AN ABSTRACT OF THE THESIS OF

Mary Rose Gradoville for the degree of Master of Science in Ocean, Earth and Atmospheric Sciences presented on March 15, 2013.

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Abstract approved:

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Biological di-nitrogen ($N_2$) fixation is a key process in open-ocean ecosystems, where the new nitrogen (N) provided by marine diazotrophs can support a large fraction of primary productivity and carbon (C) drawdown. Recent laboratory studies have shown that elevated $pCO_2$ enhances the rate of $N_2$ fixation by select laboratory isolates of *Trichodesmium* and *Crocosphaera*. While both $N_2$-fixing cyanobacteria, these groups differ widely in their cell size, maximal growth rates, and diel periodicity of $N_2$ fixation. It is unclear whether the $CO_2$ enhancement shown by these species in laboratory settings will hold for diverse diazotrophic communities under natural ocean environments. The aim of this thesis was to investigate how open ocean diazotrophs respond to elevated $pCO_2$ and whether this response is modulated by environmental conditions. I used laboratory and field approaches to address this problem. Laboratory experiments were designed to test the impact of changing $pCO_2$ on growth, C and N accumulation by *Crocosphaera* strain WH8501. Elevating $pCO_2$ above present-day conditions increased growth rates and net C and N accumulation rates. Monitoring C and N accumulation over a diurnal cycle showed that cultures had a distinct rhythm of

C and N accumulation; elevating $pCO_2$ changed this pattern by extending the period of high N accumulation rates. However, the ecologically relevant question is not how a single organism responds to elevated $pCO_2$ in a controlled setting, but rather how diverse microbial communities will respond in their natural habitats. Accordingly, field experiments were conducted on three cruises at Station ALOHA in order to study how natural assemblages of *Trichodesmium* and co-associated organisms respond to elevated $pCO_2$. In contrast to the laboratory results, we observed no consistent $pCO_2$ enhancement of C or $N_2$ fixation rates on these cruises. This response was not affected by amending seawater with P or Fe or by changing the light levels of deckboard incubations. Ultimately, DNA sequences from *Trichodesmium* colonies provided evidence suggesting that biological diversity of marine diazotrophs in our samples is the underlying reason for the lack of $pCO_2$ enhancement observed in the field. In other words, $pCO_2$ may upregulate metabolic processes in certain species but not others, leading to no measurable $CO_2$ enhancement of $N_2$ fixation by real-environment, diverse diazotrophic assemblages.

# A study on the effects of enhanced $pCO_2$ on open ocean diazotrophic assemblages

by

Mary Rose Gradoville

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Mary Rose Gradoville, Author

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# CONTRIBUTION OF AUTHORS

Ricardo Letelier and Angelicque White contributed intellectually to the experimental design, interpretation of data, and editing process for Chapters 2 and 3. Matthew Church and Daniela Böttjer contributed intellectually to the experimental design and data interpretation for the phylogenetic analyses in Chapter 3. Matthew Church also contributed to the editing process for Chapter 3.

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# 1. MARINE DIAZOTROPHY IN A HIGH CARBON DIOXIDE WORLD

## 1.1 Introduction

### *1.1.1 Nitrogen fixation: a key process for the global production of organic matter*

The availability of nitrogen (N) is a powerful control of biological production. As a major component of nucleic acids, proteins, and other cellular components, N is needed in large proportions for all known life forms: on average, N composes 6.25% of the dry weight of cells (Bothe et al. 2006). In terrestrial and aquatic systems alike, reservoirs of carbon dioxide ($CO_2$) and water, the substrates for oxygenic photosynthesis, are often vast compared to the pool of bioavailable N. Indeed, N availability limits productivity in a wide variety of ecosystems (Vitousek et al. 2002). Thus, N plays a strong role in global biogeochemical cycles and carbon (C) transformations.

The N cycle is unique in that the vast majority of N exists in a form that is biologically unavailable for most organisms. In both the atmosphere and the oceans, the major pool of N is in the form of di-nitrogen gas ($N_2$), which most life forms are not able to utilize because of its strong triple bond. All other major forms of N are termed 'fixed N' and cycle in terrestrial and aquatic systems through a series of microbially-mediated processes (Fig. 1-1). The process of $N_2$ fixation is crucial because it is the gateway for $N_2$ to enter this cycle. Without humans, $N_2$ fixation is only accomplished through lightning strikes and biological $N_2$ fixation. Lightning contributes a relatively small portion of the natural global $N_2$ fixation (Galloway et al. 1995); the largest fraction is accomplished biologically via a reduction-oxidation (redox) reaction that can be described generically in the form:

$$N_2 + 10H^+ + 8e^- + 16ATP \rightarrow 2NH_4^+ + H_2 + 16ADP + 16Pi \quad (1)$$

where Pi represents inorganic phosphate, ATP represents adenosine triphosphate, ADP represents adenosine diphosphate, and the sources of protons ($H^+$) and electrons ($e^-$), and thus the production of reduced hydrogen, depend on the acid-base and redox conditions of the reaction environment. This process is energetically expensive and can only be carried out by a select group of prokaryotes, termed diazotrophs, who possess the catalyzing enzyme nitrogenase. After fixed $N_2$ has been converted to $NH_4^+$, it may cycle as a variety of biologically usable compounds before being converted back to $N_2$ through denitrification or anaerobic ammonium oxidation by other groups of microorganisms (anammox; Fig. 1-1). Thus, the balance of $N_2$ fixation and denitrification plus anammox and the extent to which they are temporally and spatially separated can have large impacts on primary productivity, biogeochemistry and C flux.

Humans have exerted a significant impact on the N cycle, especially in terrestrial systems. This phenomenon began 6500 years ago when ancient societies began to cultivate leguminous plants that harbor $N_2$ fixing microorganisms within root nodules, increasing the concentration of biologically available N in soils and enhancing agricultural productivity. In the early twentieth century, $N_2$ fixation was further exploited through the development of the Haber-Bosch process, which industrially produces $NH_3$ from $N_2$ and $H_2$. Today, the anthropogenic inputs of fixed N through cultivation, Haber-Bosch, and the N released as a byproduct of burning fossil fuels are nearly equivalent to, or have exceeded, the combination of all 'natural' $N_2$ fixation (Galloway et al. 2004). Meanwhile, the 'natural' terrestrial $N_2$ fixation is decreasing due to land use changes. In contrast to terrestrial environments, many open ocean ecosystems are less influenced by human activities and rely solely on microbial and physical processes for the input of new N input (Fig. 1-1). Several lines of evidence suggest that marine $N_2$ fixation rates in the ocean had been greatly underestimated until very recently (Zehr et al. 2001, Mohr et al. 2010a). Because of the vital links between C and N cycles, and in the face of anthropogenic changes to

both of these cycles, understanding the controls of $N_2$ fixation is of critical importance.

### *1.1.2 Marine nitrogen fixation: importance, players, and controls*

Marine $N_2$ fixation is generally thought to be the most important in oligotrophic, open-ocean ecosystems, where warm, stratified, relatively high-light surface waters appear to favor diazotrophic growth (Capone et al. 1997). These regions comprise the largest biomes on the planet and together are responsible for nearly half of global primary production (Field et al. 1998). In pelagic marine environments, production by photoautotrophs is limited to the euphotic zone, which often contains low concentrations of fixed N and other essential nutrients. Though primary production can also be limited by phosphorus (P) or iron (Fe) availability, N limitation is considered the main nutrient limiting production in many open-ocean ecosystems (Capone 2000). Biologically available N can be supplied to surface waters through physical mixing of deeper, nutrient-rich waters, or through biological $N_2$ fixation. Though physical mixing was long thought to be the dominant source of new fixed N into the euphotic zone through the upwelling of nitrate, the discovery that the bloom-forming filamentous cyanobacterium *Trichodesmium* was capable of fixing $N_2$ (Dugdale 1961) began to change this view. Some early studies have estimated that *Trichodesmium* alone is responsible for more than half of new production in some regions (Karl et al. 1997, Capone et al. 2005, Mahaffey et al. 2005). The importance of biologically mediated $N_2$ fixation in the open ocean was further appreciated with the discovery of *Richelia*, a group of heterocystous $N_2$-fixing cyanobacteria that lives as a symbiont of marine diatoms (Villareal 1992).

Over the past two decades, genetic tools and methodological advancements have further revolutionized our understanding of marine $N_2$ fixation. Scientists have begun to use the *nifH* gene, which encodes the iron protein subunit of nitrogenase, to directly target $N_2$-fixing microorganisms. These efforts have shown that open ocean diazotrophs are much more diverse than previously recognized, and include the unicellular cyanobacterial groups A and B (termed UCYN A and UCYN B,

respectively), heterocystous cyanobacteria, proteobacteria, and members of the diverse, typically anaerobic group Cluster III, in addition to the traditionally recognized *Trichodesmium* spp. (Zehr et al. 1998, Zehr et al. 2001, Church et al. 2005b). The unicellular cyanobacterial group in particular has been shown to occupy broad oceanic ranges (Moisander et al. 2010) and contribute substantially to overall $N_2$ fixation rates (Montoya et al. 2004). The magnitude of global marine $N_2$ fixation has also been a matter of recent discussion: direct $N_2$ fixation rates have historically been lower than geochemically-derived estimates, leading to questions of whether the marine N budget is in steady state (Mahaffey 2005). The discovery of new classes of open-ocean diazotrophs, along with the recognition of a possible methodological rate underestimation (Mohr et al. 2010a), together suggest that marine $N_2$ fixation rates may be substantially higher than previously recognized.

With this new appreciation for the diversity and functional importance of marine diazotrophs, there is an increased need to constrain the environmental controls of community $N_2$ fixation rates. Some of these controls are well-established for *Trichodesmium*, but may not hold for other groups of diazotrophs. For instance, *Trichodesmium* inhabit tropical and subtropical waters with a limited temperature range (mean annual temperature >~24°C), but unicellular $N_2$-fixing cyanobacteria seem to have broader thermal tolerances (Moisander et al. 2010). Likewise, light is a powerful control of $N_2$ fixation by cyanobacteria, but may not be as important in regulating $N_2$ fixation by heterotrophic groups such as Cluster III. Oxygen concentrations also regulate $N_2$ fixation: the nitrogenase enzyme is permanently inactivated by oxygen (Gallon 1992). Marine diazotrophs circumvent this problem through different methods; while some heterotrophic groups such as species from Cluster III are strict anaerobes, diazotrophic cyanobacteria not only reside in oxygenated water, but also generate intracellular oxygen through photosynthesis. These organisms separate oxygen evolution from $N_2$ fixation by spatial or temporal decoupling of the processes and other methods such as respiration (Gallon 1992). Nutrient limitation is another important control; there is substantial evidence that $N_2$

fixation in certain oceanic regions is limited by the availability of P (Sañudo-Wilhelmy et al. 2001) and Fe (Moore et al. 2001) in particular.

Recently, the availability $CO_2$ required for photosynthesis has been proposed as another control of marine $N_2$ fixation (Hutchins et al. 2009). Marine phytoplankton are not generally considered to be C limited because the total pool of inorganic C is in great excess to pools of other nutrients. However, as discussed below, recent evidence may be changing this view. In this context, the issue regarding how the elevated partial pressure of $CO_2$ ($pCO_2$) affects phytoplankton, and $N_2$-fixing cyanobacteria in particular, becomes relevant due to anthropogenically driven changes in the ocean C chemistry.

### *1.1.3 Ocean acidification*

Human activities, such as the burning fossil fuels and deforestation, are adding $CO_2$ to the atmosphere at unprecedented rates. Atmospheric $CO_2$ levels have increased from 280 μatm before the industrial revolution to a current level of 385 μatm (Equation 3): (IPCC 2007). Current projections indicate that atmospheric $CO_2$ will roughly double by the year 2100 (IPCC 2007) and may reach 2000 μatm by the year 2300 (Caldeira and Wickett 2003). The ocean helps to buffer these atmospheric changes by taking up approximately 26% of anthropogenic $CO_2$ emissions (Le Quéré et al. 2009) After gaseous $CO_2$ ($CO_{2(g)}$) dissolves in seawater (Equation 2), the resulting aqueous form ($CO_{2(aq)}$) reacts with water to form carbonic acid, which dissociates in acid-base reactions (Equation 3) whose equilibria control the dissolved inorganic C (DIC) partitioning into species of $CO_{2(aq)}$, bicarbonate, and carbonate ions:

$$CO_{2(g)} \rightleftharpoons CO_{2(aq)} \quad (2)$$

$$CO_{2(aq)} + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \rightleftharpoons 2H^+ + CO_3^{2-} \quad (3)$$

In sum, these anthropogenically driven changes to ocean chemistry result in increased $pCO_2$, decreased pH, and decreased carbonate ion concentrations: these consequences are termed “ocean acidification” (OA). The effects of OA are concentrated in surface

seawaters, where forecasts suggest that pH will drop by 0.3-0.4 units by the year 2100 (Caldeira and Wickett 2003).

OA has been implicated in a suite of changes for marine diversity and biological rate processes. One notable effect of OA has been shown for calcifying organisms such as corals, coccolithophores, and shellfish. Because OA decreases the concentration of carbonate ions, it becomes increasingly difficult to precipitate $CaCO_3$ shells and skeletons under high $CO_2$ conditions; OA ultimately slows calcification rates for some organisms (Doney et al. 2009). The reduced pH of seawater can also have direct biological impacts, for instance, by shifting the proportions of $NH_3$ and $NH_4^+$ and by affecting the sensory system of certain fish species (Simpson et al. 2011).

Another predicted effect of OA is a potential upregulation of processes by photoautotrophs that use $CO_2$ as a substrate. Humans actions have made a very small change in the total DIC content of the oceans: a total of ~110 Pg DIC have been added to the oceanic reservoir of ~38,000 Pg DIC (Sabine et al. 2004) Even in the surface ocean where the effect of OA is the most extreme, total DIC has only changed by ~1.6% (Sabine et al. 2004). However, because of the shifts to the C system equilibria discussed above, these small changes in total DIC can produce much larger changes in carbon speciation (Fig. 1-2). Under the current ocean pH of ~8.1, aqueous $CO_2$ ($CO_{2(aq)}$) constitutes only ~1% of total DIC. However, by the year 2100, the concentration of $CO_{2(aq)}$ is expected to approximately double (IPCC 2007). It is this higher concentration of $CO_{2(aq)}$ that some researchers have suggested may increase metabolic rates for certain phytoplankton groups, including some diazotrophic groups of cyanobacteria (Hutchins et al. 2009).

### *1.1.4 $CO_2$ as a possible control of marine carbon and nitrogen fixation*

$CO_2$ is the substrate used by photoautotrophs, including marine phytoplankton, in the crucial ecological process of C fixation. Because photosynthesis requires $CO_2$, all phytoplankton must become C limited at some threshold $CO_{2(aq)}$ value. The

ecologically relevant question is whether marine phytoplankton are currently limited by the $CO_2$ levels within their environment. This is a topic of debate within the scientific community (Beardall et al. 2009, Hutchins et al. 2009, Joint et al. 2011).

The first step in C fixation by oxygenic photoautotrophs is the carboxylation of $CO_2$ by the enzyme ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO), a reaction which can be rate-limited under the current atmospheric $CO_2$ levels. RuBisCO evolved under the high $CO_2$, low $O_2$ conditions of the Archean Eon (Buick 1992); consequently, it has a low affinity for $CO_2$ and catalyzes the competing oxygenase reaction when $O_2$ is present. This problem is not helped by the fact that $CO_2$ accumulates slowly within cells because of the slow diffusivity and the relatively small concentration of $CO_{2(aq)}$ in seawater. Though the various forms of RuBisCO have different catalytic efficiencies, the RuBisCO forms present in most phytoplankton groups are not saturated under typical marine $pCO_2$ (Badger et al. 1998).

Nearly all phytoplankton species address the problem of RuBisCO's inefficiency by using active processes to concentrate $CO_2$ at the site of carboxylation. These processes, termed carbon-concentrating mechanisms (CCMs) primarily involve the active uptake of $CO_2$ and $HCO_3^-$ and increasing the speed of their interconversion. There is a large diversity in CCM proteins and their efficiency in the different phytoplankton groups that relates to differing evolutionary histories (Badger et al. 2002, Reinfelder 2011). For example, cyanobacteria, as the first oxygen-evolving organisms, evolved under high $CO_2$ conditions and consequently the cyanobacterial RuBisCO is particularly inefficient. It is thought that cyanobacteria evolved effective CCMs in response to a large decrease in atmospheric $CO_2$ and increases in $O_2$ ~400 million years ago; these CCMs allow cyanobacteria to achieve efficient photosynthesis under present $CO_2$ conditions (Badger et al. 2002). The presence of CCMs in nearly all phytoplankton species examined has led to the conclusion that, in general, $CO_2$ supply does not limit aquatic photosynthesis and growth in nature (Falkowski and Raven 2007).

Despite these theoretical considerations, recent evidence indicates that some species and assemblages do increase C fixation and/or growth rates when exposed to elevated $pCO_2$. This trend has been shown for certain monocultures of coccolithophores (Rost and Riebesell 2004), raphidophytes (Fu et al. 2008b) and cyanobacteria (Fu et al. 2007). Experiments using whole phytoplankton assemblages have shown increased C fixation rates in some cases (Riebesell et al. 2007, Tortell et al. 2008) but not in others (Tortell et al. 2002).

