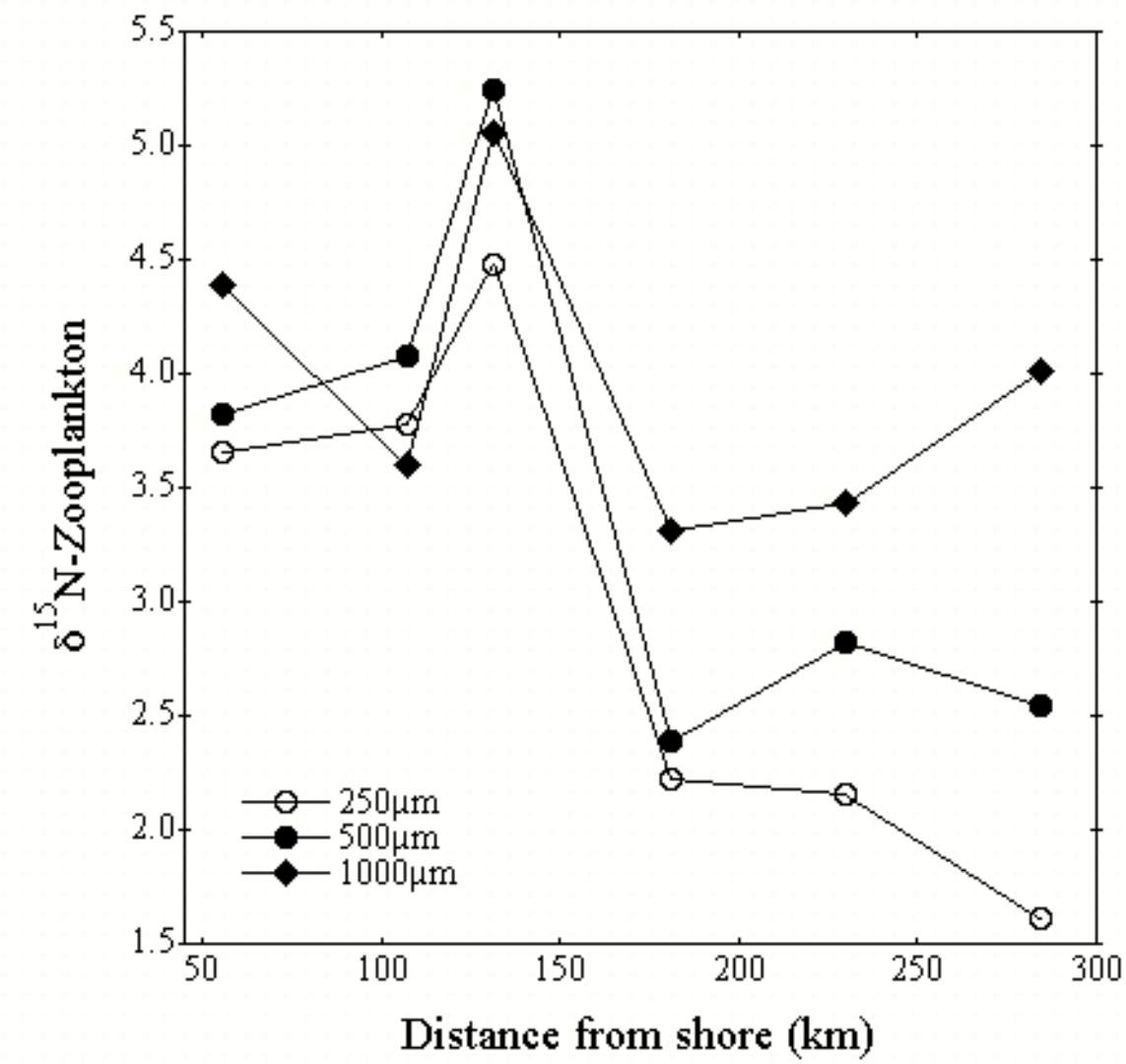

Figure 12 - $\delta^{15}N$ of the zooplankton as a function of the distance from shore.

shows that as much as 60% of the C in the zooplankton may come from *Trichodesmium* POC and the remaining 40% from particulate POC (Figure 14).

