N_2 fixation by subsurface populations of *Trichodesmium:* An important source of new nitrogen to the North Atlantic Ocean

By

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Abstract *Trichodesmium*, a genus of diazotrophic cyanobacteria, is an important contributor to the marine nitrogen (N) and carbon (C) cycles. The extent to which *Trichodesmium* dinitrogen (N_2) fixation contributes to the marine N cycle has been modeled based on abundance data and rate estimates from surface populations. However, recent data show that *Trichodesmium* populations have a broad vertical distribution. The presence of previously unaccounted for subsurface populations suggests that past estimates of the contribution of new N by *Trichodesmium* to the North Atlantic may be artificially low. Herein, culture and field studies were combined to examine trends in N_2 fixation in discrete surface and subsurface *Trichodesmium* populations in the western North Atlantic. Surface populations were dominated by the raft colony morphology of *Trichodesmium* and surface N_2 fixation rates ranged from (33 to 156 μmol h^{-1} mol C^{-1}). Subsurface populations were dominated by the puff colony morphology. Subsurface N_2 fixation was typically detectable, but consistently lower than surface population rates (9 to 88 μmol h^{-1} mol C^{-1}). In an analysis of the entire field dataset, N_2 fixation rates varied

non-linearly as a function of *in situ* irradiance. This trend in N_2 fixation versus *in situ* irradiance is consistent with field and culture observations in the literature (Bell et al., 2005; Capone et al., 2005), however other models that predict N_2 fixation based on light predict higher subsurface N_2 fixation than what was detected in this study. In culture, N_2 fixation in *Trichodesmium* was proportional to light level over the range of irradiances tested (10 to 70 μmol quanta m^{-2} s^{-1}) and over long and short time scales, suggesting subtle changes in the light field could depress subsurface N_2 fixation. Since the subsurface samples were dominated by the puff colony morphology, it is unclear if the subsurface N_2 fixation rates are the result of the intrinsic responses of different species of *Trichodesmium*, or light driven population segregation within a single species, among other possibilities including the effects of temperature and nutrient availability. Regardless, the subsurface rates presented herein indicate that N_2 fixation by subsurface populations represents an undersampled source of new N to the western north Atlantic. This result is consistent with the findings of Davis and McGillicuddy (2006), who suggest that subsurface populations of *Trichodesmium* increase the average N_2 fixation rate in the North Atlantic by 2.9 to 3.3 times over estimates based solely on surface estimates (Davis and McGillicuddy, 2006).

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Rationale

Nitrogen fixation in Trichodesmium – The extensive nutrient deficient waters of the subtropical ocean, including the western North Atlantic, are key drivers in the global carbon (C) cycle, responsible for fixing 5-6 Gt C y^{-1}, accounting for approximately 50% of the 10-11 Gt C yr^{-1} estimated for global oceanic C drawdown (Emerson et al. 1997). Export production is fueled by new nutrients entering the system and stimulating production. In the case of nitrogen (N), sources of new N can include atmospheric deposition, deep mixing and eddy-diffusion processes, as well as biological dinitrogen (N_2) fixation by diazotrophs (Dugdale and Goering, 1967). As they are recognized as important in the global N cycle, diazotrophs and studies of their distribution and relative contribution to global N-cycling are numerous (Lipschultz et al., 1996; Church et al., 2005a,; Church et al., 2005b; Fong et al., 2008). The majority of these studies have identified a few key microbial taxa driving N flux in the oligotrophic oceans. The filamentous non-heterocystous cyanobacterium *Trichodesmium* is one genus that is consistently identified as an important N_2 fixing microbe in oligotrophic oceans (Capone et al., 1997; Capone et al., 1998; Karl et al., 2002), especially in the western North Atlantic where it may contribute up to 30 mg N m^{-2} day^{-1} to the new N pool, which equals or exceeds the vertical flux of nitrate to the surface (Capone et al., 2005).

There are six species of *Trichodesmium*, which are traditionally separated on the basis of physical characteristics and colony morphology: *T. contortum, T. erythraeum,*

T. hildebrandtii, T. tenue, T. thiebautii, and *T. pelagicum* (Hynes, 2009). Sequencing studies of 16s rRNA, *nifH, hetR,* and the ITS region along with fingerprinting of the HIP1 region in *Trichodesmium* have shown low genetic diversity between species of *Trichodesmium* (Ben-Porath et al., 1993; Orcutt et al., 2002; Lundgren et al., 2005). Colony morphology is often used to classify field populations at the species level, although there may be little correlation between colony morphology and genetically assigned species identifications (Lundgren et al., 2005). Raft colonies, in which the *Trichodesmium* filaments align in parallel are typically classified as *T. erythraeum*. Puff colonies, in which individual filaments align radially, tend to be classified as *T. thiebautii* in the western North Atlantic. *T. erythraeum* is found in high abundance at the Great Barrier Reef, and the type strain IMS101 was isolated from coastal North Carolina. *T. thiebautii* is thought be the dominant species in the Northern Hemisphere (Hynes, 2009). However, species distributions are not well defined and undoubtedly overlap, which is the case at the Bermuda Atlantic Time-series Station (BATS). Genetic studies indicate that *T. thiebautii* is present year round at BATS, with an increase in abundance during the summer, when *Trichodesmium* spp. abundances peak. *T. erythraeum* is also found at BATS during the summer months (Hynes, 2009). In areas where they are found, *Trichodesmium* is typically the dominant diazotroph. A recent study by Goebel et al. (2010) examined the distribution and quantities of *nifH* genes, which encodes nitrogenase reductase, during a transect across the North Atlantic. They found that 93% of all *nifH* genes detected during the transect were from *Trichodesmium* spp. (Goebel et al., 2010).

This suggests that *Trichodesmium* may be the primary species contributing to N_2 fixation in the North Atlantic.

N_2 fixation in *Trichodesmium* has a strong diel signal, with maximum nitrogenase activity during the midpoint of the light period (Saino and Hattori, 1978; Chen et al., 1998). The maximum N_2 fixation rate is affected by a complicated mixture of factors, including light (Capone et al., 2005; Breitbarth et al., 2008), phosphorus (P) and iron (Berman-Frank et al., 2001; Sañudo-Wilhelmy et al., 2001). In the western North Atlantic, the dominant paradigm is that P limits N_2 fixation (Sañudo-Wilhelmy et al., 2001).

Deep populations of Trichodesmium: A great deal of work has focused on quantifying the contribution of *Trichodesmium* to the fixed N pool (Reynolds et al., 2007; Goebel et al., 2010). Many of these studies utilized surface net tows or satellite data to estimate the size of the *Trichodesmium* population in near surface waters and extrapolate its contribution to global N fluxes (Marumo et al., 1974; Tassan, 1995; Capone et al., 1997; Subramaniam, 2002; Westberry et al., 2006). However, it is increasingly recognized that there are populations of *Trichodesmium* deeper than the near surface populations that are accessible using these methods. Recently, Davis and McGillicuddy (2006) utilized a video plankton recorder to determine the distribution of *Trichodesmium* across the North Atlantic basin. They found that *Trichodesmium* colonies were distributed much more broadly in the water column than previously estimated, with colonies as deep as 120 m. The deep vertical distribution of *Trichodesmium* in the water column suggests that using

surface net tows and satellite data to estimate the population may miss a significant portion of the community. Davis and McGillicuddy (2006) also noted that *Trichodesmium* colony morphology structured vertically in the water column. They noted a greater abundance of rafts in the upper 50 m of the water column, with a more even distribution of puffs throughout the photic zone. Post et al. (2002) also noted a greater abundance of rafts at the surface relative to puffs, which dominated the lower half of the photic zone in the Red Sea.