Interestingly, the largest $CO_2$ enhancement of phytoplankton metabolism has been observed not for C fixation rates, but for $N_2$ fixation rates by an isolate of *Trichodesmium*. Several laboratory studies have shown this trend for *Trichodesmium erythraeum* strain IMS101, which has been observed to increase $N_2$ fixation rates between 35% and 138% and C fixation rates between 23% and 40% with a doubling of current atmospheric $pCO_2$ (e Ramos et al. 2007, Hutchins et al. 2007, Levitan et al. 2007, Kranz et al. 2009). Explorations into the mechanism behind this enhancement show that elevated $pCO_2$ does not increase gross photosynthesis by *Trichodesmium* (Kranz et al. 2010). Rather, elevated $pCO_2$ has been linked to changes in the CCM (Kranz et al. 2009, Kranz et al. 2010); under elevated $pCO_2$, *Trichodesmium* appears to be able to reallocate energy from CCMs to other cellular processes including $N_2$ and C fixation (Kranz et al. 2011). As discussed above, $N_2$ fixation by marine diazotrophs including *Trichodesmium* is of critical importance in oligotrophic open-ocean ecosystems, where the new N they supply can support up to half of new production and C export (Karl et al. 1997). If the response of *Trichodesmium* IMS101 is reflective of *in situ* diazotrophic communities, OA could be expected to increase $N_2$ fixation and consequently net primary productivity in many N-limited regions. This may possibly lead to increased C export, resulting in a negative feedback to climate change (Hutchins et al. 2007).

However, the actual effect that ongoing OA will have on global marine $N_2$ fixation remains speculative. Under laboratory conditions, *Trichodesmium* strains IMS101 and GBRTRLI101, as well as *Crocosophaera watsonii* strain WH8501, have

all shown enhanced C and $N_2$ fixation under elevated $pCO_2$ (e Ramos et al. 2007, Hutchins et al. 2007, Levitan et al. 2007, Fu et al. 2008a). In contrast, recent studies have shown that there is considerable variability in the carbon affinity between different strains for both of these genera (Garcia et al. 2013, Hutchins, pers. comm.). Furthermore, elevated $CO_2$ has a negative effect on growth and $N_2$ fixation by the heterocystous cyanobacterium *Nodularia spumigena* (Czerny et al. 2009). Recent field experiments have yielded mixed results: while two studies targeting *Trichodesmium* colonies observed a $CO_2$ enhancement of $N_2$ fixation (Hutchins et al. 2009, Lomas et al. 2012), other experiments using whole diazotrophic assemblages have found no relationship between $pCO_2$ and $N_2$ fixation rates (Law et al. 2012, Böttjer, in prep.). There are many possible reasons for the mixed responses to $pCO_2$, including differences in community composition, physiology, environmental conditions, or experimental methodologies. These contrasting results need to be reconciled in order to accurately predict whether global $N_2$ fixation will increase with future OA.

## 1.2 Thesis objectives

The aim of this thesis is to increase understanding of the effects of OA on open-ocean $N_2$ fixing communities. I used both laboratory and field experiments to approach this problem. Initially, I did work in the laboratory testing the effect of $pCO_2$ on isolates of *Trichodesmium* and *Crocosphaera* and comparing responses using different experimental methods from the lab and field. Chapter two shows one example of an experiment I performed in the laboratory, where effects of elevated $pCO_2$ on the growth and metabolic rates of a *Crocosphaera* isolate were observed over several days of diurnal sampling. Chapter three presents the results from experiments performed on three cruises in the North Pacific Subtropical Gyre. These experiments tested the effects of enhanced $pCO_2$ on $N_2$ and C fixation by natural assemblages of *Trichodesmium* and associated organisms. I further tested whether

these effects were modulated by environmental conditions or experimental methodology, and related results to the diversity of the community.

My results indicate that in the laboratory, elevated $pCO_2$ stimulates growth, C and N accumulation in *Crocosphaera* WH8501, and also changes the diurnal pattern of N accumulation. However, in the field, I found no evidence for $pCO_2$ enhancement of C or $N_2$ fixation by isolated *Trichodesmium* colonies. Results from sequencing *Trichodesmium* colonies suggest that diazotrophic diversity in the field is the underlying reason that elevated $pCO_2$ did not enhance $N_2$ or C fixation. That is to say, we have hypothesized that different strains of $N_2$ fixing organisms are saturated for C and $N_2$ fixation at variable $CO_2$ levels. Mixed assemblages of strains with variable growth responses to $CO_2$ will ultimately lead to a flexible response to OA. In our experiments, the diverse diazotrophic community appears to have masked any $CO_2$ enhancement of a particular *Trichodesmium* strain.

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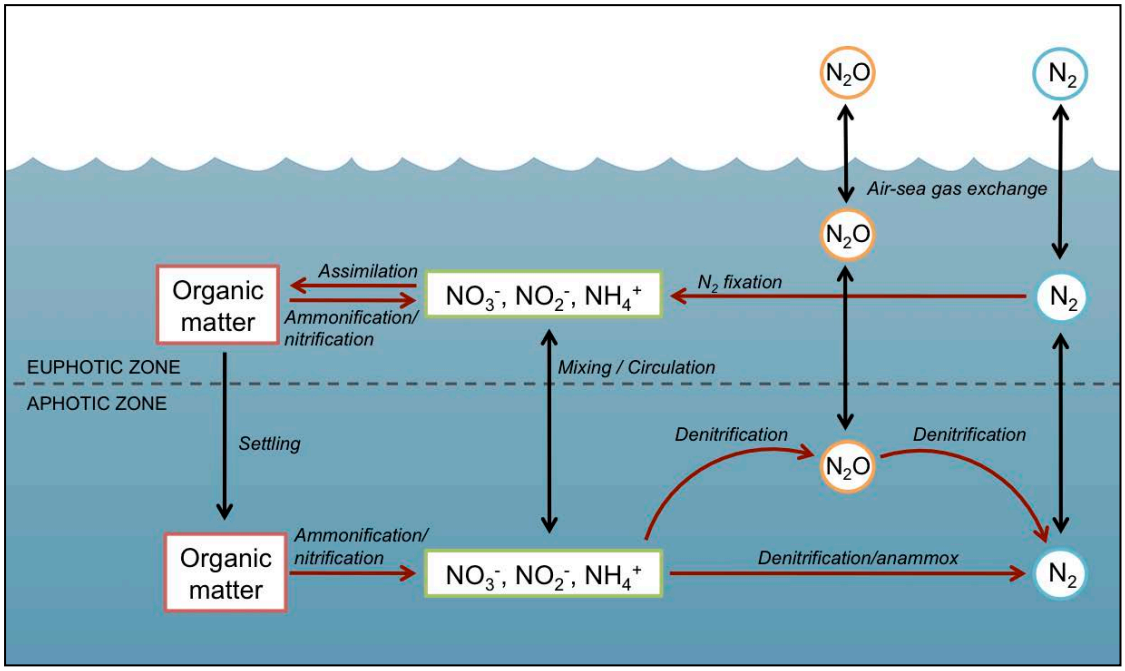


**Figure 1-1:** A schematic showing a simplified view of the nitrogen cycle in open ocean ecosystems. Physical processes are indicated with black arrows; biological processes are indicated with red arrows. Modified from Gruber (2008).

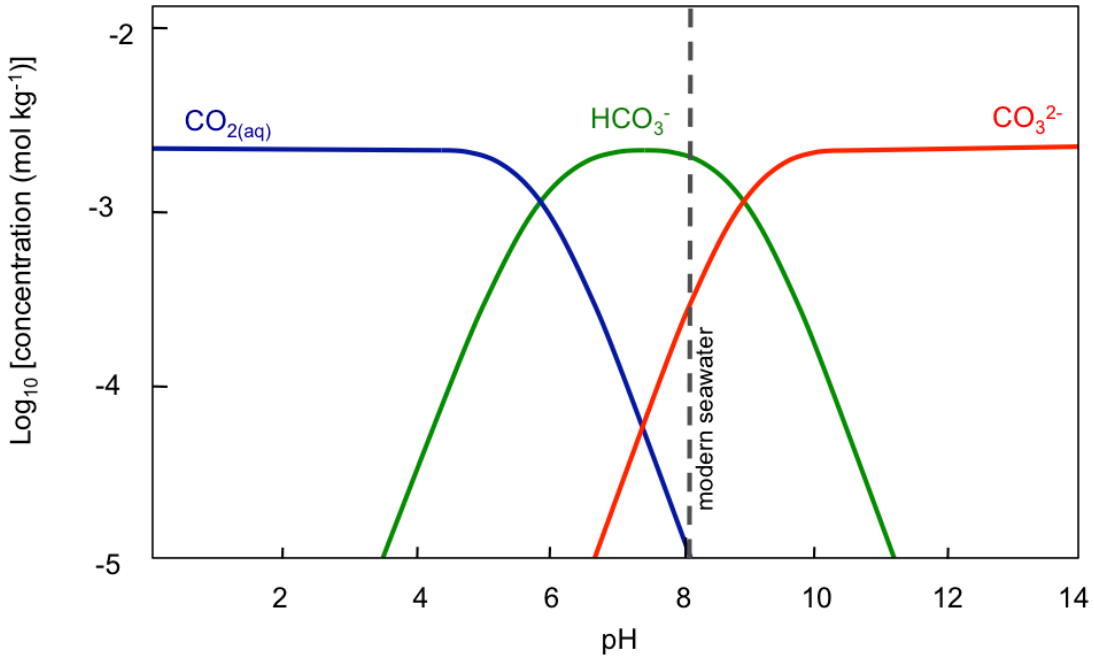


**Figure 1-2:** Concentrations of $CO_{2(aq)}$, $HCO_3^-$, and $CO_3^{2-}$ as a function of pH in seawater. Reproduced from Ridgwell and Zeebe (2005).

# Physiological effects of enhanced carbon dioxide in *Crocosphaera* cultures

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# 2. PHYSIOLOGICAL EFFECTS OF ENHANCED CARBON DIOXIDE IN *CROCOSPHAERA WATSONII* CULTURES

## 2.1 Abstract

We investigated the effects of elevated $pCO_2$ on cultures of the unicellular nitrogen-fixing cyanobacteria *Crocosphaera watsonii* WH8501. Cultures were grown in nutrient replete media under high light intensity and conditions approximating current $pCO_2$ (~400 μatm) as well as $pCO_2$ levels corresponding to low and high-end estimates for the year 2100 (~750 and 1000 μatm). Following acclimation to $pCO_2$ levels, the concentrations of particulate carbon (C), particulate nitrogen (N), and cells were measured over the diurnal cycle for a six day period. Cultures showed a distinct diurnal pattern of net C and N assimilation, with particulate C:N molar ratios reaching as high as 22:1 during the light period. During the dark period, particulate carbon concentrations decreased substantially, likely due to the use of intracellular carbohydrate reserves to fuel high $N_2$ fixation rates. Consistent with previous studies, we found that elevating $pCO_2$ above current levels significantly increased net C fixation, N accumulation, and growth rates. Rates of net C fixation and N accumulation increased by 18% and 20% at 750 μatm, and by 47% and 63% at 1000 μatm, respectively. Elevating $pCO_2$ also drove an increase in the cell number density (by 35% at 750 μatm and 63% and 1000 μatm) decreased the average size of cells, possibly as a result of an increase in cell division rates. Furthermore, under elevated $pCO_2$, the proportion of N accumulation occurring early in the dark period increased. This study gives the first glimpse into how $pCO_2$ affects diurnal C and N dynamics in *C. watsonii*.

KEY WORDS: Ocean acidification · *Crocosphaera* · Unicellular nitrogen-fixing bacteria · Nitrogen fixation · Diurnal cycle

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## 2.2 Introduction

Human actions including the burning of fossil fuels are releasing substantial quantities of $CO_2$ into the atmosphere: atmospheric $pCO_2$ has increased 50% relative to preindustrial levels, and is forecasted to double by the year 2100 (IPCC 2007). The dissolution of anthropogenic $CO_2$ into the world's oceans is increasing surface seawater $pCO_2$, decreasing pH, and changing the inorganic carbon (C) speciation, a phenomenon collectively termed "ocean acidification" (OA). Future OA is expected to have a suite of consequences for biological diversity, elemental cycling, and atmospheric feedbacks (Doney et al. 2009).

Among the predicted effects of OA is a potential upregulation of metabolic processes by certain phytoplankton groups (Riebesell et al. 1993). Phytoplankton, like terrestrial plants, use the enzyme ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) to fix inorganic C. Because RuBisCO has a poor affinity for $CO_2$, most phytoplankton species supplement the passive diffusion of $CO_2$ across the cell membrane by actively concentrating inorganic carbon at the site of carboxylation with C concentrating mechanisms (CCMs). It has been proposed that in high $CO_2$ oceans, increased diffusion of $CO_2$ into cells may directly enhance rates of photosynthesis or may allow for the reallocation of energy from CCMs to other cellular processes (Raven 1991). Indeed, elevated $pCO_2$ has been shown to stimulate C fixation by

monocultures of coccolithophores (Rost and Riebesell 2004), raphidophytes (Fu et al. 2008b), and cyanobacteria (Fu et al. 2007), and by some natural phytoplankton assemblages (Riebesell et al. 2007, Tortell et al. 2008).

One organism that seems to especially benefit from elevated $pCO_2$ is the di-nitrogen ($N_2$) fixing cyanobacterium *Trichodesmium.* A group of filamentous, non-heterocystous cyanobacteria, *Trichodesmium* are abundant in tropical and subtropical open-ocean ecosystems, where they can contribute up to 50% of the new N supporting primary productivity (Karl et al. 2002, Mahaffey et al. 2005). Recently, several laboratory studies using *Trichodesmium* strain IMS101 have shown that a doubling of $pCO_2$ increases C fixation by 23%-40% and $N_2$ fixation by 35%-138% (e Ramos et al. 2007, Hutchins et al. 2007, Levitan et al. 2007, Kranz et al. 2009). Mechanistically, elevating $pCO_2$ has been linked to changes in the CCM of *Trichodesmium* (Kranz et al. 2009, Kranz et al. 2010). It is thought that under high $pCO_2$, a down-regulation of the CCM allows for the reallocation of ATP and reductants to the processes of $N_2$ fixation, particulate organic N production, and particulate organic C production (Kranz et al. 2010, Kranz et al. 2011).

Far less is known about the effect of OA on other diazotrophic taxa. $N_2$ fixing cyanobacteria have diverse CCMs (Price et al. 2008) and metabolic strategies, which may result in different responses to elevated $pCO_2$. One characteristic that differentiates groups of $N_2$ fixing cyanobacteria is their strategy for separating the oxygen evolved through photosynthesis from the enzyme nitrogenase, which catalyzes biological $N_2$ fixation and is irreversibly inactivated by oxygen (Gallon 1992). Heterocystous cyanobacteria circumvent this problem by fixing $N_2$ exclusively in specialized cells called heterocysts, which lack photosystem II (Haselkorn 1978). The heterocystous cyanobacterium *Nodularia spumigena* has shown a negative response to elevated $pCO_2$; this lack of enhancement may be due to the spatial separation of photosynthetic energy generation and $N_2$ fixation (Czerny et al. 2009). Another strategy to protect nitrogenase from oxygen is to temporally separate photosynthesis and $N_2$ fixation by restricting these processes to the light and dark periods,

respectively. This strategy is used by many $N_2$ fixing unicellular cyanobacteria, which have been recently recognized as an important source of new bioavailable N in open oceans (Zehr et al. 2001, Montoya et al. 2004) and include the cultured representative *Crocosphaera watsonii*. As for *Trichodesmium* IMS101, a recent study found that *C. watsonii* strain WH8501 increases rates of C and $N_2$ fixation at elevated $pCO_2$ (Fu et al. 2008a).

*Crocosphaera* and *Trichodesmium* both appear to benefit from elevated $pCO_2$ despite the differing mechanisms by which they link C and N metabolism. In *Crocosphaera*, the energy needed to fix $N_2$ is generated photosynthetically and stored primarily as carbohydrate granules (Dron et al. 2012a); respiration of these organic C reserves allows for $N_2$ fixation in the dark. In contrast, *Trichodesmium* fixes $N_2$ during the light period by temporally separating maximum rates of photosynthesis and $N_2$ fixation and through a spatial separation of the two processes (Berman-Frank et al. 2001, Finzi-Hart et al. 2009). Simultaneous C and $N_2$ fixation allows for the direct transfer of energy between processes; though respiration may provide some energy for $N_2$ fixation in *Trichodesmium*, the majority of ATP and reductants come directly from photosynthesis. (Bergman et al. 1997, Kranz et al. 2011). In addition to changes in the CCM, *Trichodesmium* appears to respond to elevated $pCO_2$ through physiological changes such as increasing the ratio of photosystem I to photosystem II (Levitan et al. 2010b) and/or extending the phase of $N_2$ fixation (Kranz et al. 2010). It is unknown whether *Crocosphaera*, which uses such a different metabolic strategy for $N_2$ fixation, shares similar mechanisms for $CO_2$ enhancement.

Though we have recently begun to understand the mechanism of $CO_2$ enhancement for *Trichodesmium*, no previous research has focused on how $pCO_2$ affects C and $N_2$ fixation by the ecologically significant unicellular cyanobacteria *Crocosphaera*. Understanding the mechanism of $CO_2$ enhancement is crucial for accurate predictions of how OA will affect other groups of diazotrophs, and ultimately, diverse diazotrophic communities. Here, we present results from an experiment in which cultures of *Crocosphaera watsonii* strain WH8501 were

subjected to a range of $pCO_2$, and net C fixation, N accumulation, and growth were tracked over several diurnal cycles.