N_2-fixation Rates

We used a colony size of 200 trichomes colony^{-1} (Carpenter, et al. 1977) to compute trichome specific N_2-fixation rates, which ranged from 1.3 x 10^{-4} nmol N·trichome^{-1}·h^{-1} to 2.6 x 10^{-2} nmol N·trichome^{-1}·h^{-1} with a mean of 3.4 x 10^{-3} ± 7.6 x 10^{-4} nmol N·trichome^{-1}·h^{-1} (mean ± SE, N = 45) over the entire range of I_o. The maximal volumetric rate of N_2-fixation ranged from 4.1 x 10^{-3} µmol N·L^{-1}·h^{-1} to 4.5 x 10^{-1} µmol N·L^{-1}·h^{-1}, with a mean of 8.0 x 10^{-2} ± 0.09 (mean ± SE, N = 45). The rate of N_2-fixation by *Trichodesmium* showed significant photoinhibition at full surface irradiance (I_o) and maximal rates at 0.50 I_o (50% of surface irradiance) (Figure 13), which occurred at approximately 15 to 20 m depth in the water column. At full surface irradiance, N_2-fixation rates were almost 20% lower, from 9.0 x 10^{-3} µmol N·L^{-1}·h^{-1} to 4.5 x 10^{-2} µmol N·L^{-1}·h^{-1} with a mean of 2.4 x 10^{-2} ± 7.0 x 10^{-3} µmol N·L^{-1}·h^{-1} (mean ± SE, N = 6).

Incubations attached to an in situ array deployed at station 4 and allowed to incubate for the entire light day showed a similar pattern of decreasing N_2-fixation activity with decreasing light intensity (Figure 13). Bottles were placed at depths of 5, 15, 30, 50, and 70m on the array. To complete the depth profile, we added the mean N_2-fixation rate at 100% light intensity from our incubator experiments to our in situ profile (Figure 13). The complete profile shows surface photoinhibition of N_2-fixation and a maximum N_2-fixation rate at the 50% light intensity. We used a least-squares parabolic

regression of N_2-fixation rate as a function of light intensity with our conservative estimate of the vertical distribution of colony abundance at each station to estimate areal N_2-fixation rates for stations 1 through 4 (Figure 8). The areal fixation rates range from 47.2 μmol $N \cdot m^{-2} \cdot d^{-1}$ to 119 μmol $N \cdot m^{-2} \cdot d^{-1}$ with a mean of 84.5 ± 17.7 μmol $N \cdot m^{-2} \cdot d^{-1}$ (mean ± SE, N=4) at our four study stations. The area of the open circle (Figure 8) is proportional to the areal N_2-fixation rate at each station. We did not find a trend for increasing N_2-fixation rates with increasing distance from shore. The highest N_2-fixation rates were found at station 3, one of the three deep chlorophyll max stations farthest from shore (Figure 8).