Accounting for new estimates of *Trichodesmium* in the subsurface, N_2 fixation rates in the North Atlantic could be 2.9-3.3 times higher than net-tow based estimates using a rectilinear model for light dependent N_2 fixation and 2.7-5.0 times higher than current estimates using a hyperbolic model for light dependent N_2 fixation (Davis and McGillicuddy, 2006). If these estimates are borne out by field observations, subsurface *Trichodesmium* N_2 fixation could account for discrepancies between estimates of new N required to sustain primary production and measured fluxes of N from surface N_2 fixation and abiotic processes (Davis and McGillicuddy, 2006). While these estimates for the contribution of subsurface populations to oceanic N cycling are tantalizing, the rate of N_2 fixation for these subsurface populations has yet to be measured *in situ*. This work focuses on a combination of culture and field work to examine subsurface N_2 fixation in *Trichodesmium*.

METHODS

Culture Studies

T. erythraeum strain IMS101 was grown in RMP growth media (Webb, 2001), composed of 75% 0.2 μm filtered Sargasso Sea water and 25% Millipore Q-water (Millipore, Bedford, MA). Water was heated to boiling in a microwave oven in Teflon containers. Chemical additions to the media were push filtered through 0.2 μm syringe filters. The final concentrations in the media were $1.5x10^{-6}$ M EDTA, $8x10^{-6}$ M phosphoric acid, $5x10^{-8}$ M ferric citrate, $1x10^{-7}$ $MnSO_4$, $1x10^{-8}$ M $ZnCl_2$, $1x10^{-8}$ M $NaMoO_4$ $1x10^{-10}$ M $CoCl_2$, $1x10^{-10}$ M $NiCl_2$, $1x10^{-10}$ $NaSeO_3$, and 1.5 μg vitamin B_{12}/L (Webb, 2001). Cultures were grown in Nalgene polycarbonate flasks (Nalgene, Rochester, NY) at 25°C on a ramping 12:12 l:d. During the first hour of the light cycle, the light level ramped from 0 to 30 μmol quanta m^{-2} s^{-1}, and ramped from 30 to 0 μmol quanta m^{-2} s^{-1} over the last hour of the light cycle. The ambient photon flux was ~30 μmol quanta m^{-2} s^{-1} for routine cultivation, and adjusted as indicated for experimental treatments. Culture experiments were designed to determine the effect of low light levels on N_2 fixation.

The effect of different irradiance levels – To examine light acclimation effects, the response of *T. erythraeum* strain IMS101 to chronic low light levels was analyzed. Triplicate 150 mL cultures were grown at one of three light levels, 70, 30 or 10 μmol quanta m^{-2} s^{-1}. Growth was measured every other day using an Aquafluor

handheld fluorometer (Turner Designs, Sunnyvale, CA). At mid-log phase all cultures were harvested for determination of nitrogenase activity (see below).

The effect of rapid changes in irradiance – The response of *T. erythraeum* IMS101 to sudden changes in light intensity was evaluated. Here 1.2 L of RMP media was inoculated with *T. erythraeum* IMS101 in mid log phase and grown at 30 μmol quanta m^{-2} s^{-1}. Growth was monitored using an Aquafluor handheld fluorometer. The cultures were grown until mid-log phase, at which point they were split into triplicate 200 mL flasks and incubated at 30, 10, or 0 μmol quanta m^{-2} s^{-1}. The cultures were split in the morning before the lights in the incubator turned on, under red lamps. N_2 fixation assays were performed 24 and 48 h after switching to the lower light level. The 10 μmol quanta m^{-2} s^{-1} light level was reached by wrapping two layers of neutral density screen around the culture flasks. Cultures were wrapped in two layers of black garbage bags to achieve total darkness (light meter readings oscillated between -0.05 and 0.02 μmol quanta m^{-2} s^{-1}, at the detection limit of the light meter). N_2 fixation was measured using the acetylene reduction assay on cells harvested 1 h before the midpoint of the light period.

Acetylene reduction assay

Nitrogenase activity, or N_2 fixation rate, was measured using the acetylene reduction assay described by Capone and Montoya (2001), using a 3:1 ratio for converting acetylene reduction to N_2 reduction. For lab based assays, triplicate incubations of mid

log phase cultures of *T. erythraeum* strain IMS101 were performed with 30 mL of sample in acid clean 60 mL polycarbonate bottle capped with a Teflon Silicon septum (I-Chem, Rockwood, TN). Assays were run for 2 h and designed to intersect the midpoint of the light cycle, when N_2 fixation rates were highest. 0.3 mL syringe samples were run on a Shimadzu GC-8A gas chromatograph (Shimadzu, Columbia, MD). After the assay was completed, 100 µL subsamples were taken, and a cell count was performed to normalize assay results for differences in the biomass yield of high light and low light culture experiments. Cell counts were done using an AxioPlan 2 epifluorescence microscope (Zeiss, Thornwood, NY). Individual filaments were examined under 20x magnification and the AxioVision software (V 4.1, Zeiss, Thornwood, NY) was used to determine the average length of the cells composing that filament. All filaments in 100 µL culture were counted and measured. The cell length was then used to calculate the cell concentration (Bell and Fu, 2005; Fu and Bell, 2003). Laboratory rates are reported as nmol N_2 fixed $cell^{-1}$ h^{-1}. One-way analysis of variance (ANOVA) tests (with a post hoc Tukey test applied to the data sets as noted) and Student's t-tests were applied to the culture N_2 fixation rate data to determine statistically significant differences ($p < 0.05$).

For field assays, 10-20 sorted and washed net or pump collected *Trichodesmium* colonies were assayed after being resuspended in 30 mL 0.2 µm filtered local seawater in a 60 mL polycarbonate bottle. Incubations were performed at *in situ* light levels, up to 400 µmol quanta m^{-2} s^{-1} (Table 1), the highest light level possible in the shipboard Percival incubator (Percival Scientific). The temperature in the incubator was kept at 28.5°C. Assays were conducted from approximately 1 h before local midday to

approximately 1.5 h post local midday. When biomass yields were high enough, triplicate incubations were performed. In the field, nitrogenase activity was normalized to moles C. Colonies were filtered onto a combusted GF/F filter using vacuum filtration and CHN analysis was performed by the Marine Biological Laboratory Stable Isotope Laboratory, Woods Hole, MA using standard protocols. Values for field samples are reported as μmol N_2 fixed h^{-1} mol C^{-1}. Statistical significance ($p < 0.05$) was determined using one-way ANOVA.

Table 1: Cruise information and physiochemical parameters for OC469.

Date	Station Number	Latitude x Longitude	Pump Depth (m)		Temperature (°C)		*In Situ* Light Level (µmol quanta m^{-2} s^{-1})		Incubation Light Level (µmol quanta m^{-2} s^{-1})		Mixed Layer Depth (m)
			Surface	Subsurface	Surface	Subsurface	Surface	Subsurface	Surface	Subsurface	
4-Oct-10	1	34.79723°N x 66.52867°W	20	60	26.28	26.40	380	75	390		64
6-Oct-10	3	28.5652°N x 65.08885°W	20	60	26.96	25.06	600	210	395	140	32
7-Oct-10	4	29.00323°N x 66.0154°W	9	60	26.48	24.20	210	120	240	115	37
8-Oct-10	5	29.0174°N x 66.0123°W	8	60	26.59	24.83	275	70	300	65	30
10-Oct-10	6	25.50213°N x 67.27695°W	8	60	28.00	27.71	500	200	500	200	45*
11-Oct-10	7	22.50218°N x 67.29367°W	6	80	28.40	27.72	210	200	310	200	25*
12-Oct-10	8	21.25293°N x 64.00508°W	8	70	28.40	26.50	1500	200	400	160	37*
15-Oct-10	10	15.2193°N x 54.69137°W	6	60	29.13	28.70	1300	300	400	270	45*
16-Oct-10	11	13.41388°N x 51.31435°W	6	50	29.07	29.00	1500	400	400	400	40*
17-Oct-10	12	11.86387°N x 48.43662°W	6	60	29.34	26.88	1600	250	400	250	45*
18-Oct-10	13	11.53623°N x 51.32265°W	6	50	29.42	28.44	2000	350	400	350	10*
19-Oct-10	14	12.15608°N x 54.46175°W	6	50	29.67	27.00	1500	325	400		10*
21-Oct-10	16	12.87168°N x 56.33887	6	60	29.58	28.11	1500	220	400	220	15*
Average±Standard Deviation					28.26±1.26	27.05±1.59	1157±812	244±102	368±52	206±104	

*: Stations where the mixed layer depth was in dispute based on the temperature and salinity profiles. Generally speaking, at these stations, there appeared to be a stratified layer overlaying a less recent mixed layer.