## 2.3 Methods

### *2.3.1 Culture conditions*

Unialgal stock cultures of *Crocosphaera watsonii* strain WH8501 were grown in 0.2 μm filtered, nitrogen-free YBCII medium supplemented with 40 μmol $L^{-1}$ $K_2HPO_4$ (Chen et al. 1996). Cultures were not axenic, but heterotrophic bacterial counts were kept at low levels (1.35-2.08 x $10^5$ cells $mL^{-1}$). Light was provided using cool white fluorescent bulbs set on a 12:12 light/dark cycle, with the light period starting at 08:00 and ending at 20:00. Stock cultures were grown at 24°C and 250 μmol quanta $m^{-2}$ $s^{-1}$ at ambient $pCO_2$. For the experiment, cultures were grown at 30°C and 1000 μmol quanta $m^{-2}$ $s^{-1}$ as measured by a Biospherical light meter. Cultures were stirred at least once a day with magnetic stir bars to minimize cells sticking to the glass. Target $pCO_2$ levels of ~400 (416), ~750 (757), and ~1000 (969) μatm were achieved by gently bubbling cultures (50 mL $min^{-1}$, Cole Parmer flow meter) with commercially prepared air/$CO_2$ mixtures (Airgas). Parent cultures were grown at these $pCO_2$, light, and temperature conditions for seven days (~3-4 generations) before rates were measured.

### *2.3.2 Experimental Design*

*Crocosphaera* cultures were grown under $pCO_2$ treatments of 400, 750, and 1000 μatm and monitored over a six-day period. Triplicate bottle replicates were used for each $pCO_2$ treatment. To initiate the experiment, parent cultures in exponential growth were diluted into 2L glass bottles filled with 0.2 μm filtered media pre-equilibrated to the target $pCO_2$. Initial $CO_2$ equilibration was verified by measuring gas outflow using a LI-840 LI-COR® gas analyzer (Biosciences). All bottles were gently bubbled throughout the experiment with air/$CO_2$ mixtures at 50 mL $min^{-1}$.

Bottles were sampled daily at 14:00 (days 0-2) or 02:00, 08:00, 14:00, and 20:00 (days 3-5) for particulate carbon (PC), particulate nitrogen (PN), chlorophyll *a* (chl *a*), cell counts through flow cytometry (FCM), *in vivo* chlorophyll fluorescence, and pH.

### *2.3.3 Analytical Measurements*

For PC/PN and chl *a* measurements, 5-50 mL of culture (depending on cell density) was was filtered onto glass fiber filters (GF/F, Whatman), using pre-combusted GF/F filters for PC/PN. Samples were immediately frozen at -80°C (PC/PN) or -20°C (chl *a*). PC/PN samples were dried at 60°C overnight, packaged into silver and tin capsules, and analyzed using a Carlo Erba elemental analyzer. Acetanilide (71.09% C and 10.36% N by weight) served as a standard, and filter blanks were <10% of total C and N content. Chl *a* was extracted in 90% acetone at -20°C for 48 hours and analyzed using a Turner Model 10-AU fluorometer (Strickland and Parsons 1972). Ammonia concentrations were measured from GF/F filtrate with a Technicon AutoAnalyzer II™, using an indophenol blue method modified from US EPA (1983).

*Crocosphaera* and heterotrophic bacteria cell abundances were determined by flow cytometry. Three-mL aliquots from each bottle were pipetted into 4 mL cryovials and fixed with freshly prepared paraformaldehyde with a final concentration of 1% (volume volume$^{-1}$). Samples were inverted and allowed to sit in the dark for approximately ten minutes before being frozen at -80°C. For analysis of *Crocosphaera* cell abundance, samples were thawed and kept on ice in the dark before being spiked with a known number of 3-μm Polysciences Fluoresbrite™ yellow-green beads and run on a Becton-Dickinson FASCaliber flow cytometer with a 488 nm laser. *Crocosphaera* cells and beads were distinguished from other particulate matter by their side light scatter (SSC) and fluorescence in orange wavelengths (FL2). The bead count determined the volume of sample run, and thus the concentration of *Crocosphaera* cells. A similar method was used to enumerate the background heterotrophic bacteria in these cultures. The samples were spiked with Fluoresbrite™

1-μm beads, stained with SYBR Green I according to the method of Marie et al. (1997), and differentiated by their SSC and green fluorescence (FL1).

The maximum fluorescence (Fm) of samples was determined daily using a cuvette WATER-ED PAM (Heinz Walz, Effeltrich, Germany). This PAM fluorometer uses Light Emitting Diodes (LEDs) at 650 nm for measuring light and 660 nm for actinic light and saturating pulses. Samples were acclimated to the dark for at least 20 minutes before measurements were taken. YBCII media was used as a blank to account for background fluorescence.

The pH of each bottle was measured 3-4 times per day with a VWR sympHony electrode calibrated with VWR buffers (NBS scale). Alkalinity of YBCII medium was previously calculated using the program CO2calc (Robbins et al. 2010) with the $CO_2$ constants from Mehrbach et al. (1973) refit by Dickson and Millero (1987), using inputs of DIC and $pCO_2$ which were measured according to the methods of Bandstra et al. (2006). $pCO_2$ values were calculated from the pre-determined TA and the pH measured during the experiment using CO2calc.

### *2.3.4 Rate calculations*

Growth rates were calculated for each of the biomass parameters measured: cell abundance, PC, PN, chl *a*, and *in vivo* fluorescence. For six-hour time intervals (as in Fig. 2-7), growth rates (μ) were calculated assuming an exponential growth rate of the form $N(t) = N_0 e^{\mu(t-t0)}$, where N represents cell abundance and t represents time. Overall growth rates throughout the experiment, as in Table 2-1, were calculated as the linear regression of natural logarithm (ln) transformed values at the 14:00 time-points from days 1-3. This time interval was chosen so that growth rates were calculated for the interval in which cultures were growing exponentially (see Fig. 1B). Day 0 was excluded because the FCM samples from day 0 were unavailable.

Percentage increases of accumulation rates in elevated $pCO_2$ treatments were derived from changes in cell abundance, PN, and PC over time (between days 1 and

3), relative to the ambient (400 μatm) treatment. Percentage increase was calculated according to the equation:

$$\text{Percentage increase} = 100 \times \frac{\Delta_{\text{treatment}} - \Delta_{\text{ambient}}}{\Delta_{\text{ambient}}}$$

Where $\Delta_{\text{treatment}}$ is the biomass accumulation rate in an elevated $p\text{CO}_2$ treatment and $\Delta_{\text{ambient}}$ is biomass accumulation rate at 400 μatm. Concentrations were normalized to day 1 before calculating percentage increase. This normalization eliminates the possibility of biasing percent increase through unequal starting concentrations and exponential growth.

### *2.3.5 Statistics*

The effect of the independent variable ($p\text{CO}_2$) on each of the dependent variables (C cell$^{-1}$, N cell$^{-1}$, SSC, molar C:N, and growth rates (μ) specific to cell number, PC, PN, chlorophyll *a*, and *in vivo* fluorescence) were assessed through one-way ANOVA. Differences between $p\text{CO}_2$ treatments were determined using a Tukey Honest Significance Test (HST) of multiple comparisons. In some cases, two-way ANOVA were used to test the effects of two independent variables, $p\text{CO}_2$ and diurnal sampling timepoint (08:00, 14:00, 20:00, and 02:00) on the dependent variables. Tukey HST was also used to test the differences between $p\text{CO}_2$ treatments. All measurements reported here are averages from three replicate bottles. Statistics were determined using the program R (R Development Core Team 2011).

## 2.4 Results and Discussion

Our study tested how enhanced $p\text{CO}_2$ affects the growth and C and N dynamics in *Crocosphaera* strain WH8501. In agreement with a previous study (Fu et al. 2008a), we found that net C fixation, N accumulation, and growth rates were all positively correlated with $p\text{CO}_2$ (Fig. 2-1). We also observed changes in the daily pattern of N accumulation with $p\text{CO}_2$, similar to the elongated period of $N_2$ fixation under high $p\text{CO}_2$ reported for *Trichodesmium* (Fig. 2-2). Furthermore, the relatively

high light levels and resultant high growth rates used produced strong diurnal changes in C and N assimilation (Fig 2-3), providing us with some unique insights concerning concerning *Crocosphaera* physiology as a function of $pCO_2$.

### *2.4.1 Diurnal rhythm*

For unicellular diazotrophic cyanobacteria like *Crocosphaera*, the processes of photosynthesis and $N_2$ fixation are temporally separated yet energetically connected, producing a distinct daily pattern of C and N dynamics. In *Crocosphaera*, this pattern is regulated by a suite of genes that are differentially expressed over the light and dark periods (Shi et al. 2010b) and controlled by a circadian clock (Pennebaker et al. 2010). In our study, we observed clear diurnal patterns in net C fixation, N accumulation, and cell division by *Crocosphaera* strain WH8501 that were common to all $pCO_2$ treatments.

The PC content of *Crocosphaera* cultures fluctuated widely between the light and dark periods, indicating high rates of photosynthesis and respiration (Fig. 2-3A, 2-4B). During the light period, PC concentrations increased between 48% and 216%. In the dark, a significant portion of this fixed C was lost; PC concentrations decreased between 17 and 79%. This substantial loss of PC in the dark is consistent with previous studies of *Crocosphaera* and likely reflects the respiration of carbohydrate reserves to fuel $N_2$ fixation (Mohr et al. 2010b, Dron et al. 2012a, Dron et al. 2012b).

The daily variability in cell size (as inferred from FCM SSC) mirrors the diel cycle of PC accumulation and loss. Both cell size and cellular C increased during the light period and decreased during the dark period (Fig. 2-4B,D). A similar diurnal cell size fluctuation was reported by Mohr et al. (2010b) and Dron et al. (2012b); however, in the latter study, cell size also briefly decreased during the light period following cell division around the sixth hour of light. Though our data shows that cells divided during the first half of the light period (Fig. 2-3C), the course sampling resolution in our study obscured observation of changes in cell size following division (Fig. 2-4D). It should be noted that FCM SSC is a proxy for cell size but is

also influenced by shape and granularity (Sherr et al. 2001); thus, SSC patterns could reflect changes in cell shape and C granule content.

Net N accumulation by *Crocosphaera* was restricted to the dark period, when PN concentrations increased between 48% and 93% (Fig. 2-3B, 2-4C). During the light period, PN decreased slightly, between 1% and 8% (Fig. 2-5). This N utilization loss is low compared to similar observations in *Trichodesmium*, where releases of recently fixed N during exponential growth reached 80–90% (Mulholland and Bernhardt 2005), and to previous estimates that *Crocosphaera* can release 23–67% of recently fixed N (Dron et al. 2012b). The small proportion of N loss measured in our study may be partially explained by our method, which only measured net PN accumulation or loss over six-hour time intervals; our method does not provide information on gross N fixation, thus our estimates correspond to a lower boundary of N release. Direct measurements of $NH_4^+$ accumulation throughout the experiment also indicate small proportions of N released: $NH_4^+$ accumulation accounted for only ~1% of total N fixed (data not shown). Some fixed N may have been released as organic compounds such as glutamate, but dissolved organic N was not measured in this experiment. Additionally, as our cultures were not axenic, some fixed N released from cells may have been taken up by heterotrophic bacteria. However, heterotrophic bacterial abundances within these cultures were relatively low, ranging from 1.35–2.08 x $10^5$ cells $mL^{-1}$. This corresponds to only 0.03%–0.12% of total biovolume, assuming an average heterotrophic bacterial diameter of 0.7 μm and *C. watsonii* WH8501 diameter of 2.7 μm (Mohr et al. 2010b, Dron et al. 2012b). Our combined observations of low PN loss during the day, low $NH_4^+$ accumulation and low heterotrophic bacterial abundances suggest that *Crocosphaera* did not release a large portion of fixed N during this study.

Together, the high rates of C fixation, respiration, and PN accumulation led to large fluctuations of the particulate C:N ratio over the daily cycle: molar C:N ratios in this study ranged from 5.5-22.1 (Fig. 2-4A). Previous studies have reported much less dramatic daily fluctuations in *Crocosphaera*, with C:N content of 6.5–8.5 (Mohr et al.

2010b) and 4.96–8.76 (Dron et al. 2012b). One major difference between our study and these previous studies is the light conditions: we grew *Crocosphaera* cultures at 1000 μmol quanta $m^{-2}$ $s^{-1}$, levels that are over ten times the daily light flux used by Mohr et al. (2010b) and Dron et al. (2012b), in order to ensure for maximum rates of growth, C, and $N_2$ fixation. Indeed, growth rates in our study were ~0.5 $d^{-1}$, over twice as high as cell specific growth rates reported in Dron et al. (2012b). The deviations in C:N were largely driven by changes in PC content. The increased PC during the light period reflects both C fixed for cellular growth and C fixed into carbohydrates to fuel $N_2$ fixation and other cellular processes. Additionally, a fraction of PC accumulated during the light period may have been subsequently exuded from cells: *Crocosphaera* WH8501 cultures have been shown to release ~10% of total C content daily as extracellular polymeric substances (Dron et al. 2012b).

It is interesting to note that despite the large deviations of C:N during the light period, cultures always came back to a relatively consistent C:N ranging from ~5.5–8 (Fig. 2-4A) at the 08:00 sampling point. This range encapsulates the 6.6 C:N ratio expected from Redfield stoichiometry (Redfield 1958). The particulate C:N of *Crocosphaera* cultures consistently returned to this ratio at 08:00 even in unbalanced growth conditions, when the magnitude of photosynthesis, respiration, and $N_2$ fixation were changing between days.

*Crocosphaera* cultures were not in balanced growth for the full time period of this experiment. Cultures grew exponentially for the first four days, and rates began to decline in day four (Fig. 2-3B). As the artificial medium used contained an ample supply of macro and micronutrients (Chen et al. 1996), the decrease in growth was likely due to self-shading or a high $O_2$:$CO_2$ ratio during the photoperiod. The imbalanced growth is especially apparent in the daily-normalized diurnal patterns of C and N dynamics (Fig. 2-6). As the experiment progressed and cell densities increased, there were declines in both the magnitude of PC increase during the light and PC decrease in the dark, indicating decreasing rates of both photosynthesis and respiration (Fig. 2-6). The decreasing overall percentage of PC loss in the dark (Fig. 2-5) is

further indication of decreasing respiration rates. In contrast, PN loss during the light period increased through the experiment, though percentages lost were relatively small (1-8%) (Fig. 2-5). Accompanying the declining growth rates were decreases in the magnitude of diurnal increase in C:N, C cell$^{-1}$, and FCM SSC (Fig. 2-4).

The increase in cell density over time also affected the stability of the carbon chemistry with $pCO_2$ treatments (Fig. 2-7). Though the $pCO_2$ within each treatment was relatively stable for the first 24 hours of the experiment, by day 5, the 1000 μatm treatment had fluctuated between 1216 and 96 μatm (Fig. 2-5). Despite these extreme fluctuations, elevated $pCO_2$ treatments always had higher $pCO_2$ values than the 400 μatm treatment, with the exception of the final time point (Fig. 2-5). The high cell densities towards the end of the experiment were confounding, as some parameters (e.g. PC loss) varied more between days than between $pCO_2$ treatments. Yet even with these changing conditions, we found that $pCO_2$ significantly affected the growth and diurnal patterns of N accumulation in *Crocosphaera*.

### *2.4.2 Effects of $pCO_2$ on growth, C fixation, $N_2$ fixation, and cell size*

Consistent with previous studies of *Crocosphaera* WH8501 (Fu et al. 2008a) and *Trichodesmium* IMS101 (e Ramos et al. 2007, Hutchins et al. 2007, Levitan et al. 2007, Kranz et al. 2009), we found that elevating $pCO_2$ increased growth, net C fixation, and N accumulation in *Crocosphaera* cultures (Fig. 2-1). Growth at 1000 μatm was significantly higher than at 400 μatm for growth rates specific to cell abundance, PC, PN, and *in vivo* fluorescence (Table 2-1). There was also a significant difference between growth at 750 μatm and 400 μatm for growth rates specific to cell abundance and *in vivo* fluorescence. There was no significant difference in chlorophyll *a* growth rates between ambient and elevated $pCO_2$, likely because these measurements had the largest coefficient of variation between replicates (7-10%) (Table 2-1).

Comparing PC and PN accumulation rates from our study with net $N_2$ and C fixation rates reported by Fu et al. (2008a) (via $^{15}N$ and $^{14}C$ assimilation) across $CO_2$

treatments shows that both studies observed similar magnitudes of enhancement at ~750 μatm compared to ~400 μatm (Fig. 2-1). However, while Fu et al. (2008a) did not test $pCO_2$ above ~750 μatm, our study indicates that net C fixation, N accumulation, and growth rates were further enhanced when grown at 1000 μatm (Fig. 2-6, Table 2-1). Higher $pCO_2$ treatments would need to be tested to determine whether *Crocosphaera* WH8501 is saturated at 1000 μatm. It should also be noted that a recent study by Garcia et al. (2013) found that elevating $pCO_2$ did not significantly affect growth, C, or $N_2$ fixation rates for *Crocosphaera* strains WH0401 and WH0402; these strains appear to be fully saturated under present-day $pCO_2$ conditions. Thus, the $CO_2$ enhancement of *Crocosphaera* WH8501 metabolic rates observed in our study may not reflect the response of other *Crocosphaera* isolates or natural communities of Group B unicellular cyanobacteria.