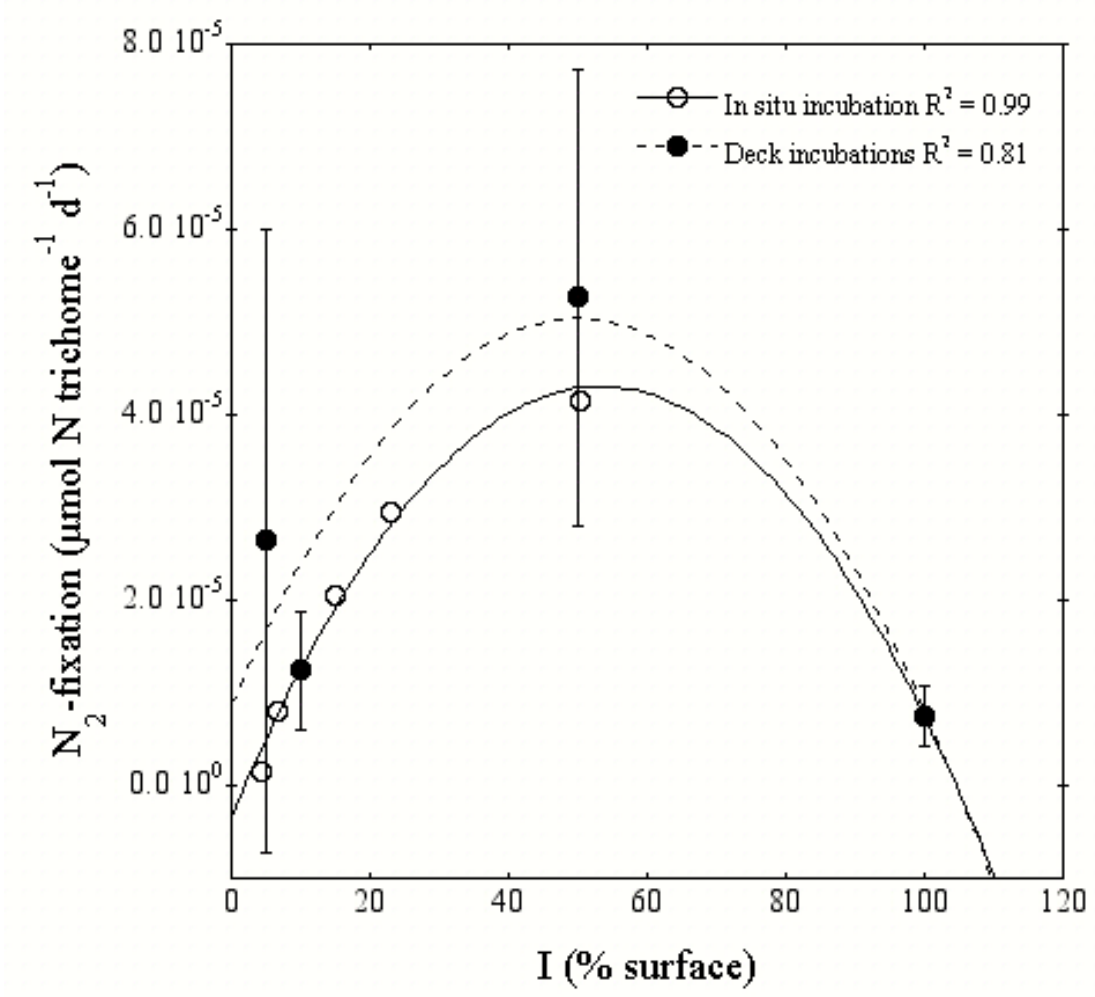

Figure 13 – *Trichodesmium* N_2-fixation as a function of I_o (% of surface irradiance).

Discussion

Trichodesmium was clearly abundant at each station on our cruise, except station 8, with depth integrated trichome concentrations ranging from 1.8×10^3 trichomes·m^{-2} to 4.3×10^4 trichomes·m^{-2} with a mean of $1.2 \times 10^4 \pm 8.5 \times 10^3$ trichomes·m^{-2} (mean ± SE, N = 5 for stations 1-5). Conditions that favor development of *Trichodesmium* blooms (Carpenter 1983, Capone, et al. 1998, Eleuterius, et al. 1981) are prevalent in the Gulf of Mexico particularly during the summer months, making the Gulf an ideal environment for this colonial cyanobacterium. Though blooms are unpredictable and patchy in distribution, when they occur the resulting increased DIN and DON may contribute to an increase in primary production (Carpenter, et al. 1991, Capone, et al. 1997, Karl, et al. 1997), increased secondary production (Landry, et al. 2001), and enhanced heterotrophic bacterial production (Biddanda, et al. 1997).

N_2-fixation rates measured at four stations within the bloom show significant spatial heterogeneity and no trend in N_2-fixation rate with distance from shore (Figure 8). Vertically integrated rates at the four stations where we had depth profiles of rate