Field collection methods and studies

Field samples of *Trichodesmium* were collected for analysis during October, 2010 aboard the RV Oceanus on cruise OC469, which followed a cruise track from Woods Hole, MA to Barbados (Figure 1).

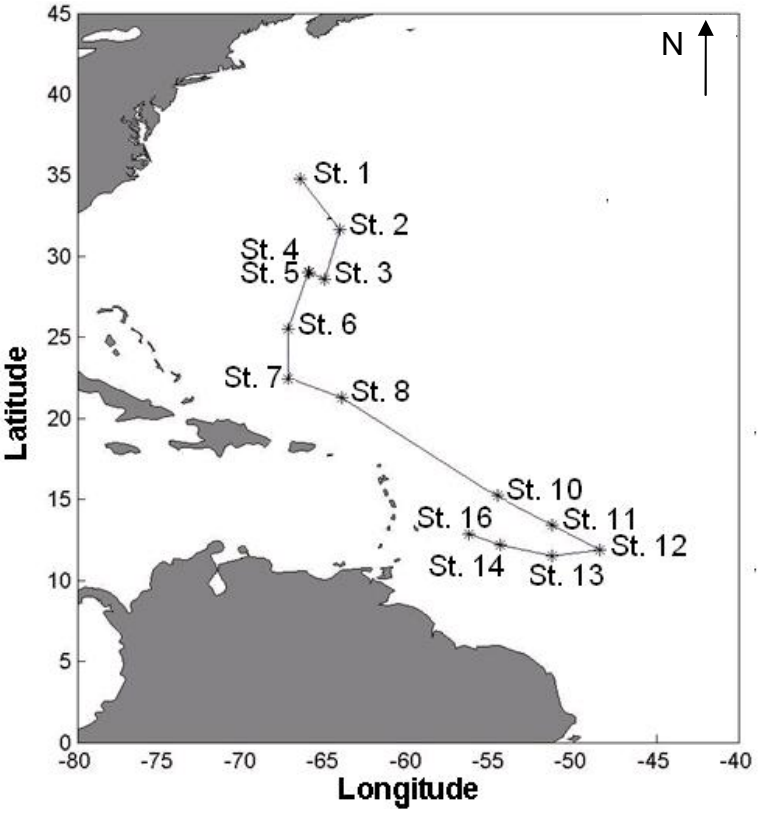

Figure 1: Leg one of cruise OC469, from Woods Hole, MA to Bridgetown, Barbados from October 2-22, 2010. Stations (*) are labeled 1-16.

In the field, colonies were isolated using two different methods. Samples for physiological parameters other than N_2 fixation rate were obtained from the surface water by hand-towing a 130.0 μm plankton net with a 25 m line. Surface and subsurface populations of *Trichodesmium* were sampled discretely for N_2 fixation using a pump-

based approach utilized for sampling subsurface phytoplankton populations in the Gulf of Maine (Anderson et al., 2005). *Trichodesmium* pump and net sampling were performed from 0900-1100 h EST. Pump depths were chosen based on observed mixed layer depth during a 100 m cast immediately prior to the surface pump and abundance data from a towed video plankton recorder when available. Actual sampling time varied to ensure incubations could be performed at local midday, which was determined using The National Oceanic and Atmospheric Administration Sunrise/Sunset Calculator (http://www.srrb.noaa.gov/highlights/sunrise/sunrise.html). This temporally adaptive method was utilized to ensure that N_2 fixation assays always coincided with local midday, when *Trichodesmium* N_2 fixation rates have been shown to be highest (Saino et al., 1978). Colonies were transferred from the cod end of the net or pump to a plastic beaker and rapidly sorted inside, shaded from direct sunlight. Colonies were picked using Pasteur pipettes and washed twice in 0.2 μm filtered local seawater (Webb, 2007). The washing process took approximately 15 minutes. When biomass permitted, triplicate flasks were assayed for nitrogenase activity.

Response to rapid changes in light level – To understand the response of *Trichodesmium* to abrupt changes in the light field, a light switching experiment was undertaken at Station 11. *In situ* surface irradiance at this station was 1500 μmol quanta m^{-2} s^{-1}, and *in situ* subsurface irradiance was 400 μmol quanta m^{-2} s^{-1}. At this station, colonies from the surface pump were split into six 60 mL acid washed polycarbonate filters, three of which were incubated at 400 μmol quanta m^{-2} s^{-1} and three were incubated at

170 μmol quanta m^{-2} s^{-1} for the duration of the N_2 fixation assay. This light level was chosen to ensure a strong response from the surface cultures, and is within the range of subsurface irradiances observed. All treatments were incubated at 28.5°C. To determine statistically significant differences between rates, one-way ANOVA tests were performed ($p < 0.05$).

Assessing morphological differences in N_2 fixation – To understand differences in N_2 fixation driven by differences in *Trichodesmium* puff and raft colony morphology, surface pump collected populations of *Trichodesmium* were separated at Station 10 on the basis of morphology. Replicates were incubated at 400 μmol quanta m^{-2} s^{-1}. Triplicate N_2 fixation assays were performed for each morphology and incubated for 2.5 h, spanning midday. A Student's t-test was used to determine whether rates were statistically significantly different ($p < 0.05$)

Alkaline phosphatase activity assays

Colonies of *Trichodesmium* were isolated and washed after collection using a 130 μm plankton net. Colonies were filtered onto a 5 μm 47 mm polycarbonate filter and stored at -20°C prior to analysis. Alkaline phosphatase (AP) activity was measured using a fluorometric assay (Dyhrman and Ruttenberg, 2006), that has commonly been used for *Trichodesmium* in the western North Atlantic (Orchard et al. 2010). Colony morphologies were assayed individually and the measured AP activity rates were averaged to determine the *Trichodesmium* AP activity for each station. The relationship between AP activity and

N_2 fixation was linear, and a linear regression was applied. The Pearson correlation coefficient was calculated to determine correlation.

RESULTS

Culture results

The effect of irradiance on N_2 fixation – T. erythraeum IMS101 was cultured at different irradiance levels to understand the effect of light acclimation over several generations on the N_2 fixation rates of *Trichodesmium.* The mean rates for the three treatments were analyzed using a one-way ANOVA, and were found to be significantly different from every other treatment ($p < 0.001$) The overall trend showed N_2 fixation rate increasing with increasing light (Figure 2).

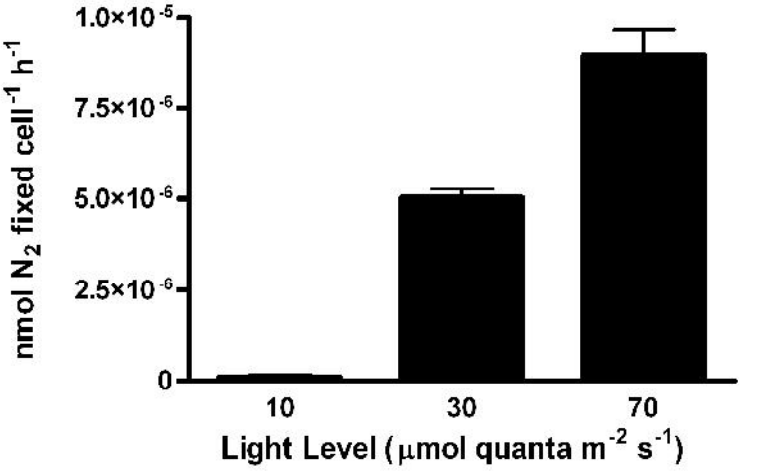

Figure 2: N_2 fixation rates of *T. erythraeum* IMS101 normalized to cell number and plotted as a function of light intensity sustained for several generations. Data are from triplicate flasks. N_2 fixation rates in each treatment were significantly different ($p < 0.001$) from the other treatments over the range of intensities tested. Significance was determined using one-way ANOVA. Bars represent ±1 standard deviation.