One surprising finding from our study is that elevating $pCO_2$ produced smaller average *Crocosphaera* cells. Three internally consistent lines of evidence support this conclusion. First, 400 μatm treatments had significantly lower average PC and PN content per cell than 1000 μatm treatments (Tukey HST, $p < 0.001$ for both) and 750 μatm treatments (Tukey HST, $p < 0.01$ for both) (Fig. 2-4B,C). Second, flow cytometry SSC, a proxy for size (as well as shape and granularity) was significantly higher in 400 μatm treatments than in 1000 μatm or 750 μatm treatments (Fig. 2-4D, Tukey HST, $p < 0.01$ for both). Finally, comparing the volumetric increase of cells, PC, and PN through the experiment showed that elevating $pCO_2$ had a stronger effect on cell division than on PC accumulation (Fig. 2-1). Our finding of smaller average *Crocosphaera* cells in elevated $pCO_2$ treatments was unexpected, as elevated $pCO_2$ has been previously reported to increase the diameter of *Crocosphaera* cells (Fu et al. 2008a). One possible explanation is that the smaller average cell size is the result of higher cell division rates at higher $pCO_2$, and thus a dominance of post-division cells in the assemblage rather than a smaller diameter of cells within a certain stage of the cell cycle.

### *2.4.3 Effects of $pCO_2$ on diurnal dynamics*

Monitoring changes in the PN content of *Crocosphaera* cultures through the diel cycle showed that elevating $pCO_2$ changed the temporal pattern of N accumulation (Fig. 2-2B). While $pCO_2$ did not affect N accumulation rates in the second half of the dark period (One-way ANOVA, $p > 0.05$), N accumulation rates were significantly higher at 1000 μatm than at 400 μatm in the first half of the dark period (Tukey HST, $p = 0.039$). Previous studies have found that *Crocosphaera* WH8501 accomplishes the majority $N_2$ fixation in the second half of the dark period (Mohr et al. 2010b, Dron et al. 2012b) and in a study by Dron et al. (2012b), $N_2$ fixation was not detected until after 4-5 hours of darkness. Our results suggest that at higher $pCO_2$, this rhythm is changed and a substantial fraction of $N_2$ fixation is accomplished earlier in the dark period (Fig. 2-2B).

This finding gives clues to the specific physiological changes responsible for the observed increases in $N_2$ fixation by *Crocosphaera*. For *Trichodesmium*, $pCO_2$ has been shown to extend the period of $N_2$ fixation (Kranz et al. 2010) but not increase the protein pool of the nitrogenase enzyme (Levitan et al. 2010a, Levitan et al. 2010b); thus, elevated $pCO_2$ seems to increase the activity per protein, possibly through post-translational modification and/or allocating more energy to nitrogenase activity (Kranz et al. 2011). Because elevating $pCO_2$ does not increase gross rates of photosynthesis (Kranz et al. 2010), this extra energy is presumed to come from a redistribution of ATP and NADPH from the CCM. Though our sampling regime did not provide temporal resolution that is fine enough to determine whether $N_2$ fixation in elevated $pCO_2$ treatments began earlier in the dark period, the change in the temporal pattern of $N_2$ fixation shown here for *Crocosphaera* can be explained by an extended period of $N_2$ fixation, as has been shown for *Trichodesmium* (Kranz et al. 2010). This suggests that the physiological responses of these organisms to elevated $pCO_2$ could be the result of a similar cellular mechanism.

Though elevated $pCO_2$ increased the magnitude of net PC accumulation (Fig. 2-1), $CO_2$ did not appear to affect the diurnal timing of PC accumulation and loss (Fig.

2-2A). This result was surprising: we would expect the higher N accumulation rates we observed in elevated $pCO_2$ treatments during the first half of the dark period to be temporally coupled to increased respiration of carbohydrate reserves. The six-hour time intervals in our study may have been insufficient to resolve this respiration signal, especially given that some PC loss at night is due to exudation and respiration fuelling processes other than $N_2$ fixation.

The imbalanced growth at the end of the experiment complicates interpretations of the effects of $pCO_2$ on cellular C and N retention. Our study did not measure differences between gross and net C and $N_2$ fixation rates, but tracking changes in PC in the dark and PN in the light provided information about the net losses during those time periods (Fig. 2-5). Both N and C retention displayed greater variation between days than between $pCO_2$ treatments. The decrease in net C loss during the dark period as the experiment progressed likely reflects lower respiration rates as growth slowed (Fig. 2-5, Fig. 2-3B). PN retention showed the opposite trend: we observed a slight increase in N loss toward the end of the experiment (Fig. 2-5). In *Trichodesmium*, $pCO_2$ has been shown to affect gross:net $N_2$ fixation rate ratios under certain light conditions (Garcia et al. 2011). Though the highest rates of N loss in this study correspond to elevated $pCO_2$ treatments, they also correspond to highest total PN concentrations, when cultures may have been experiencing light limitation or oxidative damage. Thus, our results on the effect of $pCO_2$ on N retention by *Crocosphaera* are inconclusive.

## 2.5 Conclusions and ecological implications

The high growth rates achieved in our study provide some unique insights into the physiology of *Crocosphaera*. The C:N ratio of cultures fluctuated between 5.5 and 22.1, a much larger range than had been previously reported for *Crocosphaera* (Fig. 2-4A). The large increase in C:N through the light period was presumably driven by the storage of carbohydrate reserves to fuel $N_2$ fixation and other metabolic processes.

Interestingly, this pattern shifted when growth rates began to decline on day four. The magnitude of PC accumulation and loss decreased toward the end of the experiment, likely due to decreased respiration as growth slowed. One notable consequence of the high rates of growth, photosynthesis and respiration is that the $pCO_2$ was not constant within any treatment. Daily fluctuations became extreme toward the end of the experiment; on day six $pCO_2$ fluctuated between 1216 and 96 μatm in the 1000 μatm treatment (Fig. 2-7). Though the $pCO_2$ shifts can be confounding, they also provide insight into the physiological tolerances of this organism. *Crocosphaera* cultures benefitted from elevated $pCO_2$: growth rates and net C and N accumulation were all the highest in the high $pCO_2$ treatment (Fig. 2-1). However, this organism was still able to actively grow when $pCO_2$ fluctuated to values lower than the concentration at the last glacial maximum (~150 μatm).

Our study also contributes to our understanding of how *Crocosphaera* may respond to OA and the underlying mechanism of this response. Initial experiments have suggested that OA may increase rates of C and $N_2$ fixation by certain groups of nitrogen-fixing bacteria (Hutchins et al. 2009). Our results, in agreement with previous studies of *Trichodesmium* strain IMS101 and *Crocosphaera* strain WH8501, showed that elevating $pCO_2$ enhanced rates of net C and N accumulation by a *Crocosphaera* isolate under laboratory conditions. Further, we found that elevated $pCO_2$ appears to change the diurnal pattern of $N_2$ fixation by *Crocosphaera*. This finding is a first step toward understanding the mechanism of $pCO_2$ stimulation of $N_2$ fixation by this organism.

Elucidating the mechanism of $CO_2$ enhancement of nitrogen-fixing cyanobacteria has an ecological relevance. First, physiological understanding can improve our interpretation of results from common experimental methodologies. For example, under laboratory settings, *Trichodesmium* increases $N_2$ fixation rates without concomitant increases in the concentration of *nifH* gene transcripts or nitrogenase proteins (Levitan et al. 2010b). Thus, *nifH* based approaches may not be the most effective way to test the effects of $pCO_2$ on $N_2$ fixation in the field.

Perhaps more importantly, understanding the mechanism of $CO_2$ enhancement is critical to predicting whether the trend of enhanced C and $N_2$ fixation shown by *Trichodesmium* and *Crocosphaera* isolates in laboratory conditions will be reflective of natural $N_2$ fixing communities. Recent field studies using natural diazotrophic communities have shown mixed responses to elevated $pCO_2$ (Hutchins et al. 2009, Gradoville, in prep., Böttjer, in prep., Law et al. 2012, Lomas et al. 2012). Furthermore, there appears to be a diverse response to $pCO_2$ even within taxa: certain strains of *Trichodesmium* and *Crocosphaera* do not show any response to elevated $pCO_2$ (Garcia et al. 2013, Hutchins, pers. comm.). Understanding the full mechanism of $pCO_2$ enhancement, its plasticity within strains, and its variability between strains, species, and taxa is key to predicting whether global rates of $N_2$ fixation will increase under future scenarios of OA.

## 2.6 Literature cited


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**Table 2-1:** Growth rates specific to each measured biomass parameter at a range of $pCO_2$. Tukey HST *p*-values are provided for comparisons among $pCO_2$ treatments. Growth rates were calculated as a linear regression of natural ln normalized values from the 14:00 time point of days 2-4, when cultures were growing exponentially. Standard deviations of three replicate bottles are presented in parentheses.

| | **Specific Growth rate μ ($d^{-1}$)** | | | **Tukey HST *p*-value** | |
|---|---|---|---|---|---|
| | 400 ppm | 750 ppm | 1000 ppm | 400ppm vs. 1000 ppm | 400 ppm vs. 750 ppm |
| Cell abundance | 0.45 (0.02) | 0.54 (0.02) | 0.60 (0.02) | 0.0002 | 0.003 |
| Particulate Nitrogen | 0.54 (0.02) | 0.60 (0.05) | 0.71 (0.04) | 0.003 | 0.2 |
| Particulate Carbon | 0.58 (0.02) | 0.63 (0.06) | 0.71 (0.06) | 0.04 | 0.44 |
| Chlorophyll *a* | 0.60 (0.06) | 0.72 (0.05) | 0.72 (0.07) | 0.12 | 0.14 |
| *in vivo* fluorescence | 0.46 (0.04) | 0.56 (0.01) | 0.62 (0.02) | 0.001 | 0.014 |

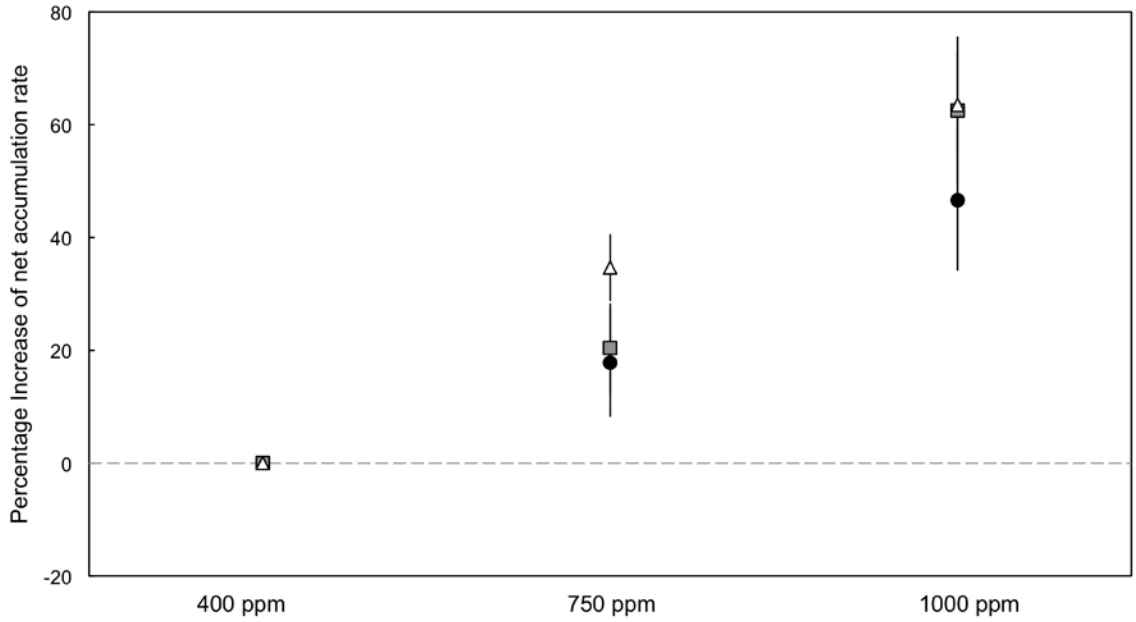


**Figure 2-1:** Percentage increase in net accumulation rate of particulate carbon (black circles), particulate nitrogen (grey squares) and cell number (white triangles) as a function of $pCO_2$, relative to ambient levels (400 μatm). Data were normalized to the day 1 14:00 time point (as in Fig. 1) and accumulation rates were calculated as the rate of change between the 14:00 time point of days 1 and 3, while cultures were in exponential growth. Error bars represent the propagated error of standard deviations of triplicate bottles.

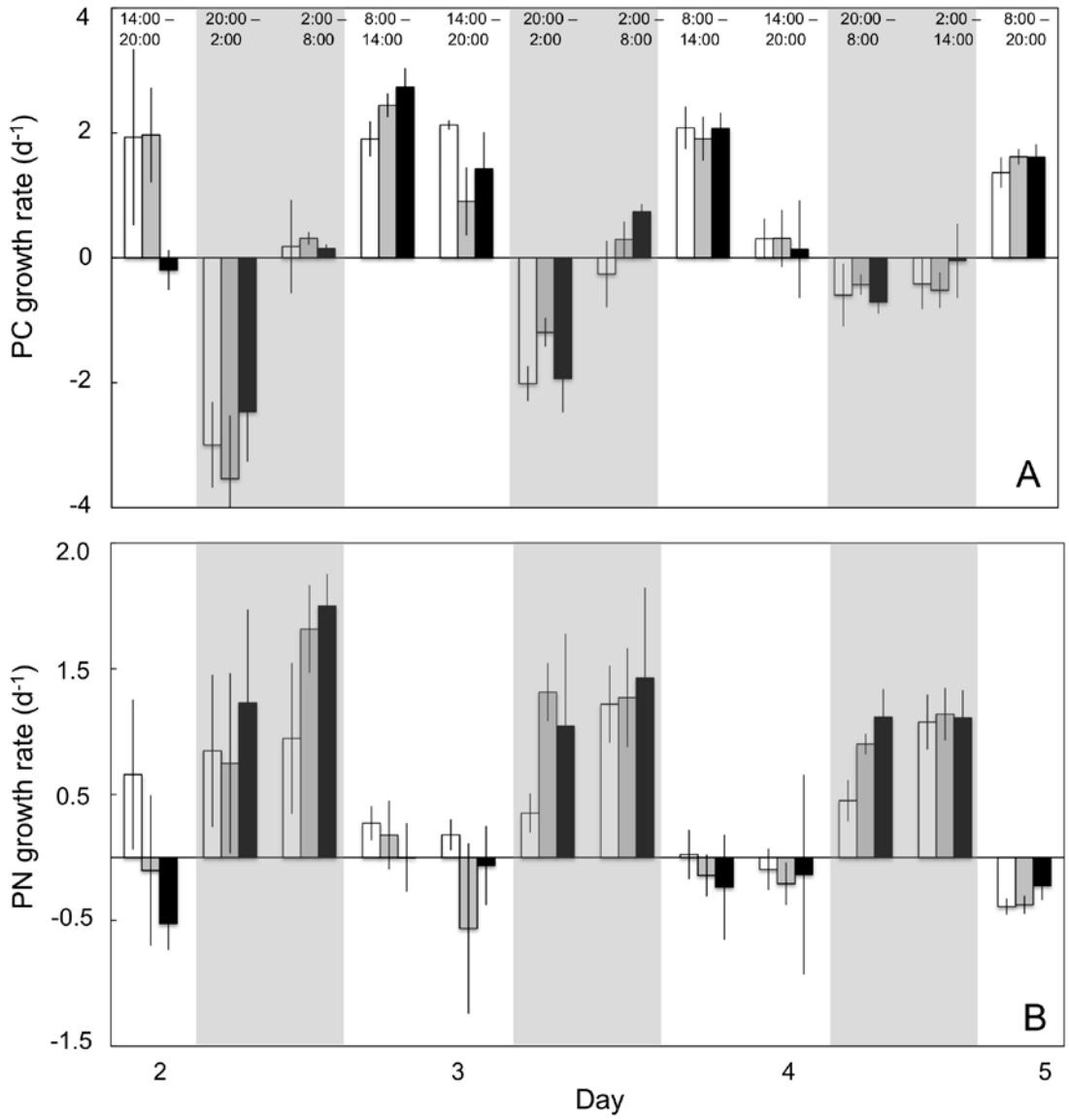


**Figure 2-2**: Particulate carbon (A) and particulate nitrogen (B) growth rates between sampling time points through the diurnal sampling period of the experiment. White, grey, and black bars represent *Crocosphaera* cultures bubbled with 400 µatm, 750 µatm, and 1000 µatm $pCO_2$, respectively. Shaded areas represent the dark periods. Error bars represent standard deviations of triplicate bottles.