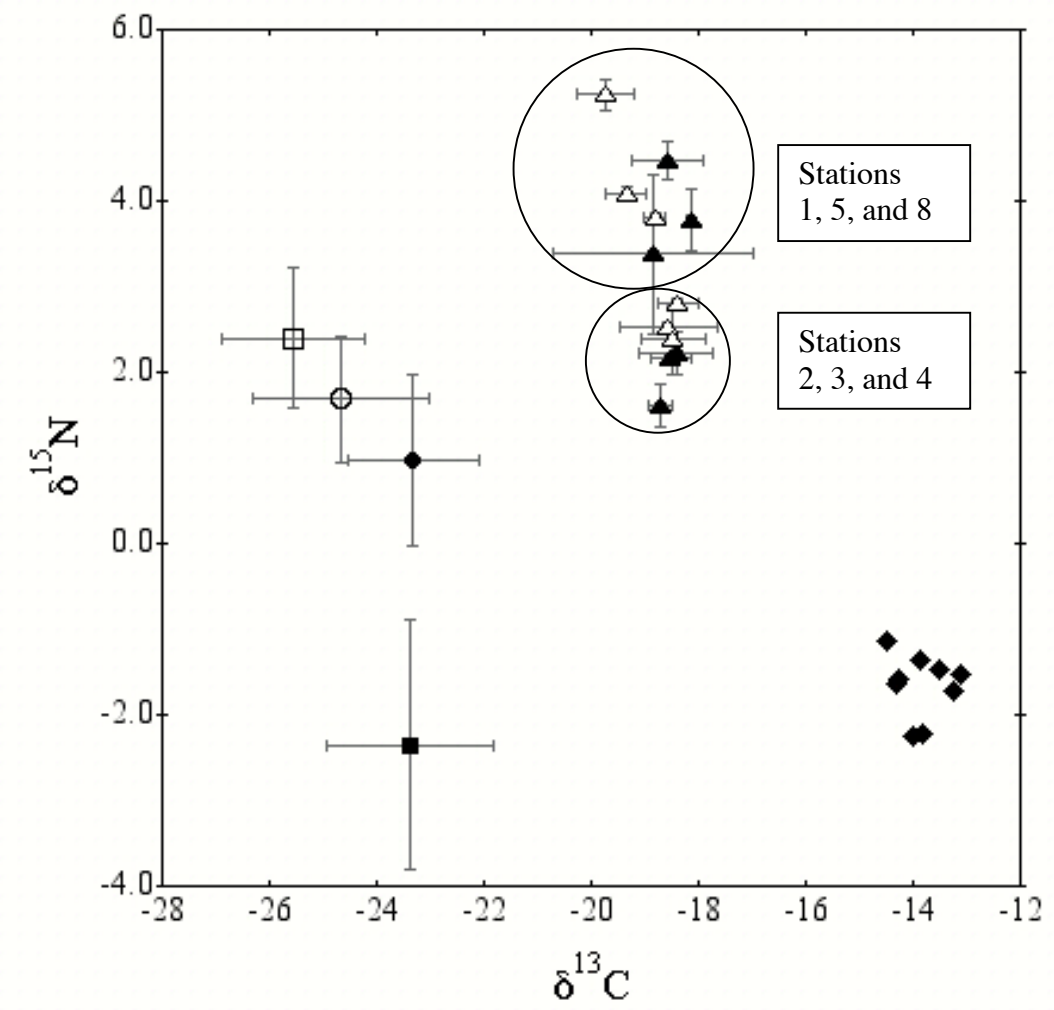

Figure 14 – Cross plot of $\delta^{15}N$ as a function of $\delta^{13}C$ for *Trichodesmium*, particulate organic matter, and zooplankton.

Legend: *Trichodesmium* (filled diamonds), surface POM stations 2, 3, and 4 (open square), surface POM stations 1 and 5 (open circle), 20 m POM stations 2, 3, and 4 (filled circle), 20 m POM station 1 and 5 (filled square), 250 µm size fractioned zooplankton (filled triangles), and 500 µm size fractioned zooplankton (open triangles).

measurements fall within the range of values for *Trichodesmium* in other oligotrophic basins (Table 3).

While recognizing that ideally *Trichodesmium* would be collected at each of the in situ light levels and then incubated at the corresponding light level, time constraints as well as patchy vertical *Trichodesmium* distribution in the water column dictate that surface samples must be used for this type of assay. N_2-fixation of surface-collected *Trichodesmium* samples incubated in situ declined with depth (Figure 13), with the highest N_2-fixation rates at the 50% light level at roughly the same depth as the maximal trichome concentration (15m). *Trichodesmium* can regulate its buoyancy (Walsby, et al. 1978, Carpenter, et al. 1979, Villareal, et al. 1990, Villareal, et al. 2003) and may avoid photoinhibition of both C and N_2-fixation by aggregating at a depth that maximizes N_2-fixation (Carpenter, et al. 1977). At greater depths, photosynthesis is light limited, which in turn reduces the energy available for N_2-fixation, leading to the observed decrease in N_2-fixation rate with depth. We used a parabolic regression to describe the relationship between N_2-fixation and light intensity with a maximum located at 50% surface irradiance (Figure 13). The same parabolic pattern was shown for both our in situ and our deck incubations, therefore validating the use of the regression from the in situ incubations to compute depth integrated areal N_2-fixation rates.

DON leakage or excretion (Glibert, et al. 1994, Capone, et al. 1994, Bronk, et al. 2000) is an important sink for newly fixed nitrogen and a potential source of new nitrogen to non-diazotrophic phytoplankton. Our particulate nitrogen $\delta^{15}N$ vertical profiles show generally lower values in the upper water column at stations 2, 3, and 4

relative to stations 1 and 5 in the upper water column (Figure 10). Surface and 20m particulate $\delta^{15}N$ signatures generally decrease with increasing distance from shore. The smallest zooplankton fractions (250µm and 500µm) have lower $\delta^{15}N$ in areas where N_2-fixation was prevalent (Figure 12). Both the PN and zooplankton data indicate that the nitrogen available to the non-diazotrophic phytoplankton is isotopically depleted and consistent with significant inputs of recently fixed nitrogen.