Response to rapid changes in irradiance – When cultures grown at 30 μmol quanta m^{-2} s^{-1} were shifted to a lower irradiance (10 μmol quanta m^{-2} s^{-1}) or to complete darkness the N_2 fixation rate in both treatments decreased. The N_2 fixation rates were significantly different between triplicate experimental treatments within a timepoint (analyzed using one-way ANOVA; $p = 0.003$ at 24 h and $p = 0.001$ at 48 h), however rates did not significantly change between 24 and 48 h (analyzed using a one-way ANOVA with a post hoc Tukey test) (Figure 3). The culture shifted from 30 μmol quanta m^{-2} s^{-1} to 10 μmol quanta m^{-2} s^{-1} showed a 47% decrease in N_2 fixation rate relative to the 30 μmol quanta m^{-2} s^{-1} light level control after 24 h (Figure 3). The cultures shifted from

Figure 3: N_2 fixation in *T. erythraeum* IMS101 normalized to cell number and plotted as a function of light intensity. All cultures were grown at 30 μmol quanta m^{-2} s^{-1} until mid log phase, at which point triplicate flasks were switched to 0, 10 or 30 μmol quanta m^{-2} s^{-1}. Differences in rate were analyzed using one-way ANOVA and a post hoc Tukey test. All rates were significantly different from each other treatment within a timepoint ($p<0.005$), but did not significantly change from 24 to 48 h. Error bars represent ±1 standard deviation. Treatments with statistically significant rate differences are noted by different letters (a, b, c).

30 μmol quanta m^{-2} s^{-1} to darkness decreased 87% in N_2 fixation rate after 24 h (Figure 3). The culture shifted from 30 to 10 μmol quanta m^{-2} s^{-1} decreased 88% in N_2 fixation rate after 48 h, and the treatment left in darkness for 48 h decreased 92% relative to the 30 μmol quanta m^{-2} s^{-1} control 48 h into the experiment (Figure 3).

A few interesting trends are evident in a comparison of the two independent light effects experiments. In the first experiment, cultures were entrained at 10, 30 and 70 μmol quanta m^{-2} s^{-1} over the course of several generations in culture. In the second experiment, cultures grown at 30 μmol quanta m^{-2} s^{-1} until mid log phase were shifted abruptly to 10 or 0 μmol quanta m^{-2} s^{-1}. In the second experiment, the N_2 fixation rate at 10 μmol quanta m^{-2} s^{-1} was higher than the N_2 fixation rate at the same irradiance in the first experiment, where several generations were adapted to that light level. Based on the first long term experiment, a 98% decrease in activity would be anticipated after shifting the culture grown at 30 μmol quanta m^{-2} s^{-1} culture to 10 μmol quanta m^{-2} s^{-1}. However, the abrupt shift in irradiance only reduced N_2 fixation by 47%.

Field results

N_2 fixation in subsurface populations – N_2 fixation in field populations of *Trichodesmium* was measured during October 20l0 on cruise OC469 in the western North Atlantic (Figure 1). Temperatures on the cruise ranged between approximately 24 and 29.5°C (Table 1). The average surface temperature was 28.26±1.26°C and the average subsurface temperature was 27.03±1.52°C. Colony morphology generally structured vertically in the water column, with rafts being most abundant at the surface and the puff morphology

dominating at depth. Sampling for the subsurface populations was adaptive. The absolute depth for the subsurface pump varied daily, ranging between 50 and 80 m (Table 1), and was typically below the predicted mixed layer.

In situ photosynthetically active radiation (PAR) for samples from the surface pumps ranged from approximately 200 to 2000 μmol quanta m^{-2} s^{-1} (Table 1). N_2 fixation rates at the surface ranged from 33 to 156 μmol N_2 fixed h^{-1} mol C^{-1} (Table 1). In contrast, PAR for N_2 fixation samples taken from subsurface pumps ranged from approximately 70 to 400 μmol quanta m^{-2} s^{-1} (Table 1). N_2 fixation rates from subsurface samples ranged from 9 to 88 μmol N_2 fixed h^{-1} mol C^{-1} (Table 2; Figure 4). Regardless of the depth of the subsurface pump, N_2 fixation rates for *Trichodesmium* colonies isolated from surface pumps showed significantly higher N_2 fixation rates than subsurface colonies at all stations (p = 0.0007) (Table 2, Figure 4).

Table 2: . Biological assay measurements for OC469.

Station Number	N_2 Fixation Rate (μmol N_2 h^{-1} mol C^{-1})			Alkaline Phosphatase Activity (nmol P h^{-1} $colony^{-1}$)$^{\kappa\ \alpha}$
	Surface§	Subsurface §	Net Collected$^{\alpha}$	ND
1	48.6	ND	ND	ND
3	34.4±12.0	16.5	ND	0.50±0.54
4	32.8	NS	6.2	0.74±0.51
5	156.6±21.6	NS	82.4±52.7	0.88±0.41
6	42.6	18.6	129.9±12.2	0.45±0.07
7	124.1±42.8	20.6	122.5±19.8	0.84±0.21
8	89.7±30.6	18.9±0.2	106.1±14.8	0.35±0.22
10	98.4±13.5	41.0±12.0	103.2±4.7	0.49±0.30
11	78.1±26.6	9.3±0.2	116.4±28.0	0.43±0.07
12	39.7±22.5	NS	155.9±27.1	0.25±0.02
13	125.9±25.3	24.9±9.2	140.4±87.1	0.45±0.08
14	71.6±12.9	ND	91.9±38.9	0.38±0.12
16	149.3±90.4	87.9±62.6	302.5±128.1	0.26±0.03
Average±Standard Deviation	91.7 ±49.05	31.3±30.80	127.4±79.60	0.50±0.29

§: Colonies collected via a pump based sampling approach.

α: Colonies were gathered using a 130 μm plankton net hand towed with a 25 m line.

κ: Morphologies were assayed for alkaline phosphatase activity separately and then averaged to generate the activity for each station.

ND: Insufficient or no biomass collected for assay.

NS: Measured N_2 fixation rates could not be resolved from zero.

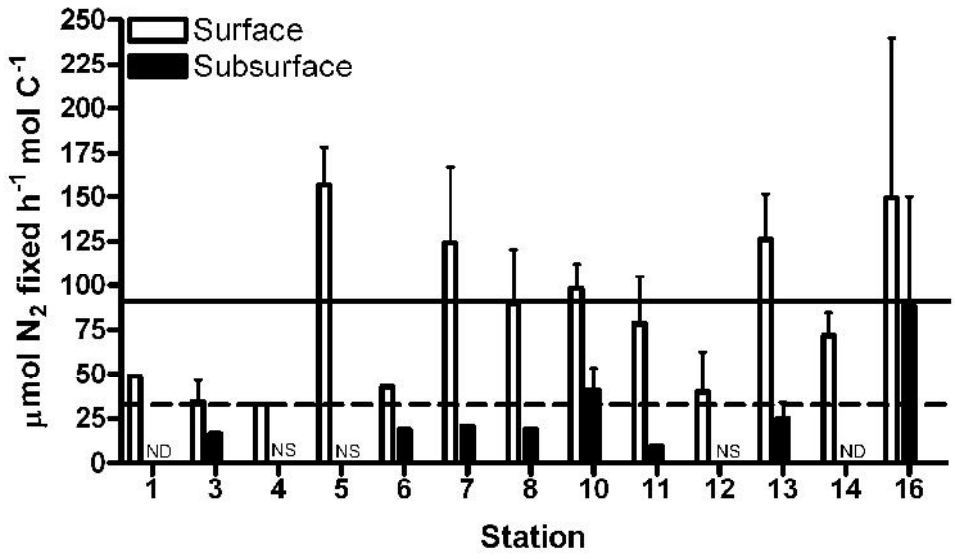

Figure 4: *Trichodesmium* N_2 fixation rate normalized to C plotted at each station along the cruise transect. Surface pump depths ranged from 6-20 m and subsurface pump depths ranged from 50-80 m. N_2 fixation assays were run in triplicate when biomass yields from the pump were high enough; stations without error bars indicate stations where there was only one replicate. Stations marked "NS" indicate stations where N_2 fixation rates were too low to be statistically detectable. Stations marked "ND" indicate stations where no deep N_2 fixation data was available, typically due to low pump biomass. The means of surface and subsurface N_2 fixation were significantly different ($p = 0.0007$). Bars represent ± 1 standard deviation. The dashed line indicates the average N_2 fixation rate for pump collected subsurface *Trichodesmium*, and the solid line indicates the average N_2 fixation rate for pump collected surface *Trichodesmium.*