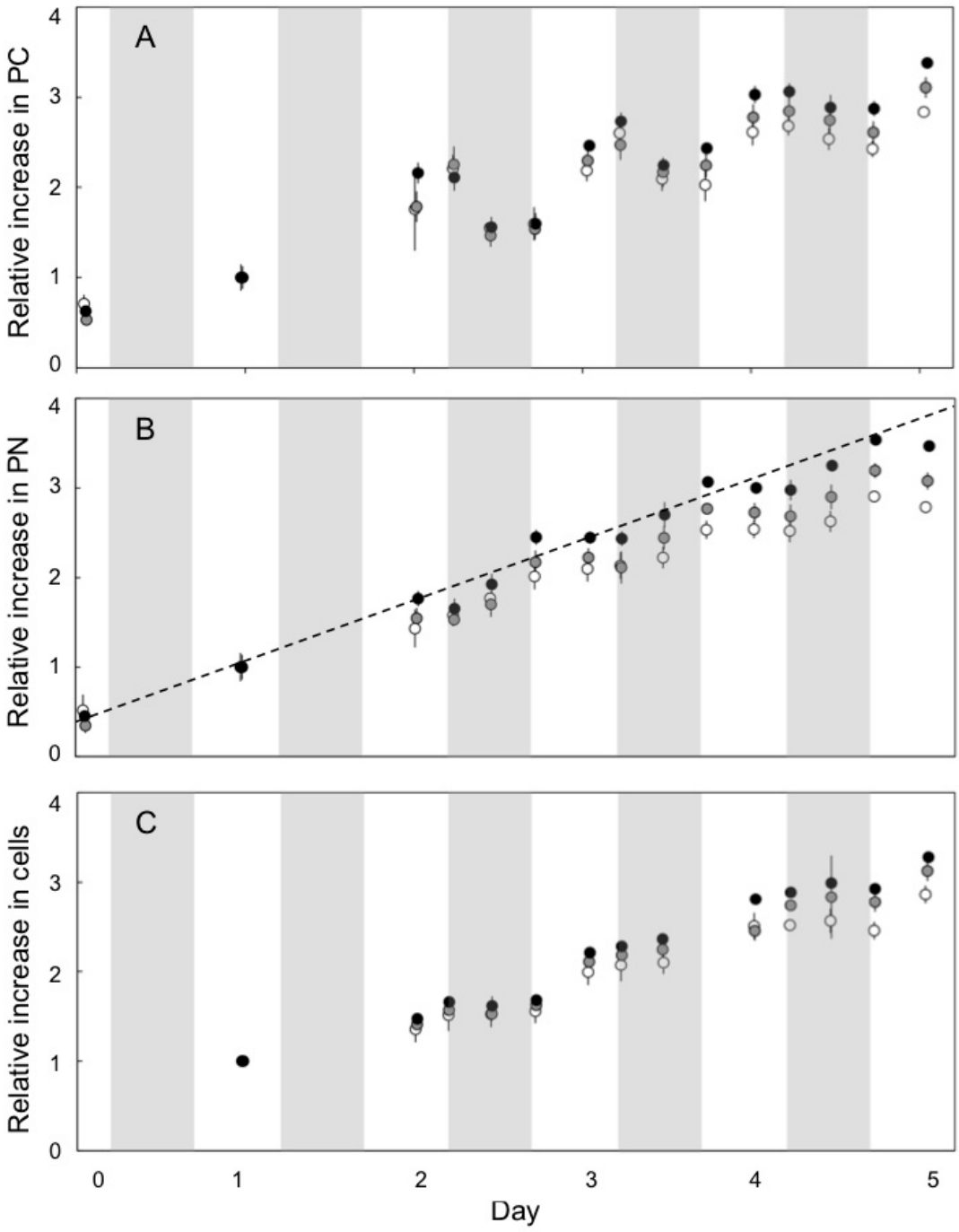


**Figure 2-3:** Particulate carbon (A), particulate nitrogen (B), and cell abundance (C) of *Crocosphaera* cultures bubbled at 400 µatm (clear), 750 µatm (grey), and 1000 µatm $pCO_2$ (black). Data are from volumetric measurements that were ln transformed and normalized to day 1. Shaded areas represent the dark periods. Error bars represent standard deviations of triplicate bottles. Dotted line in (B) displays exponential growth in 1000 µatm 14:00 timepoints through the first four days of the experiment.

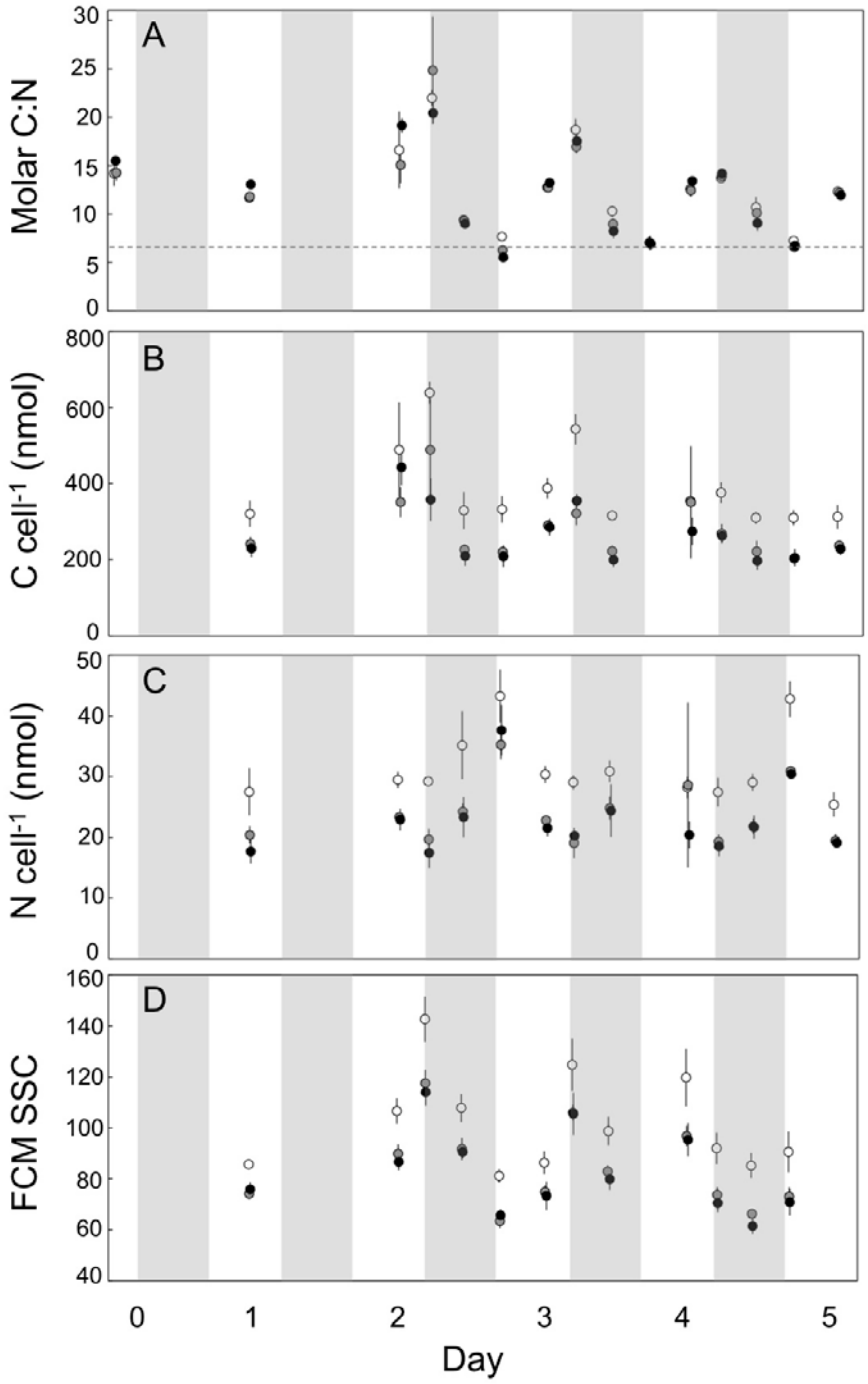


**Figure 2-4:** Molar C:N ratio (A), C cell$^{-1}$ (B), N cell$^{-1}$ (C), and flow cytometry side scatter, a proxy for cell size (D), of *Crocosphaera* cultures bubbled at 400 µatm (clear), 750 µatm (grey) and 1000 µatm $pCO_2$ (black). Shaded areas represent dark periods. Error bars represent standard deviations of triplicate bottles. The dotted line in (A) shows the 6.6 molar C:N ratio expected from Redfield stoichiometry.

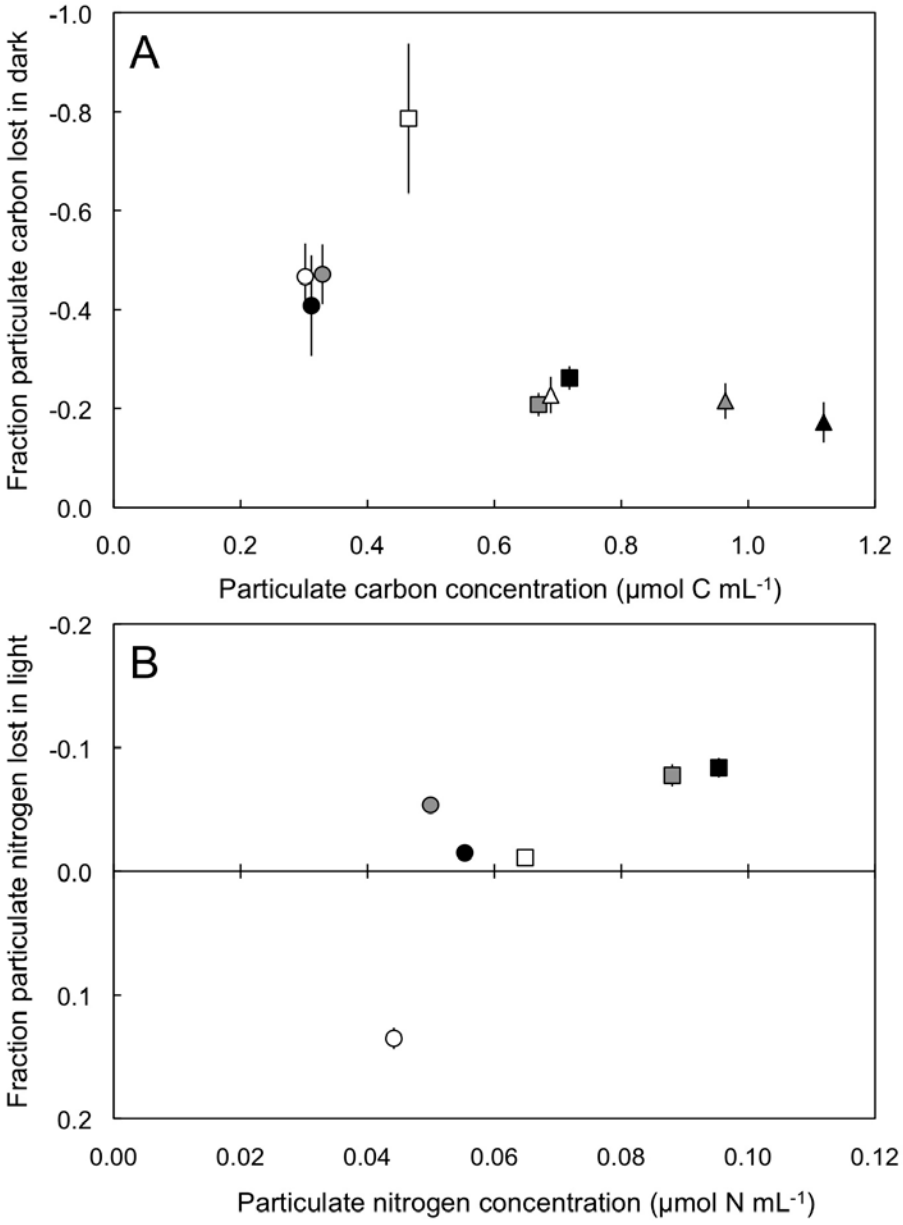


**Figure 2-5:** Fraction of particulate carbon lost in the dark (A) and particulate nitrogen lost in the light (B) periods in *Crocosphaera* cultures bubbled with 400 µatm (white), 750 µatm (grey), and 1000 µatm (black) $pCO_2$, as a function of biomass. Circles indicate measurements on day 3, squares on day 4, and triangles on day 5. Negative values indicate fractional decreases in concentrations of PC (during the dark) or PN (during the light). Error bars indicate standard deviations of triplicate bottles.

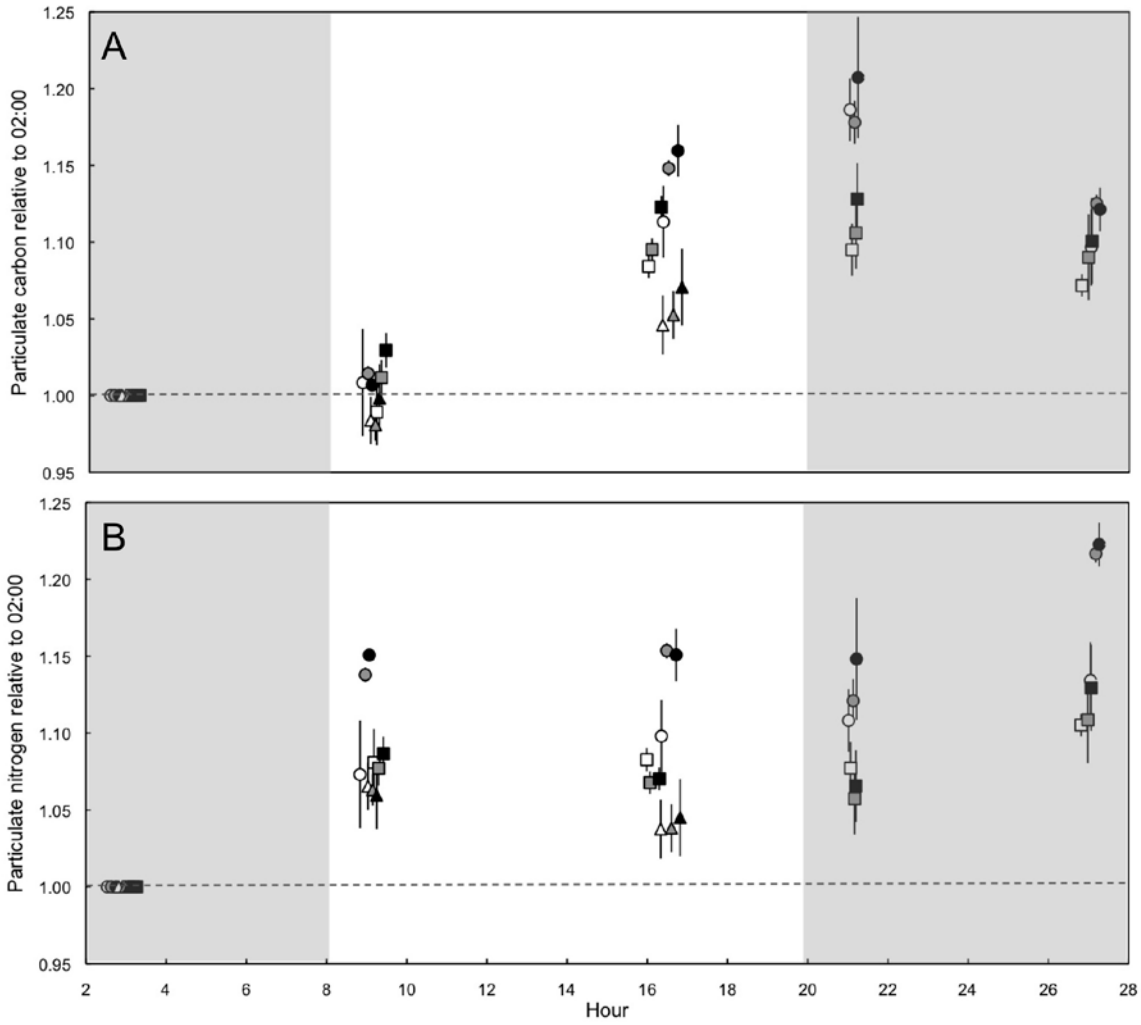


**Figure 2-6:** Relative concentrations of particulate carbon (A) and particulate nitrogen (B) through the diurnal sampling period of the experiment (days 2-5). Colors represent $pCO_2$ treatments (white = 400 µatm, grey = 750 µatm, black = 1000 µatm) on day 3 (circles), day 4 (squares) and day 5 (triangles). Values are ln transformed and normalized to the 02:00 time point of each respective day. Error bars represent standard deviations of triplicate bottles.

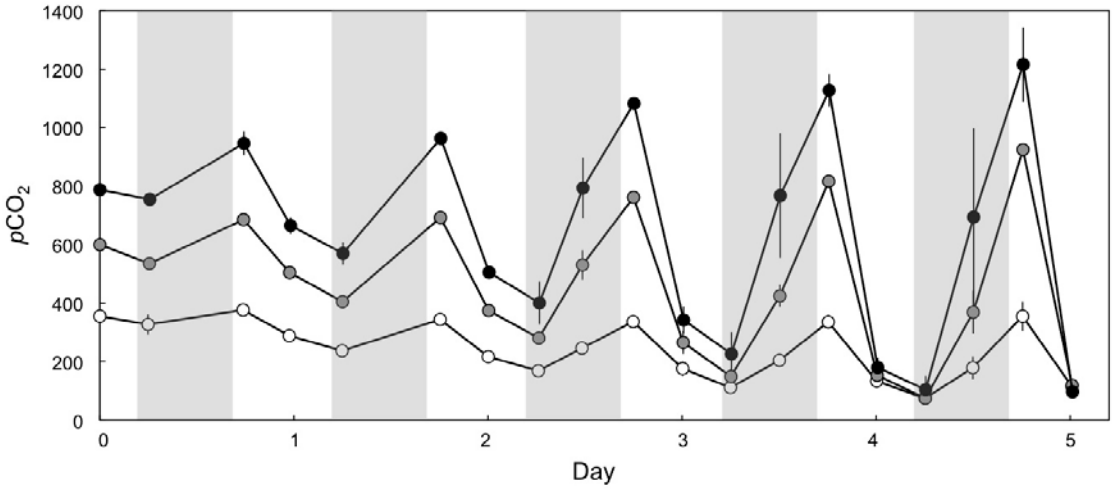


**Figure 2-7:** $pCO_2$ (in µatm) of *Crocosphaera* cultures bubbled with 400 µatm (white), 750 µatm (grey), and 1000 µatm (black) air/$CO_2$ mixtures through the course of the experiment. Shaded areas represent the dark periods. Error bars represent standard deviations of triplicate bottles.

# Diversity trumps Acidification: No carbon dioxide enhancement of *Trichodesmium* community nitrogen or carbon fixation at Station ALOHA.

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# 3. DIVERSITY TRUMPS ACIDIFICATION: NO CARBON DIOXIDE ENHANCEMENT OF NITROGEN OR CARBON FIXATION BY *TRICHODESMIUM* ASSEMBLAGES AT STATION ALOHA.