A third characteristic marker of diazotrophy is the low $\delta^{15}N$ of the DIN. In a system dominated by diazotrophy the dissolved organic pool can become isotopically depleted. Because microbial nitrifiers are subsequently degrading a pool of depleted N, the product of this nitrification will also be depleted. Typical deep(mid)water N signatures are 4-6 ‰ (Liu, et al. 1989, Sigman, et al. 1997) but at stations 2 and 4 at 200m, the signature is 0.5 and 1.5 ‰, respectively. DIN with an isotopic signature of even 1.5 ‰, the higher value in the range, at 200m depth is considerably lower than would be expected for water at a depth that would be potentially mixing with deep(mid) water nitrate. This isotopic signature may be indicative of a diazotrophic source of nitrogen in the water column.

Table 3: Summary of *Trichodesmium* N_2-fixation measurements for oligotrophic waters. Acetylene reduction (AR) estimates are based on a 3:1 reduction ratio for conversion to N_2-fixation rates.

Location	Dates	Mean Areal Rate (μmol N m^{-2} d^{-1})	SE	N	Method	Citation
Southwestern N. Atlantic 0°-24°N, 45°-66°W	Nov 1964	41	7.6	19	$^{15}N_2$ uptake	(Goering, et al 1966)
	May 1965	108	24	17		
Caribbean, 12° to 22°N		161	20	12	AR	(Carpenter, et al 1977)
BATS	1995-1997	41		14	$^{15}N_2$ uptake	(Orcutt, et al 2001)
N. Pacific, 21°N, 159°W	1972	134		2	AR	(Gundersen 1976)
East China Sea, 10°-25°N		126	49	32	AR	(Saino 1977)
HOT/ALOHA	1990–1992	84	43	8	AR	(Karl, et al 1997)
Arabian Sea, 7-10°N	May 1995	35	7.4	9	AR	(Capone, et al 1998)
Arabian Sea, 10°N bloom	May 1995	99	25	5	AR,	(Capone, et al 1998)
Coastal Tanzania (bloom – upper 0.5 m)	1975-1999	59			AR	(Lugomela, et al 2002)
Gulf of Mexico	July 2000	85	18	4	$^{15}N_2$ uptake	This study

There are few known direct consumers of *Trichodesmium* (O'Neil 1998, Bottger-Schnack, et al. 1989, Calef, et al. 1966). However, the $\delta^{13}C$ isotope results from our 5 study stations imply that much of the zooplankton C may be coming from *Trichodesmium* (Figure 14) either by direct ingestion or by the incorporation of *Trichodesmium*-derived DIC. *Trichodesmium* has a characteristically heavy C isotope signature (Carpenter, et al. 1997, Minagawa, et al. 1986, Wada, et al. 1976), -13.86 ‰, when compared to the POC, which at our study stations has a $\delta^{13}C$ of approximately -25.00 ‰. Two distinct carbon sources with characteristic $\delta^{13}C$ isotope signatures allow us to employ a two source mixing model to determine the potential fate of *Trichodesmium* C and mass balance calculations indicate that as much as 60% of the zooplankton C is coming from *Trichodesmium* and the remaining 40% from POC (Figure 14).

While the relative importance of diazotrophic unicellular cyanobacteria in supplying new nitrogen to the oligotrophic ocean is becoming increasingly clear (Montoya, et al. 2004, Dore, et al. 2002, Falcon, et al. 2004, Zehr, et al. 2001), *Trichodesmium* remains an important supplier of "new" nitrogen to the areas in which it is found (Montoya, et al. 2002, McClelland, et al. 2003). Its capacity to fix nitrogen has been studied in many tropical and subtropical regions of the world ocean, yet the Gulf of Mexico has been virtually un-represented in regards to *Trichodesmium* N_2-fixation even though it is commonly found there (Biddanda, et al. 1997, Eleuterius, et al. 1981). To our knowledge, our data are the first published *Trichodesmium* N_2-fixation rates in the Gulf of Mexico. Our sampling, though spatially limited, produced areal N_2-fixation rates for the Gulf of Mexico comparable to N_2-fixation rates measured in other oceanic basins

(Table 3). Because the Gulf of Mexico is easily accessible and typically has extensive surface blooms of *Trichodesmium* each summer, it provides an ideal location for field studies of *Trichodesmium* biology and its role in oceanic biogeochemistry.