N_2 fixation in the subsurface populations accounted for between 11 and 60% of the surface population N_2 fixation (Figure 5). Davis and McGillicuddy (2006) used two models (Capone et al., 2005; Orcutt et al., 2001), one rectilinear and one hyperbolic, which predict subsurface percent N_2 fixation rate relative to the surface based on the percent of surface light level. The *in situ* light levels from cruise OC469 were used to determine expected percent N_2 fixation rate at subsurface depths and compared to the observed data (Figure 5).

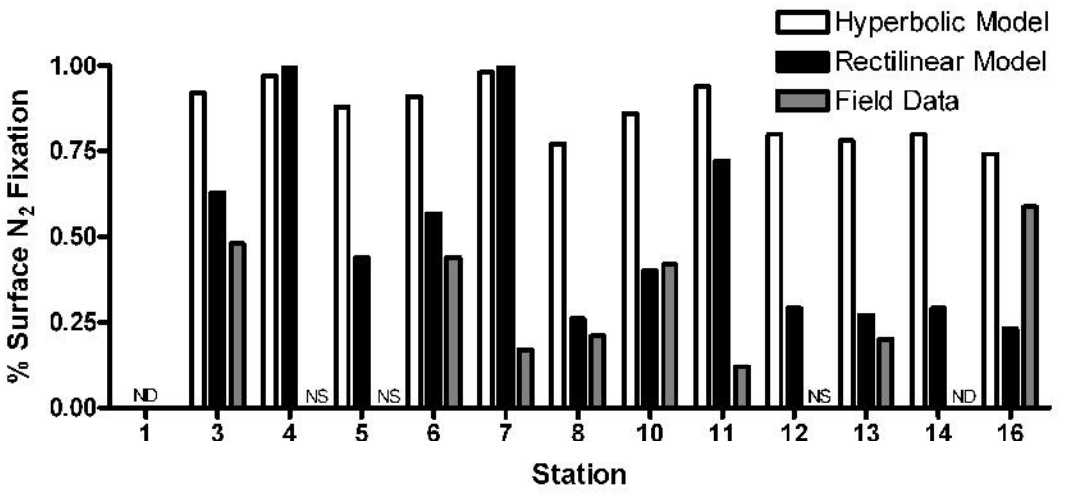

Figure 5: Percent surface N_2 fixation of subsurface *Trichodesmium* populations determined using the rectilinear (Orcutt et al., 2001) and hyperbolic (Capone et al., 2005) model for N_2 fixation as a function of percent surface light intensity for each station. Grey bars represent measured subsurface N_2 fixation as a fraction of surface N_2 fixation. Stations marked "NS" did not have significant subsurface N_2 fixation. Stations marked "ND" did not have subsurface N_2 fixation rates for comparison. Percent N_2 fixation was significantly different from field results when comparing to results generated from the hyperbolic model ($p = 0.0001$). There was no statistical difference between field data and results generated using the rectilinear model ($p = 0.4120$).

Based on the surface and subsurface light intensities measured on transect OC469, it was predicted that subsurface N_2 fixation would range between 74 and 97% using the hyperbolic model (Capone et al., 2005) relating N_2 fixation and light intensity. This was significantly different from the observed values, determined using a one-way ANOVA, ($p = 0.0001$). The rectilinear model relating N_2 fixation and light intensity (Orcutt et al., 2001), predicts that subsurface N_2 fixation rates would range between 23 and 100% of surface N_2 fixation (Figure 5). There was no statistical difference ($p = 0.4120$) between observed N_2 fixation rates and the rates predicted by the rectilinear model.

There was a significant ($p = 0.0075$) non linear, relationship between *in situ* irradiance and N_2 fixation levels (Figure 6A). Subsurface rates tended to fall slightly below the non-linear fit to the pooled surface and subsurface data. N_2 fixation assays were as close to *in situ* light levels as possible, but incubator light levels only reached 400 µmol quanta m^{-2} s^{-1}. Because short term changes in light level caused changes in N_2 fixation rates, N_2 fixation rate versus incubation light level was plotted in Figure 6B, where surface N_2 fixation tended to cluster at higher light intensities than subsurface samples.

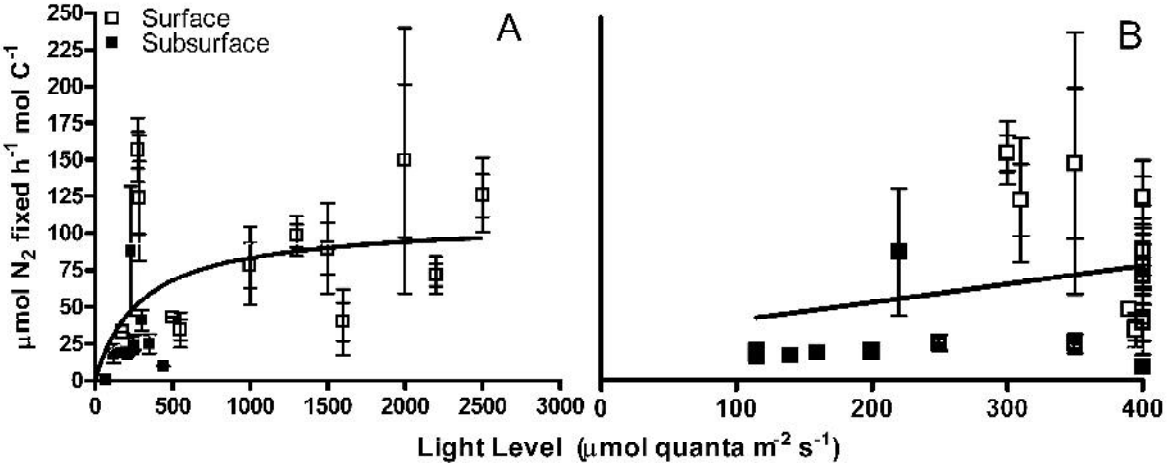

Figure 6: *Trichodesmium* N_2 fixation plotted versus *in situ* light level (A) and incubation light level (B). N_2 fixation assays were run in triplicate when *Trichodesmium* biomass from surface and subsurface pumps was available. Subsurface incubations were run at approximately *in situ* light levels for all stations. Surface incubations were run at approximately *in situ* light levels, up to 400 µmol quanta m^{-2} s^{-1}, the highest irradiance possible in the incubator. When N_2 fixation was plotted against *in situ* light level the best-fit line to the data was a non-linear hyperbolic equation of the form N_2 fixation rate = B_{max}*(Light level)/(K_d + Light level) where B_{max} = 108.1±19.58 and K_d = 297.4± 173.7 (A). When N_2 fixation was plotted against incubator light level, surface and subsurface samples tended to cluster separately. The relationship when N_2 fixation was plotted against incubator light level was not significant ($p = 0.1400$). Error bars represent ± 1 standard deviation.