## 3.1 Abstract

Marine diazotrophs including the filamentous cyanobacteria *Trichodesmium* are important sources of biologically available nitrogen (N) for oligotrophic open-ocean regions. Recent laboratory studies show that certain strains of *Trichodesmium* increase di-nitrogen ($N_2$) and carbon (C) fixation rates when exposed to elevated carbon dioxide ($CO_2$) conditions and it has been suggested that global $N_2$ fixation may increase under future scenarios of anthropogenic ocean acidification. However, the response of natural diazotrophic communities to elevated $pCO_2$ remains unclear. In this study, we conducted eleven independent $CO_2$ manipulation experiments using natural *Trichodesmium* colonies isolated on three cruises in the North Pacific Subtropical Gyre. $N_2$ and C fixation rates of these colonies were compared over $CO_2$ conditions ranging from ~180 μatm (last glacial maximum atmospheric $pCO_2$) to ~1500 μatm (predicted for ~year 2200). Our results showed that elevated $CO_2$ had no consistent, significant effect on rates of *Trichodesmium* colony $N_2$ or C fixation. Moreover, this response was not modulated by phosphorus or iron amendments or by light level. Sequencing the *hetR*, ITS, 16S, and *nifH* genes of *Trichodesmium* colonies revealed a diverse community of *Trichodesmium* and other colony-associated organisms. The composition of the *Trichodesmium* colonies shifted from day to day on the cruise, but over half of total sequences were phylogenetically closely related (>99% *hetR* sequence similarity) to isolate H9-4 of *T. erythraeum*, which has shown no response to elevated $CO_2$ conditions in previous laboratory experiments. Furthermore, our incubations included a substantial number of organisms other than *Trichodesmium* with the metabolic capacity for $N_2$ and C fixation. Our results suggest that the biological diversity within our samples is the underlying reason for the lack of

an observed $CO_2$ enhancement of $N_2$ or C fixation rates. Our results are in agreement with previous experiments in the North and South Pacific subtropical gyres, which have reported no effect of elevated $pCO_2$ on whole-community $N_2$ fixation rates. This may be a common feature of natural diazotrophic assemblages under realistic environmental conditions.

KEY WORDS: Ocean acidification · *Trichodesmium* · Nitrogen fixation · Station ALOHA

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## 3.2 Introduction

Burning fossil fuels and other human actions are adding carbon to the atmosphere at unprecedented rates (e.g. Caldeira and Wickett 2003). This increase in atmospheric $CO_2$ propagates into the ocean, increasing the $pCO_2$ of seawater and causing a suite of changes to ocean chemistry, collectively termed ocean acidification (OA; e.g. Doney et al. 2009). Among the predicted effects of OA is a possible upregulation of cellular processes by carbon (C)-limited phytoplankton (Riebesell et al. 1993, Hutchins et al. 2009). Because almost all phytoplankton species invest

energy in carbon-concentrating mechanisms (CCMs) to help saturate their low $CO_2$-affinity carboxylating enzyme, ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) (Badger et al. 1998), it has been proposed that future OA may allow certain species to increase their C fixation rates and reallocate energy from CCMs to other processes (Hutchins et al. 2007).

Cyanobacteria, which evolved under the high $CO_2$ conditions of the Archaean Eon, have an especially low $CO_2$-affinity RuBisCO compared to other phytoplankton, and may thus benefit from future elevated $pCO_2$ (Buick 1992, Beardall and Giordano 2002).There is a particular interest in the effect of OA on a class of cyanobacteria with the capacity to fix di-nitrogen ($N_2$), termed diazotrophs. The bioavailable nitrogen (N) provided by marine diazotrophs helps to fuel primary productivity, especially in oligotrophic open-ocean environments, where $N_2$ fixation can account for up to half of the new N supporting productivity in the euphotic zone (Karl et al. 1997, Karl et al. 2002). A large fraction of this fixed N is provided by *Trichodesmium*, a filamentous non-heterocystous cyanobacteria (Capone et al. 1997, Karl et al. 1997, Capone et al. 2005, Mahaffey et al. 2005).

Recently, several laboratory studies on two isolates of *Trichodesmium erythraeum* have shown enhanced $N_2$, and sometimes C fixation, when exposed to increases in $pCO_2$ (e Ramos et al. 2007, Hutchins et al. 2007, Levitan et al. 2007, Kranz et al. 2009). In addition, an isolate of *Crocosphaera watsonii*, a naturally abundant unicellular cyanobacterium, also increases $N_2$ and C fixation rates when exposed to elevated $pCO_2$ (Fu et al. 2008a, Gradoville unpublished data). In these studies, a doubling of present-day $pCO_2$ increased $N_2$ fixation rates between 35% (Kranz et al. 2009) and 138% (Levitan et al. 2007). An enhancement of global marine $N_2$ fixation in this range could have substantial consequences for the coupling of elemental cycles, ocean primary productivity, carbon sequestration, and the biological pump (Falkowski 1997).

It is unclear whether the early evidence for a $pCO_2$ enhancement of $N_2$ fixation by *Trichodesmium* and *Crocosphaera* isolates manipulated in the laboratory can be

used to predict the response of diazotrophs under natural environmental conditions. In most laboratory studies, *Trichodesmium* and *Crocosphaera* cultures are grown in an artificial medium with excess nutrients uncharacteristic of open-ocean habitats (Chen et al. 1996). These growth conditions may influence the affect of OA on $N_2$ fixation. For example, under Fe limiting conditions, OA has a negative or neutral affect on $N_2$ fixation by *Trichodesmium* and *Crocosphaera* isolates (Fu et al. 2008a, Shi et al. 2012). To date, the only study of *Trichodesmium* IMS101 grown in nutrient-amended seawater rather than YBC media (Chen et al. 1996) found that OA reduced $N_2$ fixation rates even in Fe-replete treatments, suggesting that this strain may not be C-limited when grown in seawater. Furthermore, it is not known whether the $CO_2$ enhancement trend shown for laboratory strains can be extrapolated to diverse diazotrophic communities. Though OA enhancement of $N_2$ fixation has been observed in select *Trichodesmium* and *Crocosphaera* strains, an isolate of the heterocystous cyanobacterium *Nodularia spumigena* decreases $N_2$ fixation rates in response to OA (Czerny et al. 2009). Recent evidence suggests that the $N_2$ fixation response to $CO_2$ manipulations can vary widely even within the genus *Trichodesmium*; e.g. certain laboratory strains do not exhibit the same positive response to $CO_2$ observed in the initial experiments that used IMS101 and GBR strains (Hutchins et al. 2007, Hutchins, pers. comm.).

Recent experiments investigating the responses of naturally occurring *Trichodesmium* colonies and whole diazotrophic communities to OA have yielded conflicting results. Elevated $pCO_2$ has stimulated $N_2$ fixation by isolated *Trichodesmium* colonies from the Subtropical Atlantic and the Gulf of Mexico (Hutchins et al. 2009, Lomas et al. 2012). However, the specific methodological approaches and the application of phosphorus (P) and Fe replete conditions in these experiments make ecologically-relevant interpretations difficult (Lomas et al. 2012). In contrast, unamended whole-community experiments in the North and South Pacific gyres do not display a significant relationship between $pCO_2$ and $N_2$ fixation rates

(Law et al. 2012, Böttjer, in prep.). These conflicting results paint an unclear picture of the response of natural diazotrophic communities to OA.

Accurate forecasts of future marine nutrient cycling and associated atmospheric feedbacks require an understanding of how OA will affect biological rate processes in real-environment, integrated microbial communities. A key step in this direction is determining whether the contrasting responses exhibited by *Trichodesmium* colonies and whole-water communities in previous $CO_2$ manipulation experiments are due to differences in community composition, physiology, environmental conditions, and/or experimental methodology. To this end, the aim of our present study is to characterize the effect of elevated $pCO_2$ on naturally occurring *Trichodesmium* assemblages in the oligotrophic North Pacific subtropical gyre (NPSG) under a range of nutrient and light conditions. Here, we present results of $N_2$ and C fixation rate measurements from $pCO_2$ manipulation experiments in context of the diversity of organisms used in our incubations.

## 3.3 Methods

### *3.3.1 Experimental Design*

Experiments were carried out on three cruises aboard the RV *Kilo Moana* at and near Station ALOHA (A Long-term Oligotrophic Habitat Assessment), the long term field site of the Hawaii Ocean Time-series (HOT) program ~100km north of the Hawaiian island of Oahu (Table 3-1). In all experiments, picked *Trichodesmium* colonies or whole water samples were incubated on deck under varying $CO_2$, nutrient and light regimes. C and $N_2$ fixation rates were measured, and samples of *Trichodesmium* colonies were saved for DNA extractions (Table 3-1).

*Trichodesmium* colonies were collected using a 202-$\mu$m plankton net that was hand-towed at <1 knot through the near-surface ocean (~<10m depth) for 15-20 minutes. An inoculating loop was used to gently pick colonies from the net tow material and place them into 0.2 $\mu$m filtered surface seawater. Colonies were kept in

minimal light until the initiation of the experiment (within 5 hours of collection). To prepare "media" for *Trichodesmium* incubations, surface seawater was collected with a Teflon pump and filtered through a 0.2 $\mu$m in-line polycarbonate membrane filter. The filtered seawater was dispensed into acid-washed polycarbonate carboys, where pH and $pCO_2$ was manipulated (see p*CO$_2$ manipulations and DIC measurements*) and 20 $\mu$mol $L^{-1}$ EDTA was added to prolong *Trichodesmium* activity (Burns et al. 2006). For P or Fe replete treatments, media was enriched to final concentrations of 5 $\mu$mol $L^{-1}$ $KH_2PO_4$ or 100 nmol $L^{-1}$ $FeCl_3$EDTA. Immediately preceding rate experiments, media was dispensed into acid-washed 38 mL glass serum bottles and 8-12 *Trichodesmium* colonies were added per bottle. Experiments started at sunrise and ended at sunset. Samples were incubated in flow-through deckboard incubators under blue acrylic at ~60% of sea surface irradiance. Black netting was used to shade bottles to additional light levels (~30%, ~15%, and ~8% incoming irradiance) within the incubator as measured by a Biospherical light meter. Daily integrated PAR for each treatment was calculated by trapezoidal integration of measured sea surface PAR values collected with an on deck LICOR Siltronic 2$\pi$ sensor and correcting for percent light levels of treatment incubations. Though surface seawater was continuously pumped through the incubator, HOBO temperature sensors measured a daily temperature range of 23.5-29°C in August 2012.

### *3.3.2 Carbon and nitrogen fixation rates*

On August 2010 and March 2011, $N_2$ fixation rates were measured using the acetylene reduction technique (AR) (Capone 1993). 25 mL of incubation media with the proper $pCO_2$ was dispensed into serum bottles, leaving 12 mL of headspace. Eight to twelve handpicked colonies of *Trichodesmium* were added to each bottle and the headspace was flushed with the appropriate $pCO_2$ (March 2011 cruise only), fitted with Viton septa, and sealed by crimping with aluminum caps. Acetylene ($C_2H_2$) was produced from calcium carbide (Aldrich) and injected into each bottle to produce a volumetric concentration of 10% $C_2H_2$ in the headspace. Once every 3 hours through

the incubation period, 100 μL of headspace was subsampled and ethylene ($C_2H_4$) was detected by flame ionization gas chromatography using a Shimadzu GC-8A. Samples were incubated from dawn until dusk, approximately 12 hours. After the final time point, samples were filtered onto 25mm glass fiber filters (GF/F, Whatman), frozen at -80°C, and shipped in liquid nitrogen to Oregon State University, where they were stored at -20°C. Chlorophyll *a* was extracted in 90% acetone at -20°C for 48 hours and analyzed using a Turner Model 10-AU fluorometer and the acidification method of Strickland and Parsons (1972). AR rates were calculated as a linear regression of $C_2H_4$ produced time$^{-1}$ and normalized to the chlorophyll *a* in each bottle.

C and $N_2$ fixation were measured in August 2012 using $^{13}C$ method of Legendre and Gosselin (1997) and a modification of the original $^{15}N_2$ uptake methods (Montoya et al. 1996). $^{15}N_2$ (99%) was added as enriched seawater (2% by volume) instead of direct gas injection to avoid problems with bubble dissolution (Mohr et al. 2010a, Wilson et al. 2012). Mixtures of incubation media, $^{15}N_2$ enriched seawater, and $^{13}C$ bicarbonate stock were dispensed into serum bottles using a large glass syringe to avoid air exposure. *Trichodesmium* colonies were added and the bottles were fitted with Viton septa and aluminum caps and crimp sealed, leaving no headspace. Incubations were terminated by vortexing and gently filtering colonies onto precombusted 25 mm GF/F filters. Filters were stored at -80°C and shipped in liquid nitrogen to Oregon State University, where they were dried at 60°C overnight and packaged tightly into tin capsules. Particulate carbon (PC) and nitrogen (PN) masses and isotopic composition were measured using a PDZ Europa ANCA-GSL elemental analyzer coupled to a PDZ Europa 20-20 isotope ratio mass spectrometer at the University of California Davis stable isotope facility. Fixation rates were calculated according to Montoya et al. (1996) and normalized to measured PC concentrations.

### *3.3.3 $pCO_2$ manipulations and DIC measurements*

Two methods were used to manipulate $pCO_2$ for these experiments: carbonic acid additions and gas bubbling. Both methods manipulate $pCO_2$ by increasing DIC

without altering alkalinity (Schulz et al. 2009), similar to anthropogenic OA. In laboratory experiments, we found that the method chosen had no significant effect on the response of *Trichodesmium* IMS101 to $CO_2$ levels (Gradoville unpublished data).

Carbonic acid additions were used on the August 2010 and March 2011 cruises. Equal molar concentrations of HCl and $NaHCO_3$ were made to 0.2 $\mu$m filtered seawater to give pH values of approximately 8.02 (~ambient $pCO_2$), 7.82 (~750 µatm), 7.7 (~1100 µatm), and 7.5 (~2000 µatm). Gas bubbling was used during the August 2012 cruise; for these experiments, 0.2 $\mu$m filtered seawater was bubbled for >24 hours with commercially manufactured (Airgas) artificial air/$CO_2$ mixtures to achieve $CO_2$ conditions of 152, 282, 410, 748, and 996 µatm.

The carbonate chemistry was monitored each day that an experiment was run on March 2011 and August 2012 cruises. Incubation media was dispensed into oxygen bottles or 350 mL amber glass bottles, fixed with 100-300 $\mu$L $HgCl_3$, and sealed with no headspace. Samples were analyzed for dissolved inorganic carbon (DIC) and total alkalinity (TA) either onboard or at a shore-based laboratory. For August 2010 and March 2011 cruises, DIC was estimated coulimetrically and TA was measured by acid titration. The DIC/TA calibrations were validated with certified $CO_2$ reference materials (Dickson et al. 2003). $pCO_2$ values were calculated from the DIC and TA data with the program CO2calc (Robbins et al. 2010) using the $CO_2$ constants from Mehrbach et al. (1973) refit by Dickson and Millero (1987). DIC and TA of incubation media were only measured once during the August 2010 cruise; samples were analyzed on the ship according to the methods used in March 2011. However, pH was measured each day an experiment was run in August 2010, and did not change by more than 0.1 pH unit through the cruise. For the August 2012 cruise, DIC and $pCO_2$ were measured at Oregon State University according to the methods of Bandstra et al. (2006). These measured and calculated $pCO_2$ values sometimes deviated from desired $pCO_2$ (Table 3-2), but relative differences between treatments were achieved.

### *3.3.4 Fe measurements*

For Fe measurements, 25 mL samples were saved in acid-leached HDPE bottles and immediately frozen. Samples were analyzed at the Woods Hole Oceanographic Institute according to the method of Lee et al. (2011).

### *3.3.5 DNA extraction*

Plastic inoculating loops were used to pick duplicate or triplicate sets of 20-50 *Trichodesmium* colonies into 0.2 $\mu$m filtered surface seawater. The samples were vortexed and gently filtered onto Supor membranes which were stored at -80°C until analysis. DNA was extracted using the DNeasy Plant MiniKit (Qiagen). The extraction protocol was modified from manufacturer's suggestions to include bead-beading with 0.1mm and 0.5mm glass zirconium/silica beads (Biospec products) for additional cell disruption (Paerl et al. 2008). DNA concentrations were determined by PicoGreen DNA quantification (Molecular Probes) using a Perkin Elmer 2030 multilabel plate reader. Extracted DNA was stored at -20°C.