CHAPTER 5

CONCLUSION

Trichodesmium is a quantitatively important supplier of N to the oligotrophic regions in which it is found. It is common in subtropical and tropical seas worldwide where surface aggregations can occur over enormous stretches of the ocean. Its importance to the ecology of these regions, both as a source of new nitrogen and as a physical substrate, has been established. *Trichodesmium* N_2-fixation has been studied in many of the areas in which it is found and recently in laboratory cultures, but our understanding of the ways and the extent to which environmental factors can affect N_2-fixation rate remains limited.

We established a continuous culture of *Trichodesmium* and maintained this cyanobacterium at a range of dilution rates to establish a physiological steady state with constant growth and N_2-fixation rates through time. In our continuous culture, biomass (PN) concentration decreased with growth rate and N_2-fixation increased with growth rate. The C:N:P ratio remained constant across all growth rates, suggesting balanced growth and macronutrient uptake in our cultures. Though somewhat variable, N_2-fixation rate was stable at each of our steady state growth rates and N-specific growth rate varied linearly with dilution rate, implying a closed N budget in a culture of a cyanobacterium that is known to excrete DON.

At steady state, our continuous culture provided a system with which we could investigate the effects of the presence and uptake of nitrate on N_2-fixation. Our study showed that exposure to nitrate at μm levels inhibits N_2-fixation by as much as 70%

relative to unamended controls, with an apparent saturation of inhibition at an initial nitrate concentration of 10μM. Nitrate uptake made up for the reduced rate of N_2-fixation over the light day. In fact, even though N_2-fixation was severely inhibited in cultures initially amended with more than 2.5 μM nitrate, total integrated daily N uptake in the presence of nitrate was higher than total integrated N uptake in unamended controls. While our results may not be directly related to surface waters of an oligotrophic system where appreciable amounts of combined N are rare, they shed light on the potential effects of nitrate exposure on N_2-fixation by migrating populations of *Trichodesmium*, which may encounter significant concentrations at the nutricline.

Our field efforts in the Gulf of Mexico revolved around the quantification of N_2-fixation by *Trichodesmium* in an oligotrophic basin where N_2-fixation by this diazotroph has received little attention. We combined biomass measurements (trichome abundance profiles) with in-situ N_2-fixation assays to compute areal N_2-fixation rates for the NW Gulf. Our rates for the Gulf are comparable to many of the published areal fixation rates from other oligotrophic basins. *Trichodesmium* is of course known to be important to N cycling, however in this field study we also used stable carbon isotope signatures to establish the importance of *Trichodesmium* in supplying C to higher trophic levels. Our results suggest that as much as 60% of zooplankton C in the 250 to 1000 μm size fractions came either directly or indirectly from *Trichodesmium*. Our work established that this diazotroph is important to the ecology of the water column of the Gulf of Mexico where it is frequently found and where its presence has important implications for the cycling of both N and C.

Establishment of a continuous culture was the first step towards determining the effects of growth rate and nitrate uptake on N_2-fixation rates of this globally significant cyanobacterium. Future work employing continuous cultures should include the effects of ammonium and trace metal concentration on N_2-fixation. Continuous cultures may also be used to explore the effects of phosphate concentration, temperature, and light intensity on N_2-fixation. Such studies will help to elucidate how these factors may affect N_2-fixation deep in the water column where trichomes are frequently found. Results from these culture explorations could then be used in modeling efforts to quantify *Trichodesmium* N_2-fixation on an oceanic scale. These results can also be added to models of global N_2-fixation estimates in an attempt to balance the N budget.

Balancing the N budget in the ocean has become more than just an academic pursuit. The strength of the biological pump, the main pathway for the export of CO_2 from the atmosphere to the deep ocean, is controlled by the amount of new N available to support biological production in the surface ocean. It is therefore important for us to understand how much new N enters the oligotrophic ocean in an attempt to quantify how much C can be removed. Understanding the ways in which N_2-fixation by diazotrophs is affected by commonly occurring environmental factors such as light, temperature, combined N, trace metals, etc. is a critical step in accurately quantifying the input of new N to the oligotrophic ocean via diazotrophy.

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