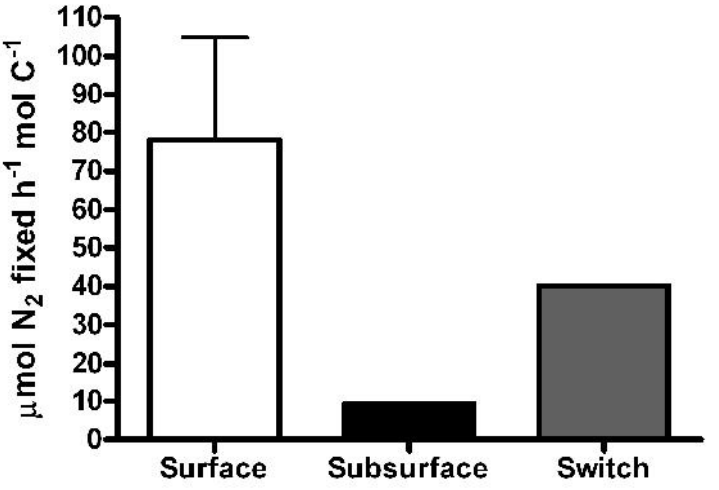

Figure 7: *Trichodesmium* N_2 fixation rates from surface, subsurface and surface populations incubated at low irradiance (switch) (isolated at 1500 μmol quanta m^{-2} s^{-1} and incubated at 170 μmol quanta m^{-2} s^{-1}). Surface and subsurface N_2 fixation assays were run in triplicate and the switch treatment was run in duplicate. Surface and deep populations were both incubated at 400 μmol quanta m^{-2} s^{-1}. Error bars represent ± 1 standard deviation. Statistical differences were assessed using a one-way ANOVA. Rate differences were statistically significant ($p = 0.01$).

Based on the hyperbolic relationship posited by Capone et al. (2005), and used by Davis and McGillicuddy (2006) to calculate subsurface N_2 fixation, the anticipated change in N_2 fixation would be an approximately 80% decrease from surface N_2 fixation. Using the rectilinear relationship presented Orcutt et al. (2001), also used by Davis and McGillicuddy (2006), N_2 fixation would be expected to decrease by approximately 75% relative to the surface. The hyperbolic curve generated from the measured rates predicts a 53% decrease in N_2 fixation (Figure 6). The observed decrease in rate was approximately 34%.

Assessing morphological differences in N_2 fixation – Puffs and rafts were distributed differently throughout the water column, with puffs dominating the subsurface populations. To determine whether or not there were intrinsic differences in N_2 fixation rate between morphotypes, surface populations were separated into raft and puff morphologies and N_2 fixation was measured at Station 10 at 400 µmol quanta m^{-2} s^{-1}. Puffs showed approximately 60% lower N_2 fixation rates relative to rafts isolated from surface net tows. Statistical significance was determined using a Student's t-test, and was determined to be significant ($p = 0.02$) (Figure 8).

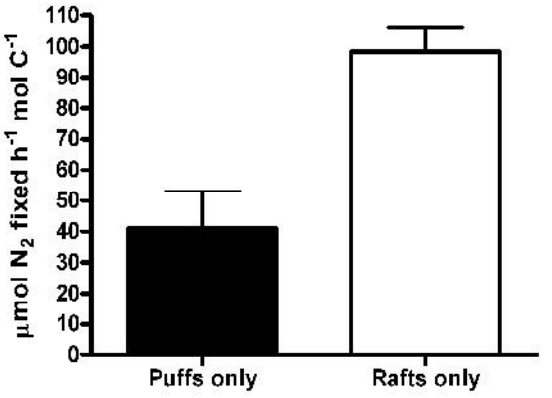

Figure 8: N_2 fixation rate for net collected puff and raft colonies of *Trichodesmium* from Station 10. Triplicate N_2 fixation assays were performed at the same light levels. The difference in rate was significant as determined using a student's t-test ($p = 0.002$). Bars represent ±1 standard deviation.

The effect of temperature on N_2 fixation – Temperature ranged from 26.3 to 29.6°C at the surface, and in the subsurface from 24.2 to 29.0°C. The relationship of temperature to N_2 fixation rate was plotted to assess the effect of changes in temperature on N_2 fixation rates. There was a significant linear correlation between N_2 fixation and temperature ($p = 0.02$) (Figure 9).

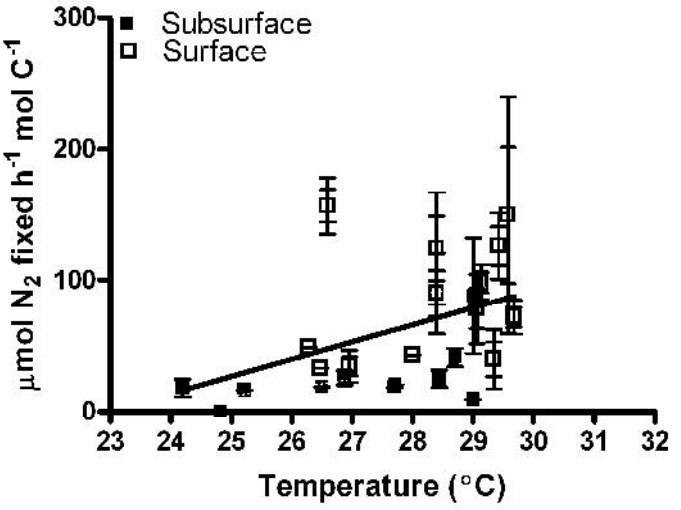

Figure 9: The relationship of *Trichodesmium* N_2 fixation rate to *in situ* temperature. N_2 fixation rates were measured from surface and subsurface pump collected samples. Temperatures were measured using a CTD aboard cruise OC469. There was a significant positive correlation between N_2 fixation and temperature (p = 0.02) the slope = 13.06 ± 4.613. R^2 = 0.1335. Error bars, where present, represent ± 1 standard deviation

Alkaline phosphatase activity and N_2 fixation – AP activity and N_2 fixation were measured on mixed colonies of puffs and rafts isolated from surface net tows to determine the effect of P availability on surface populations of *Trichodesmium*. N_2 fixation rates were significantly (p = 0.04) inversely correlated with AP activity for the stations where both assays were performed (Figure 10).

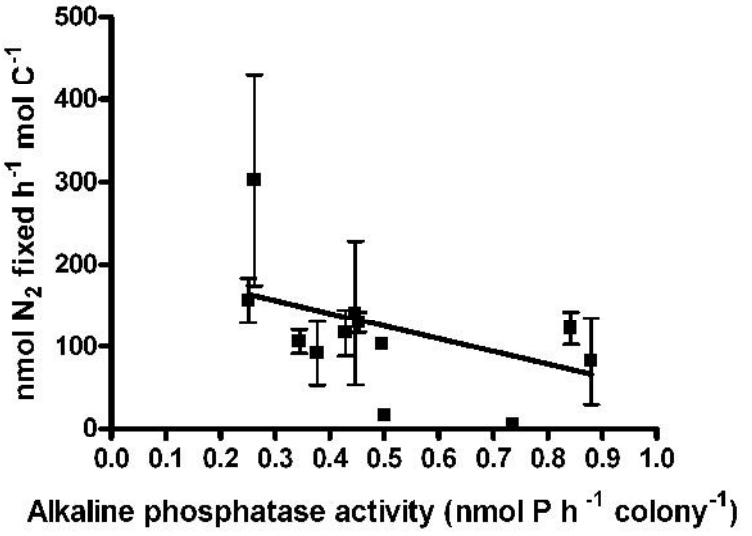

Figure 10: Net collected *Trichodesmium* N_2 fixation rates versus average alkaline phosphatase (AP) activity for all stations where both sets of data were available. AP activity assays were performed on each colony morphology and then averaged. Slope = -155.4 ± 63.96. R^2 = 0.164, p = 0.04. When present, bars represent ± 1 standard deviation

DISCUSSION

The relationship between irradiance level and N_2 fixation has been intensely studied in field and in culture (Bell et al., 2005; Breitbarth et al., 2008; Capone et al., 2005; Orcutt et al., 2001). Interest in this area has been piqued in recent years due to the finding that *Trichodesmium* populations are distributed much more deeply in the water column than previously thought (Davis and McGillicuddy, 2006). The contribution of subsurface populations of *Trichodesmium* to basin scale N_2 fixation was modeled by Davis and McGillicuddy (2006), but until this study, there has been no assessment of *in*

situ N_2 fixation rates from discrete subsurface populations of *Trichodesmium* to validate these predictions.