### *3.3.6 PCR amplification and clone library sequencing*

To analyze the diversity within the *Trichodesmium* colonies, we amplified regions of the 16S, ITS, and *hetR* genes as done by Hynes et al. (2012) and the *nifH* genes as described in Zehr and Turner (2001) (summarized in Table 3-3). PCR reactions were performed using PTC-200 Thermo Cyclers (BioRAD) with 50 μL reaction volumes. All reactions used the Platinum® *Taq* Polymerase High Fidelity kit (Invitrogen). The 16S rRNA gene was amplified in a mixture containing 1X PCR buffer, 200 $\mu$mol $L^{-1}$ dNTPs, 3% BSA, 1.5 mmol $L^{-1}$ $Mg^{2+}$, 1U *Taq*, 4 ng DNA, and 0.5 $\mu$mol $L^{-1}$ CYA-106F and CYA-781R primers (Nübel et al. 1997). 16S PCR was cycled at 95°C for 4 min; then 30 cycles of 93°C for 1 min, 57°C for 1 min, and 72°C for 1 min; followed by 72°C for 7 min. *hetR* amplification used the same reaction mixture as 16S but with the PH1 and PH2 primers (Lundgren et al. 2005). The temperature profile for amplification of *hetR* was 94°C for 6 min; then 30 cycles of

93°C for 1 min, 50°C for 1 min, and 72°C for 1 min; followed by 72°C for 10 min. Amplification of the ITS region used a mixture containing 1X PCR buffer, 200 $\mu$mol $L^{-1}$ dNTPs, 3% BSA, 3.5 mmol $L^{-1}$ $Mg^{2+}$, 1U *Taq*, 4 ng DNA, and 0.5 $\mu$mol $L^{-1}$ tri16S-1247F (Orcutt et al. 2002) and tri-23SR (Hynes et al. 2012) primers. Cycling conditions for ITS were 95°C for 2 min, 95°C; then 30 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C; and finally 10 min at 72°C. A nested PCR amplification procedure was used to amplify *nifH* genes. The first round of PCR contained 1X PCR buffer, 200 $\mu$mol $L^{-1}$ dNTPs, 3% BSA, 4 mmol $L^{-1}$ $Mg^{2+}$, 1U *Taq*, 4 ng DNA, and 1 $\mu$mol $L^{-1}$ nif3 and nif4 primers (Zani et al. 2000). This reaction was cycled at 94°C for 7 min; then 30 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min; followed by 72°C for 7 min. The second *nifH* PCR reaction used the same temperature profile and mixture ingredients but used the nif1 and nif2 primers (Zehr and McReynolds 1989) and 5 $\mu$mol $L^{-1}$ PCR product from the first reaction.

PCR products were visualized and separated on a 1% agarose gel stained with SYBR Safe DNA gel stain. Amplified products were purified using the QIAquick® MiniElute® kit and the purified PCR products were quantified as described above. Amplified gene fragments were cloned using the TOPO® TA Cloning Kit for Sequencing (Invitrogen) according to manufacturer's instructions. Briefly, PCR products were ligated into the TOPO® cloning vector and stored at -20°C before being transformed into DH5α™-T1 chemically-competent *E. coli* cells. Transformations were spread onto LB agar plates with 100 μg $mL^{-1}$ ampicillin and grown overnight at 37°C. Positive colonies were picked and grown at 37°C in CircleGrow media with 100 μg $mL^{-1}$ ampicillin. Samples were sequenced on an ABI 3100 Gene analyzer (Applied Biosystems) by the Advanced Studies in Genomics, Proteomics, and Bioinformatics (ASGPB, University of Hawaii at Manoa).

Sequences were edited and trimmed using the software Geneious . Alignments were made using ClustalW (http://clustal.org) and included sequences obtained from GenBank; accession numbers from these sequences are given in Fig. 3-6. To compare with *Trichodesmium* culture sequences from (Hynes et al. 2012), ITS sequences were

trimmed to internal primers AlaF and AlaR (Rocap et al. 2002). 16S sequences were trimmed to 630 bp; *hetR* were trimmed to 441 bp; *nifH* were trimmed to 370 bp. Sequences from our study can be found in GenBank using accession numbers XX. Phylogenetic trees were created in Geneious, using the Jukes-Cantor model and Neighbor-Joining method and *O. sancta* or *A. plantensis* sequences for outgroups. Trees were Bootstrapped 1000 times. Environmental sequences were compared with sequences from the four major *Trichodesmium* clades previously identified (Lundgren et al. 2005).

### *3.3.7 Quantitative PCR*

Quantitative polymerase chain reaction (qPCR) was used to determine the relative abundance of *nifH* genes from different groups of diazotrophs within our samples. Primers were chosen to target certain *nifH* groups identified in clone library sequencing: *Trichodesmium*, unicellular cyanobacteria (Group A and Group B), heterocystous cyanobacteria (Het1, Het2, and Het3), and the Cluster III phylotype. Descriptions of primers, probes, PCR reactions, and thermocycling conditions can be found in previous work by Church et al. (2005a, 2008).

### *3.3.8 Statistics*

The effect of $pCO_2$ and nutrient concentrations on $N_2$ and C fixation rates were determined using a one-way ANOVA. In some cases, two-way ANOVA were used to test the effects of two independent variables, $pCO_2$ and the day the experiment was conducted, on the dependent variables. No post-hoc tests were used. All experiments used triplicate incubation bottles, but occasionally issues were encountered with individual samples, resulting in duplicate samples. Only measurements with replicates are reported in this study, with the exception of the 1100 μatm puffs treatment on March 15, 2011; this treatment had no replication (Fig. 5) because all replicates were lost during shipment. All statistics were determined using the program R (R Development Core Team 2011).

## 3.4 Results

### *3.4.1 Carbon and nitrogen fixation rates*

Rates of $N_2$ fixation by *Trichodesmium* colonies varied widely between cruises, days, and bottle replicates (Fig. 3-1). In general, AR rates were higher in August 2010, ranging from 9.1-141.8 nmol $C_2H_4$ $L^{-1}$ μg chl $a^{-1}$ $hr^{-1}$, than on the March 2011 cruise when they ranged from 4.8-35.7 nmol $C_2H_4$ $L^{-1}$ μg chl $a^{-1}$ $hr^{-1}$ (Fig. 3-1A). These values are similar to previously reported rates of AR by *Trichodesmium* colonies at Station ALOHA (Letelier and Karl 1998). $^{15}N_2$ fixation rates in August 2012 ranged from 0.46-5.08 nmol N μmol $C^{-1}$ $d^{-1}$, while $^{13}C$ assimilation rates in August 2012 ranged between 72-619 nmol C $d^{-1}$ (Fig. 3-2). We chose not to normalize rates to colony number because of the known variability in size and morphology of *Trichodesmium* colonies (Luo et al. 2012). However, our range of approximately 8-12 colonies $bottle^{-1}$ gives a broad C fixation range of 0.5-6.4 nmol C labeled $colony^{-1}$ $hr^{-1}$, which is similar to previously reported *Trichodesmium* C fixation rates (Lomas et al. 2012). On all cruises, the variability of C and $N_2$ fixation rates between days was greater than the variability resulting from the differences in treatment (Fig. 3-1, 3-2). On average, the coefficient of variation for $N_2$ fixation rates in ambient $pCO_2$, ambient nutrient condition treatments was 17.4% in August 2010, 1.7% in March 2011, and 76.2% in August 2012 (Table 3-4).

Under ambient nutrient conditions, increasing $pCO_2$ did not significantly enhance $N_2$ fixation rates in any of the seven independent experiments (One-way ANOVA, $p > 0.05$). Treatments in which we lowered the $pCO_2$ below present day levels displayed a slight decrease in $N_2$ fixation; however, this trend was not statistically significant (Fig. 3-3, One-way ANOVA, $p > 0.05$). Likewise, variations in $pCO_2$ did not significantly affect C fixation rates in August 2012 (Fig. 3-2).

Additional experiments were performed to test the possibility that nutrient limitation precluded a positive response to $CO_2$ amendments. We found no evidence that *Trichodesmium* $N_2$ fixation was limited by phosphorous (P) availability during

these cruises. At ambient $pCO_2$, $N_2$ fixation rates were not enhanced by amendment with 5 $\mu$mol $L^{-1}$ $KH_2PO_4$ in March 2011 (Fig. 3-1) or by 5 $\mu$mol $L^{-1}$ $KH_2PO_4$, methylphosphonate (MPN), or amino-tris-methylene-phosphonic acid (AMP) in August 2010 (data not shown). Furthermore, there was no significant difference in $N_2$ fixation rates between ambient and elevated $pCO_2$ treatments amended with 5 $\mu$mol $L^{-1}$ $KH_2PO_4$ in March 2011 (Fig. 3-1). In the summer of 2012, ambient phosphate levels were consistently high for Station ALOHA (125-170 nmol $L^{-1}$). Therefore, these experiments were all considered to be P replete and seawater was not amended with $KH_2PO_4$.

Similar to P, Fe additions did not significantly affect rates of *Trichodesmium* C or $N_2$ fixation in our study. At ambient $pCO_2$, neither $N_2$ fixation nor C fixation were significantly different in treatments with and without added Fe (Fig. 3-2). Likewise, Fe amendments did not affect the $N_2$ fixation rates or C fixation rates in elevated $pCO_2$ treatments (Fig. 3-2, 3-3). Because all treatments were amended with EDTA, which interferes with the dissolved Fe measurements, the Fe concentrations in control treatments could not be determined. However, in two separate community experiments using whole water and no EDTA additions, control treatments had 0.54 ± 0.07 and 10.86 ± 3.93 nmol Fe $L^{-1}$. These concentrations are both substantially lower than the Fe replete treatment (100 nmol Fe $L^{-1}$), and the experiment with 10.86 nmol Fe $L^{-1}$ did not have higher $N_2$ fixation rates than the experiment with 0.54 nmol Fe $L^{-1}$ (data not shown).

Differences in the daily flux of light significantly affected *Trichodesmium* $N_2$ fixation rates in August 2012. Rates were higher at 60% incoming light (27.5-28.5 mol quanta $m^{-2}$ $d^{-1}$) than at 8% (3.7-3.8 mol quanta $m^{-2}$ $d^{-1}$) incoming light in both experiments (Fig. 3-4); however, light level only had a significant effect on $N_2$ fixation rates on August 9 (two-way ANOVA, $p = 0.00008$). The flux of light did not change the effect of $pCO_2$ on $N_2$ fixation rates. Rates were consistently higher at elevated $pCO_2$ regardless of light level on August 11 (2-way ANOVA, $p = 0.03$); on August 9, there was no significant difference in $N_2$ fixation rates between $pCO_2$

conditions (2-way ANOVA, $p > 0.05$). All light experiments were Fe replete by addition of 100 nmol $L^{-1}$ $FeCl_3EDTA$ and assumed to be P replete at ambient soluble reactive phosphorus (SRP) concentrations.

### *3.4.2 Diversity within Trichodesmium colonies*

Morphological diversity of *Trichodesmium* colonies varied between cruises (Table 3-4). In March 2011, the *Trichodesmium* community isolated from net tows included both "puff" and "tuft" morphologies. On March 14, 2011, a mixture of morphologies was used for incubations. The March 15, 2011 experiment compared the $CO_2$ response of the two morphologies with separate tuft, puff, and $pCO_2$ treatments (Fig. 3-5). For March 16 and March 20, 2011 experiments, only colonies with tuft morphologies were selected in an attempt to reduce biological variability. In August 2012, isolated *Trichodesmium* consisted of mostly small puff colonies or small, loose tufts; compact tufts were generally absent. Because of the low number of *Trichodesmium* colonies collected during net tows on this last cruise, incubations contained all morphologies present: small puffs, large puffs, and loose tufts. No data is available for the morphologies of colonies used for experiments in August 2010.

DNA samples were only collected during the August 2012 cruise. Sequencing the *hetR* gene revealed 184 clones belonging to the genus *Trichodesmium*; these clones grouped with three out of the four major *Trichodesmium* clades (Lundgren et al. 2005) (Fig. 3-6). Clones grouped within each clade shared >96% *hetR* gene sequence identity. Approximately 72% of clones grouped with *Trichodesmium* Clade I, which contains strains of *T. thiebautii*, *T. tenue* Z-1, *K. spiralis*, *K. pelagica*, and *T. hildebrandtii* (Hynes et al. 2012). The majority of these Clade I DNA sequences (54% of the *hetR Trichodesmium* sequences) were >99% similar to *T. thiebautii* strain H9-4 (Fig. 3-6). Approximately 27% of the *hetR* gene sequences grouped with Clade III, which contains *T. erythraeum* and some *T. contortum* strains (Hynes et al. 2012). Over 98% of these Clade III *hetR* genes were >99% similar to *T. erythraeum* strains IMS101 and GBRTRLI101. Less than 2% of *hetR* gene sequences grouped among the

Clade IV *Trichodesmium* (Fig. 3-6). The resulting phylogenetic representation of the 16S and ITS gene sequences showed similar patterns in *Trichodesmium* diversity as those obtained based on the *hetR* gene (Table 3-5). We focused our phylogenetic analyses on *hetR* sequences because this gene is recognized as an excellent genetic marker for distinguishing among different groups of *Trichodesmium* (Lundgren et al. 2005, Hynes et al. 2012) and because we have the largest number of sequences from *hetR* (Table 3-5).

The community composition of *Trichodesmium* in our samples varied between days (Fig. 3-7). Members of Clade IV *Trichodesmium* were only detected on August 9, 2012. August 10 and August 11 contained nearly equal fractions of Clade I and Clade III *Trichodesmium*. On August 12, *Trichodesmium* was dominated by Clade I (53 out of 58 clones sequenced). Despite this trend of increasing *Trichodesmium* Clade I by August 12th, duplicate filters revealed no significant difference in the fractions of *Trichodesmium* clades between days (2-way ANOVA, $p > 0.05$).

There was a substantial diversity of organisms other than *Trichodesmium* within samples. Each set of primers we used targeted a slightly different group of organisms (Table 3-3), and thus showed different patterns of *Trichodesmium*-associated organisms. While most of the clone library sequences from *hetR* and ITS corresponded to *Trichodesmium*, over half of the sequences from the 16S rRNA gene, which used a cyanobacterial-specific set of oligonucleotide primers, were phylogenetically related to organisms other than *Trichodesmium* (Table 3-5). Furthermore, from among the 93 *nifH* gene sequences obtained from these colonies, only ~20% appeared derived from *Trichodesmium* (Zehr et al. 2003).

To quantify the contributions of several groups of *nifH*-containing microorganisms, we performed qPCR with primers to target six different groups of $N_2$-fixing cyanobacteria and one Cluster III *nifH* gene sequence belonging to an organism previously described at Station ALOHA (Church et al. 2005a) and prevalent in our *nifH* gene clone library. The resulting qPCR data show that the ratio of *Trichodesmium* to total probed *nifH* containing organisms varied between days: on

August 9, 60% of the probed *nifH* belonged to *Trichodesmium*, on August 10, 73% were *Trichodesmium*, and on August 12, 98% were *Trichodesmium* (Fig. 3-8). Next to *Trichodesmium*, the majority of probed *nifH*-containing organisms within our samples were Group B, which is closely related to *Crocosphaera* spp., and Het1, which is closely related to *Richelia* spp. We were surprised to note the small number of Cluster III *nifH* gene copies, since sequences closely related to Cluster III represented 35% of *nifH* clone library sequences (data not shown). The different trends shown for Cluster III by clone libraries and qPCR may be due to a higher amplification efficiency for Cluster III than for other groups in the nested PCR reactions needed to amplify the *nifH* gene.

## 3.5 Discussion

During the period in which our experiments took place, the *Trichodesmium* assemblage did not exhibit a consistent response in $N_2$ or C fixation to elevated $CO_2$ conditions (Fig. 3-1, 3-2). These results are in contrast with several laboratory studies, which report a $CO_2$ enhancement of $N_2$ and C fixation by *Trichodesmium* strains IMS101 and GBRTRLI101 grown in YBCII media (e Ramos et al. 2007, Hutchins et al. 2007, Levitan et al. 2007, Kranz et al. 2009, Kranz et al. 2010, Levitan et al. 2010b, Levitan et al. 2010c, Garcia et al. 2011). Previous natural community experiments have shown mixed effects of $CO_2$ on diazotrophs: while some studies targeting *Trichodesmium* colonies have reported a $CO_2$ enhancement (Hutchins et al. 2009, Lomas et al. 2012), in other studies, there was no relationship between elevated $pCO_2$ and $N_2$ fixation rates in experiments conducted with naturally occurring diazotroph assemblages (Law et al. 2012 , Böttjer in prep.). Reconciling these conflicting results is crucial to determining whether $N_2$ fixation, a keystone process in N-limited open ocean regions, will increase at an ecologically significant magnitude under future conditions of OA.

After our initial experiments in August 2010 found that $CO_2$ enhancement did not affect $N_2$ fixation by *Trichodesmium* at Station ALOHA, we designed a series of experiments aimed at testing whether the apparent discrepancy between laboratory and field studies was due to environmental conditions such as nutrients and light, experimental methodologies, or community composition. Ultimately, the results from these experiments suggest that the diversity within the community of *Trichodesmium* and colony-associated organisms at Station ALOHA is the underlying reason for the lack a consistent, detectable $CO_2$ enhancement of $N_2$ fixation in the field. A new laboratory study has shown a six-fold range in the carbon affinity of diverse *Trichodesmium* strains (Hutchins, pers. comm.). DNA sequences of *Trichodesmium* colonies from our August 2012 cruise help us explain the lack of $CO_2$ stimulation and the overall biological variability seen in our results.

Mechanistically, enhanced $pCO_2$ has been linked to changes in the CCM of *Trichodesmium* IMS101 (Kranz et al. 2009, Kranz et al. 2010); reallocation of ATP and reductants from the CCM is thought to provide the extra energy needed for increased rates of $N_2$ and C fixation (Kranz et al. 2011). $CO_2$ also changes the ratios of certain protein pools; for example, higher $pCO_2$ increases the photosystem I: photosystem II ratio, possibly implying increased cyclic electron transport, but elevated $pCO_2$ does not appear to change the intracellular nitrogenase protein pool, suggesting that the specific activity of the nitrogenase protein increases with $pCO_2$ (Levitan et al. 2010b). Certain environmental and experimental conditions may regulate these physiological responses to $CO_2$ perturbations. For example, as light provides the ultimate source of energy for C fixation, $N_2$ fixation, and CCM operation, light levels would be expected to moderate the effect of $pCO_2$ on C and $N_2$ fixation. Indeed, light has been shown to affect the response of *Trichodesmium* to $pCO_2$, with the largest enhancements observed under low light intensities (Kranz et al. 2010, Garcia et al. 2011). Ample nutrient conditions may also be a prerequisite for $CO_2$ stimulation of C and $N_2$ fixation: *Trichodesmium* $N_2$ fixation can be limited by the availability of P and Fe (Falkowski 1997, Sañudo-Wilhelmy et al. 2001). Finally,

methodological choices may affect the $CO_2$ response; for example, elevated $CO_2$ has been shown to change the ratio of $^{15}N_2$ fixation to nitrogenase activity assayed via AR (Garcia et al. 2011), so the measurement used in the field could affect results. In light of these possibilities, we explored whether differences in environmental conditions, methodological approaches, or colony diversity might explain why we did not observe a $CO_2$ enhancement of $N_2$ or C fixation by the community of *Trichodesmium* at Station ALOHA.