Subsurface N_2 fixation

N_2 fixation assays on pump collected, discrete subsurface and surface populations showed that N_2 fixation rates in subsurface populations were on average 33% of surface rates and ranged between 11 and 60%. The absolute rates that ranged from 33 to 156 μmol N_2 h^{-1} mol C^{-1} in the surface and 9 to 88 μmol N_2 h^{-1} mol C^{-1} in subsurface populations. The surface rates of N_2 fixation are comparable to the rates detected by others in the North Atlantic (Sañudo-Wilhelmy et al., 2001). Previous work examining the vertical structuring of N_2 fixation by *Trichodesmium* colonies used net collected samples, integrating *Trichodesmium* populations through the water column (Orcutt et al., 2001; Capone et al., 2005). To our knowledge, this is the first confirmation of subsurface *Trichodesmium* N_2 fixation from discrete depths below the predicted mixed layer in the western North Atlantic. The results from this work may be extrapolated to other systems where subsurface populations have been detected (Carpenter et al., 2004).

Davis and McGillicuddy (2006) used two independent models for predicting *Trichodesmium* N_2 fixation as a function of light, a rectilinear curve (Orcutt et al., 2001), and a hyperbolic function (Capone et al., 2005). The models presented by Davis and McGillicuddy (2006) predict that N_2 fixation rates would range between 74 and 97% of surface rates using the hyperbolic model. This prediction is significantly higher than the observed fraction of surface N_2 fixation by subsurface populations, and also higher than

the extrapolated average (53%) from the light versus N_2 fixation relationship derived from the field observations. The rectilinear relationship between N_2 fixation and light intensity predicts that subsurface N_2 fixation rates would range between 23 and 100% of surface N_2 fixation for *Trichodesmium*. The large range of N_2 fixation is based on the fact that the rectilinear curve predicts 100% of N_2 fixation at only 50% surface light level. These percentages were not significantly different than the observed rates, indicating that the rectilinear curve for N_2 fixation versus light level (Orcutt et al., 2001) used by Davis and McGillicuddy (2006) could be a better model for predicting *in situ* N_2 fixation rates along a light gradient in the field. However, most of this may be driven by the larger variability, or range, in the rectilinear model. Ultimately, the best predictor of *Trichodesmium* light driven N_2 fixation may be derived from measured data.

The best-fit line for N_2 fixation rates versus light level on transect OC469 was a hyperbolic curve, where there was a significant correlation between N_2 fixation and light level. However, the hyperbolic curve data is based on *in situ* light levels. All incubations were run at *in situ* light levels within the constraints of the highest irradiance achievable on the shipboard Percival Incubator (400 μmol quanta m^{-2} s^{-1}), and even short term changes in the light field (over the course of a N_2 fixation assay – see below) can influence the rate. Plotting N_2 fixation versus incubator light level still yielded a modest positive trend between light level and N_2 fixation, although this trend was not significant. All but one of the subsurface N_2 fixation rates clustered beneath the best-fit line for the rate data plotted versus *in situ* light level. This indicates that the best-fit line generated for the field data was driven by the higher rates at the surface. Regardless of the form of the

function, there was a significant positive relationship between *in situ* light levels and N_2 fixation in the field data, and an expansion of this dataset would further confirm the best function for predicting subsurface N_2 fixation from light levels.

The fact that the measured N_2 fixation rates, roughly 33% of the surface, were lower than predicted based on light driven extrapolations implies that, while light is one of the driving factors affecting N_2 fixation in the water column, there may be factors other than light that have an effect on N_2 fixation and drive the vertical gradient of N_2 fixation rates in the field, including mixing, temperature, population heterogeneity and P availability.

Factors affecting N_2 fixation

Long term changes in irradiance related to stratification – Studies have shown an association between high *Trichodesmium* abundance and mixed layer depth, with shallower mixed layer depths favoring higher abundances of *Trichodesmium.* An association between abundance and vertical stratification has been documented in the North Pacific subtropical gyre (Karl et al., 2001), and in the North Atlantic (Sañudo-Wilhelmy et al., 2001; Tyrrell et al., 2003). This association could be related to low light intensities below deep mixed layers, which may make it difficult for *Trichodesmium* to meet the high energy requirements of N_2 fixation (Tyrrell et al., 2003). For cruise OC469, mixed layer depth was defined for each station using the temperature and density criteria of Levitus (1982). However, incongruence between the temperature and salinity profiles made the mixed layer depth difficult to resolve in most cases. Four stations, Stations 1, 3,

4 and 5 had highly resolved mixed layer depths, but only Stations 3, 4 and 5 had sufficient subsurface biomass for an N_2 fixation measurement. Subsurface N_2 fixation was undetectable at Stations 4 and 5, and was 16.5 μmol N_2 h^{-1} mol C^{-1} at Station 3. The Station 3 N_2 fixation rate was considerably lower than the average subsurface N_2 fixation rate in the field (31.3 μmol N_2 h^{-1} mol C^{-1}). The measured rate was also lower than N_2 fixation rate at Station 16 (87.9 μmol N_2 h^{-1} mol C^{-1}), where the subsurface light level was similar (220 versus 210 μmol quanta m^{-2} s^{-1}), but where the remnant mixed layer was only 15 m deep. The low rate of N_2 fixation in the subsurface of Station 3 is consistent with the population of *Trichodesmium* having been entrained at low irradiance for several generations, based on culture studies done with *T. erythraeum* strain IMS101, where acclimation to low light over several generations strongly depressed N_2 fixation rates. These data indicate that long term shifts in light level related to stratification may strongly structure N_2 fixation along vertical gradients in the field, and supports the association between *Trichodesmium* abundance and shallow mixed layers.

Short term changes in irradiance related to mixing – For Stations 6-16 along cruise OC469, active mixing may have ceased sometime prior to sampling, and mixed layer depths were hard to resolve, and were therefore likely remnants (Table 1, stations marked with a *) (D. McGillicuddy, personal communication, December 24, 2010). There was no observed trend between N_2 fixation rate and mixed layer depth for in this study. This may be due to the fact that many of the stations along the transect had very shallow mixed layers or were stratified with remnant mixed layers below the surface. Although the data

presented do not show a trend related to mixed layer depth, further research could reveal relationships structuring with seasonal mixed layers.

While there was no evidence to support a relationship between mixed layer depth and N_2 fixation, culture work suggests that a variable light regime within the mixed layer, occurring on the order of 24 hours or less, could strongly influence N_2 fixation rates. In laboratory experiments where cultures were shifted from 30 to 10 μmol quanta m^{-2} s^{-1} or 30 to 0 μmol quanta m^{-2} s^{-1} on the scale of 24-48 hours, N_2 fixation rates were significantly depressed. This is consistent with the findings of Chen et al. (1999), who reported a complete loss of N_2 fixation activity in *T. erythraeum* after shifting cultures from the control irradiance (100 μmol quanta m^{-2} s^{-1}) to complete darkness. However, *T. erythraeum* IMS101 subjected to a sudden change in irradiance maintained higher N_2 fixation rates than would be predicted from rates observed in cultures maintained at the same irradiance levels for several generations. For example, when entrained at 10 μmol quanta m^{-2} s^{-1} over several generations, N_2 fixation rates were 97% lower than rates for cultures grown at 30 μmol quanta m^{-2} s^{-1}. However, when cultures were shifted from 30 to 10 μmol quanta m^{-2} s^{-1} the N_2 fixation rate decreased by 53% over 24 hours. Regardless, these results imply that short term changes in light intensity, even within a mixed layer, could affect N_2 fixation rates. In brief, *Trichodesmium* that are actively fixing N_2 in the mixed layer may respond to transient changes in light intensity related to mixing, leading to a vertical gradient in N_2 fixation even within well mixed populations.

To understand the effect of short term changes in light intensity on field populations of *Trichodesmium*, the response of surface, pump collected field populations

to changes in light intensity was examined at Station 11. Here, the surface pump depth was 6 m. The temperature and salinity profiles at Station 11 indicated a remnant mixed layer at 45 m with a shallow stratified layer in the first 12 – 15 m. The *Trichodesmium* colonies from the surface pump were therefore not being actively mixed to depth at Station 11, where the irradiance at 6 m was 1500 μmol quanta m^{-2} s^{-1}. There was a significant reduction in N_2 fixation rate in surface samples incubated at 170 μmol quanta m^{-2} s^{-1} relative to the surface controls incubated at 400 μmol quanta m^{-2} s^{-1}, again indicating that the effect of light on N_2 fixation rate is rapid (less than 24h). In summary, light is an important factor influencing instantaneous N_2 fixation rates, likely leading to a strong vertical gradient of N_2 fixation with depth, even in the presence of mixing.

Temperature effects on N_2 fixation - One other factor that may be affecting the *in situ Trichodesmium* N_2 fixation rates is temperature. The temperature range for the subsurface samples was greater than the temperature range observed for the surface samples. At the surface, temperature ranged from 26.3 to 29.6°C, and in the subsurface from 24.2 to 29.0°C. Although all the temperatures were within the range of maximum growth rate (Breitbart et al., 2007), there was a significant relationship between N_2 fixation and temperature in the samples collected in this study. It should be noted that while the average temperature at the surface (28.26 ± 1.27°C) and in the subsurface (27.03 ± 1.52°C) were near the temperature indicated by Breitbarth et al. (2007) as the temperature at which N_2 fixation was maximal (27°C), there were several stations outside

of this range. At these stations, temperature may be affecting N_2 fixation. This relationship may most strongly affect subsurface populations of *Trichodesmium*. Temperature decreases with depth below the mixed layer, so the N_2 fixation rates of subsurface populations of *Trichodesmium* may be affected by the combined effects of decreased temperature and low light intensity. The decrease in temperature with depth could reduce N_2 fixation in subsurface populations, potentially compounding the effect of low irradiance.

Morphology and N_2 fixation – Field populations appeared to partition into two separate populations, the surface and subsurface populations, whose morphology and response to changes in irradiance differed. The surface population was dominated by the raft morphology, while subsurface populations were dominated by puffs at all stations. The distributions of the two colonies with depth was consistent with findings in the Red Sea (Post et al., 2002), where puffs were most abundant in the subsurface. This distribution could be related to differences in buoyancy between the two morphologies, physiological differences between the raft and puff morphology that favor this distribution, or species variability between the surface and subsurface colonies, although morphology is not considered a good predictor of species identity. Subsurface N_2 fixation was consistently lower than surface population rates, even at relatively high irradiance. Populations of other cyanobacteria have also been shown to structure with light level. For example, *Prochlorococcus* partitions into high and low light adapted groups (West and Scanlan, 1999), which have different requirements for light and nutrients (Rocap et al., 2003).

Synechococcus also shows niche partitioning in the Baltic Sea and off the coast of California in the oligotrophic Pacific Ocean in response to light (Stomp et al., 2004; Ferris and Palenik, 1998). To understand differences in N_2 fixation between puff and raft colonies on cruise OC469, net collected colonies of *Trichodesmium* were separated by morphology, and rafts had significantly higher N_2 fixation rates than puffs isolated and incubated at the same light levels. This finding suggests that the subsurface N_2 fixation rates, which were always dominated by the puff morphology, were lower than the surface because of intrinsic differences between morphotypes in addition to light and temperature. Like other cyanobacteria, there may be two physiologically distinct populations of *Trichodesmium,* one that inhabits the surface waters while the other inhabits subsurface waters. Niche differentiation by *Trichodesmium* has been suggested by Hynes (2009), and could represent a means through which *Trichodesmium* populations are able to most effectively utilize scarce resources, including nutrients and light. The presence of two physiologically distinct populations inhabiting different depths in the water column would prevent accurate prediction of the N_2 fixation rate for the subsurface population using data derived from surface colonies, since the two populations appear to be physiologically distinct with regards to their response to light. Additional studies that also examine the phylogenetic relationship of *Trichodesmium* from surface and subsurface populations would further elucidate possible niche differentiation between populations.

The effect of phosphorus availability on N_2 fixation – While not measured along a vertical gradient, the relationship between P availability and N_2 fixation was investigated in surface populations throughout the transect. There are many biogeochemical factors that can influence *Trichodesmium* N_2 fixation rates, including nutrient and metal availability (Tyrrell et al., 2003; Capone et al., 2005). In the western North Atlantic, P availability is increasingly recognized as a nutrient limiting both C and N_2 fixation (Sañudo-Wilhelmy et al., 2001). A common metric of P availability is AP activity. The AP enzyme allows marine microbes to hydrolyze ester bound dissolved organic P into dissolved inorganic phosphate that is available for metabolism (Perry, 1972). AP activity has been shown to correlate with P stress levels in *T. erythraeum* IMS101 (Orchard et al., 2009), and AP activity has been routinely detected in *Trichodesmium* populations from the western North Atlantic (Dyhrman, 2002; Orchard, 2010), where N_2 fixation has been shown to be limited by P in *Trichodesmium* (Sañudo-Wilhelmy et al., 2001). N_2 fixation rate was significantly inversely correlated with AP activity along the transect. This is consistent with research suggesting that P limits N_2 fixation in the North Atlantic. This has implications for the fine scale vertical and horizontal structuring of N_2 fixation rates during cruise OC469, where N_2 fixation rate may be influenced by P availability in addition to light, temperature or other factors.

Global contribution of subsurface N_2 fixation

Trichodesmium is the dominant diazotrophic cyanobacteria in the western North Atlantic (Goebel et al., 2010), and N_2 fixation by *Trichodesmium* represents a vital source of new N which fuels biological production. Current estimates of N_2 fixation by *Trichodesmium* place the global estimate in oligotrophic waters at 80-110 Tg new N yr^{-1} (Capone et al., 1997). Recent studies suggest that previous estimates of the contribution of N_2 fixation to the new N pool may underestimate the contribution of *Trichodesmium* in the western North Atlantic (Davis and McGillicuddy, 2006). Davis and McGillicuddy (2006) suggest that N_2 fixation by *Trichodesmium* in the North Atlantic may be as much as 2.9-3.3 times higher than previous estimates, based on deep abundance data gathered using a video plankton recorder and a rectilinear model of light dependent N_2 fixation from Orcutt et al. (2001). Recent work that spanned the same area sampled during this study suggests a consistent distribution of subsurface *Trichodesmium* relative to previous estimates (Davis et al., in preparation). Subsurface N_2 fixation in this study accounted for between 11 and 60% of surface N_2 fixation. This is not significantly different than the range of subsurface N_2 fixation rates predicted based on the rectilinear model (Orcutt et al., 2001) utilized by Davis and McGillicuddy (2006). As such, this distribution data (Davis et al., in preparation) and the N_2 fixation rates presented herein indicate that when subsurface populations of *Trichodesmium* in the North Atlantic are accounted for, N_2 fixation rates for the western North Atlantic could in fact be roughly 3 times higher than current estimates. If borne out by further research, these data indicate that N_2 fixation by

Trichodesmium in the western North Atlantic could be an even more important source of new N than previously thought.

Conclusions

The data presented herein show that N_2 fixation by subsurface populations of *Trichodesmium* represents an important new source of N to the western North Atlantic. This result is striking, and would benefit tremendously from further study. Future research is needed to assess the seasonality of patterns of subsurface N_2 fixation, and more work is required to deconvolute how population structure, physical forcing and biogeochemical factors are acting in concert to affect changes in N_2 fixation in surface and subsurface populations. Further studies on basin scale *Trichodesmium* species and morphotype distributions are also required to understand how N_2 fixation is structuring on both vertical and horizontal scales before global estimates can be made regarding the contribution of subsurface populations to global N_2 fixation.

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