### *3.5.1 Environmental conditions*

Nutrient limitation appears to be an important control on rates of $N_2$ fixation by *Trichodesmium* (Paerl et al. 1994, Sañudo-Wilhelmy et al. 2001) and there is evidence that *Trichodesmium* growth can be limited by the availability of P and Fe at Station ALOHA (Grabowski et al. 2008, Watkins-Brandt et al. 2011, Chappell et al. 2012). Hence, under P and/or Fe limitation, *Trichodesmium* $N_2$ and C fixation could be insensitive to a $pCO_2$ enhancement as a result of primary limitation by the availability of these nutrients. However, we found that the response of *Trichodesmium* assemblages to $CO_2$ was not significantly affected when amending treatments with these nutrients (Fig. 3-3). Because P has been shown to limit *Trichodesmium* $N_2$ fixation only when background SRP concentrations are below ~40 nmol $L^{-1}$ (Grabowski et al. 2008), the high background P observed during our cruises (Table 3-1) may explain the absence of an observed P stimulation of $N_2$ fixation.

In contrast to P, concentrations of Fe during the August 2012 cruise appear typical for Station ALOHA (see results, Boyle et al. 2005). Recent studies have suggested that the effect of $pCO_2$ on diazotrophs is particularly sensitive to Fe availability. Reducing seawater pH changes Fe speciation, decreasing phytoplankton Fe uptake and $N_2$ fixation rates under low Fe conditions (Fu et al. 2008a, Shi et al. 2010a, Shi et al. 2012). However, in our study elevated $pCO_2$ did not statistically increase $N_2$ or C fixation rates in the Fe amended treatments (Fig. 3-2).

A fundamental difference between our observations and results from previous laboratory studies is our use of natural seawater amended with only one nutrient at a time; most laboratory experiments use growth media that has been amended with a full suite of inorganic nutrients and trace metals. Thus, our observed lack of physiological enhancement under P or Fe enriched conditions could be explained by limitation of some other micronutrient. *Trichodesmium* isolates grown in amended seawater under more realistic nutrient concentrations have shown a negative response to $pCO_2$ (Shi et al. 2012) suggesting that the chemistry of the media used plays a key role when studying the response of *Trichodesmium* to OA.

As with P and Fe manipulations, changes in the daily light flux did not affect the response of *Trichodesmium* $N_2$ fixation to elevated $pCO_2$ (Fig. 3-4). These results contrast with laboratory experiments showing that $CO_2$ has a stronger effect on *Trichodesmium* $N_2$ fixation under low light conditions (Kranz et al. 2010, Garcia et al. 2011). However, direct comparisons between our study and these previous studies are difficult due to differences in the quantity and quality of light provided. In the laboratory, cultures were grown under a 12:12 light/dark cycle using white light; low light conditions were 38 (Garcia et al. 2011) and 50 μmol photons $m^{-2}$ $s^{-1}$ (Kranz et al. 2010). In contrast, our incubations took place under natural sunlight (sinusoidal cycle with clouds) with blue screening and neutral density filters for bottles being shaded. The mean daily surface irradiance (photon flux) was 975 μmol photons $m^{-2}$ $s^{-1}$, making the average light level in our lowest (8%) light treatment approximately 78 μmol photons $m^{-2}$ $s^{-1}$. Though this intensity is higher than the low-light conditions used by Kranz et al. (2010) and Garcia et al. (2011), it is still well below the light half-saturation constant reported for natural *Trichodesmium* colonies of ~300 μmol photons $m^{-2}$ $s^{-1}$ (Kana 1992). However, because we utilized a mixture of blue and neutral density screens while Kranz et al. (2010) and Garcia et al. (2011) used white light, the photosynthetically usable radiation in those earlier studies may be substantially lower than in ours. Although our study found that light was positively correlated to $N_2$ (Fig. 3-4) and C fixation rates (data not shown), the response of *Trichodesmium* $N_2$ fixation

to elevated $pCO_2$ was not affected by the daily flux of light the colonies received. At present we cannot discard the possibility that the light levels we used were insufficiently low to affect the response to $pCO_2$.

### *3.5.2 Methodological approaches*

Our results contrast not only with previous laboratory studies but also with several field experiments conducted on *Trichodesmium* colonies collected in the Subtropical Atlantic and the Gulf of Mexico. Lomas et al. (2012) found that elevating $CO_2$ increased $N_2$ fixation by *Trichodesmium* colonies in the majority of experiments run during two cruises at the Bermuda Atlantic Time-series site (BATS; 31°40'N, 64°10'W). Similarly, Hutchins et al. (2009) documented a 40-80% increase in $N_2$ fixation rates at elevated $CO_2$ during three experiments on a cruise off the West coast of Florida. Both studies used an experimental setup similar to that used for our study: *Trichodesmium* colonies were collected from a net tow, isolated, and incubated under a range of $pCO_2$. However, key differences in the experimental design, methodologies, and conditions between the present study and those of Lomas et al. (2012) and Hutchins et al. (2009) may explain the discrepancy in results.

First, differences in analytical methods complicate intercomparisons of the three *Trichodesmium* field studies. Our study used AR to measure $N_2$ fixation during the 2010 and 2011 cruises and $^{15}N_2$ uptake in 2012. Both methodologies are accepted by the scientific community (Capone and Montoya 2001), but they measure different processes: $^{15}N_2$ uptake measures the net assimilation of $N_2$ into particulate matter (presumably living cells), whereas AR measures nitrogenase activity, a measurement often equated to gross $N_2$ fixation (Montoya et al. 1996). Hutchins et al. (2009) found that the response of *Trichodesmium* depended on the method used: elevating $pCO_2$ increased $^{15}N_2$ uptake rates by 40-80%, but did not affect AR rates (Garcia et al. 2011). These results can be explained by a reduction of the $CO_2$ enhancement of gross *Trichodesmium* $N_2$ fixation rates at high light (Kranz et al. 2010), perhaps due to changes in cellular N retention (Garcia et al. 2011). In our study, all AR experiments

used high light conditions for incubations (~50% incoming irradiance), which could explain the lack of response of $N_2$ fixation to elevated $pCO_2$. However, even when based on rates of $^{15}N_2$ uptake in August 2012, the increase in $pCO_2$ did not significantly affect the resulting rates of $N_2$ fixation (Fig. 3-3).

Another difference between our study and previous field studies is the method used to normalize $N_2$ fixation rates. Typically rates are normalized volumetrically when assessing $N_2$ fixation rates in whole water samples. However, when isolating and concentrating *Trichodesmium* colonies, it is useful to normalize to a biomass indicator due to potentially large differences in colony size. While Hutchins et al. (2009) normalized $^{15}N_2$ uptake rates volumetrically, Lomas et al. (2012) used two normalization methods producing slightly different results: elevated $CO_2$ enhanced $N_2$ fixation colony$^{-1}$ in 9 out of 9 experiments but enhanced $N_2$ fixation colony $C^{-1}$ in 7 out of 9 experiments. In these experiments, Lomas et al. (2012) normalized rates per unit colony and reported a size range of 50-125 nmol N colony$^{-1}$. However, colony size in our experiments was extremely variable and incubations included a range of *Trichodesmium* morphologies and sizes, including loose tufts and large puffs. For this reason, rates in the present study are normalized to C content (for $^{15}N_2$ and C uptake experiments) or chlorophyll *a* content (for AR experiments). Each normalization strategy has its own limitations: while normalizing per unit colony likely increases the error associated with measurements due to variability in colony size, our C-based normalization method may be directly influenced by the $pCO_2$ treatments. These differences in the way we normalize rates make comparisons between studies challenging.

There are two other major differences between our study and previous field studies: the environmental nutrient concentrations and the level of sample replication. While we included separate P- and Fe-only amended treatments in several $CO_2$ manipulation experiments, Lomas et al. (2012) amended all treatments with both P and Fe. And, while Hutchins et al. (2009) did not amend treatments with nutrients, the ambient nutrient concentrations during experiments were not reported. In our study, P

and Fe amendments did not significantly affect the response of *Trichodesmium* $N_2$ or C fixation rates to elevated $pCO_2$. We also saw no evidence that *Trichodesmium* were P or Fe limited at ambient $pCO_2$, a condition which is not always the case in the NPSG (Watkins-Brandt et al. 2011, Chappell et al. 2012). Caution should be taken when interpreting the results of $CO_2$ manipulations combined with nutrient amendments, as high P and Fe concentrations are not characteristic of major *Trichodesmium* habitats (Capone et al. 1997).

Finally, differences in replication make it difficult to compare the present study to previous *Trichodesmium* field studies. We observed high variability in rates of both $N_2$ and C fixation between successive days, which is consistent with previous observations (Hutchins et al. 2009, Lomas et al. 2012). Moreover, we also observed high variability within the daily replicates: on average, the coefficient of variation in $N_2$ fixation rates for ambient $pCO_2$, ambient nutrient treatments was ~30%. Such high variability among replicate treatments likely overshadows any potential $CO_2$ related responses. While Lomas et al. (2012) showed that elevating $CO_2$ increased rates of $N_2$ fixation in all or most experiments (depending on the mode of normalization), the lack of sample replication does not allows us to assess to what extent these trends were statistically significant.

### *3.5.3 Diversity*

The relatively high coefficient of variation in rates of $N_2$ fixation among replicate treatments seemed to correlate with the morphological diversity of *Trichodesmium* colonies used (Table 3-4). Though morphology is not always an accurate predictor of *Trichodesmium* species (Hynes et al. 2012), certain *Trichodesmium* species tend to show particular morphotypes (Siddiqui et al. 1992); thus, diversity in colony morphology may provide some information on the diversity within the *Trichodesmium* assemblage. The lowest coefficient of variation for $N_2$ fixation rates (1.7%) was observed in March 2011. On this cruise, *Trichodesmium* colonies with tuft morphologies had higher overall $N_2$ fixation rates and showed a

greater response to elevated $pCO_2$ than colonies with puff morphologies (Fig. 3-5). Subsequent experiments during this cruise used only colonies with tuft morphologies and had a lower coefficient of variation. During August 2010, when no preference was given to a particular morphology, the coefficient of variation was greater (17.4%) than the coefficient of variation in March 2011 (1.7%), when only colonies with tuft morphologies were selected. Finally, the highest variation was observed in August 2012 (76.2%), when colonies of all sizes and forms, including loose tufts, were used for experiments due to the low *Trichodesmium* abundance observed in the field. It is conceivable that the high variability in rates observed in August 2012 reflects variability in the *Trichodesmium* species within incubation bottles.

In order to further explore the role that *Trichodesmium* diversity played in our observed $N_2$ and C fixation rates, we analyzed nucleic acid sequences from colonies isolated on the August 2012 cruise. These analyses provide evidence for genetic variability and its potential role in the variability in C and $N_2$ fixation rates observed on these cruises. The resulting *hetR* gene sequences revealed thirteen different groups of *Trichodesmium* with >99% sequence similarity; these groups clustered with three out of the four major *Trichodesmium* clades (Fig. 3-6). Interestingly, most of these sequences were >99% similar to isolates of two different *Trichodesmium* species that have been used in laboratory $CO_2$ manipulation experiments. Approximately 26% of *hetR* gene sequences grouped with *T. erythraeum* IMS101 and GBRTRLI101, strains that have both shown enhanced $N_2$ fixation rates at elevated $pCO_2$. However, the majority sequences (52% of total clones sequenced) grouped with the strain *T. thiebautii* H9-4 (Fig. 3-6). A recent laboratory study comparing the effect of $CO_2$ on $N_2$ fixation by several previously untested *Trichodesmium* isolates found that the response to $CO_2$ varies between isolates: $CO_2$ half saturation constants ($K_s$) ranged from 63-408 μatm, with the strain H9-4 having the lowest $K_s$ value of any strain tested. In contrast, strain IMS101 had one of the highest values measured in *Trichodesmium* (Hutchins, pers. comm.).

The dominance of *hetR* gene sequences clustering close to *T. thiebautii* H9-4 as well as the diversity of total *Trichodesmium* sequences can help explain our experimental results in the field. If the distribution of *hetR* gene sequences reflects the variability of *Trichodesmium* $CO_2$ $K_s$, the dominance of *hetR* gene sequences similar to H9-4 suggests low $CO_2$ $K_s$ in our samples. If our interpretation is correct, samples collected during August 2012 would have been dominated by *Trichodesmium* that is fully saturated at present $CO_2$ conditions; even the pre-industrial level $CO_2$ treatments (172 μatm) would have been significantly above the $CO_2$ $K_s$ reported by Hutchins et al. for H9-4.

Furthermore, the community did not consist of only H9-4, but included *Trichodesmium* resembling strain IMS101 and other phylotypes within Clades I, III, and IV of the major *Trichodesmium hetR* gene clades (Fig. 3-6). Unequal distribution of species into incubation bottles would contribute to the high variability we observed between replicates. Additionally, the differences in $N_2$ fixation rates observed between days may reflect changes in the community composition of *Trichodesmium* over space and time (Fig. 3-7). Nevertheless, a change in the dominant community can lead to a change in the mean $K_s$ observed in our samples and cause changes in $N_2$ fixation rates and response to $pCO_2$ perturbations.

It is relevant to note the diversity and metabolic capacities of organisms other than *Trichodesmium* found within colonies. Quantitative PCR showed that on August 9, 2012, *Trichodesmium* accounted for only 60% of *nifH* genes from organisms targeted in this study; this fraction increased to 73% on August 10 and 98% on August 12 (Fig. 3-8). The other major constituents were Group B, which includes *Crocosphaera* spp., and Het1, which includes *Richelia* spp. *Crocosphaera* likely did not greatly contribute to $N_2$ fixation rates, as the incubations were only during the light period and *Crocosphaera* fixes $N_2$ in the dark. We do not have data to show whether Het1 or the other *nifH* organisms (Het2, Het 3, Group A, and Cluster III) were actively fixing $N_2$ during the incubations. However, our data suggests that on August 9, organisms other than *Trichodesmium* may have contributed 40% of $N_2$ fixation. It

is also possible that our samples contained substantial fractions of other diazotrophs that we did not target with qPCR primers; thus, the percentages of *nifH* genes belonging to *Trichodesmium* and other groups presented in Figure 3-8 represent upper limits. Our incubations also contained organisms other than *Trichodesmium* with the ability to fix C; for instance, both Group B and Het1 are cyanobacteria. Additionally, of the clone library sequences from 16S primers, which targeted cyanobacteria, only 45% were *Trichodesmium* (Table 3-5). Though clone library sequences are not quantitatively reliable, this suggests that organisms other than *Trichodesmium* may have substantially contributed to C fixation during our experiments. In summary, it is important to consider that while our study targeted *Trichodesmium*, we measured community rates normalized to community biomass. Elevated $pCO_2$ did not enhance C or $N_2$ fixation rates by this community.

### *3.5.4 Ecological significance and future directions*

Our study found no significant response of *Trichodesmium* C or $N_2$ fixation rates to elevated $pCO_2$ during three cruises to Station ALOHA in the NPSG. We also observed a large variability in rates between replicate samples: the average coefficient of variation for ambient $pCO_2$, ambient nutrient condition $N_2$ fixation rate measurements averaged ~30%. Our results suggest that the biological diversity within our samples is responsible for both the lack of $CO_2$ enhancement and the overall variability in measured rates. Previous studies have shown convincing evidence for a $pCO_2$ enhancement of $N_2$ and C fixation by *T. erythraeum* IMS101 and GBRTRLI101, at least under nutrient replete laboratory conditions (e Ramos et al. 2007, Hutchins et al. 2007, Levitan et al. 2007, Kranz et al. 2009). However, our genetic analysis shows that this species was a small minority of the *Trichodesmium* assemblage in our experiments. Rather, the majority of *Trichodesmium* in August 2012 were closely related to *T. thiebautii* strain H9-4, a strain that shows no response to elevated $pCO_2$ (Hutchins, pers. comm.), and the relative abundance of different *Trichodesmium* clades changed between days. The biological diversity extends beyond

*Trichodesmium*: our *nifH* gene analysis revealed a diverse assemblage of diazotrophs associated with *Trichodesmium* colonies. It is possible that there was a slight enhancement of $N_2$ and/or C fixation by individual elements of this diverse community that our experimental setup was not able to capture. To fully disentangle diversity from a possible $pCO_2$ enhancement, future experiments would need to either use large sample sizes and more replication or target rates by certain groups of organisms, perhaps with molecular tags.

Our overall results show no evidence that community $N_2$ fixation will increase with OA. These results are in agreement with two independent studies of whole diazotrophic communities in the North and South Pacific Gyres, which have observed no increase in $N_2$ fixation with elevated $pCO_2$ (Law et al. 2012 , Böttjer, in prep.). Globally, *T. thiebautii* is often more abundant than *T. erythraeum* (Marumo and Nagasawa 1976, Carpenter and Price 1977); thus, the lack of $pCO_2$ enhancement observed in our study may represent the response of diazotrophic communities in many ocean regions. One important question that our methods were not able to address is how OA will impact diazotrophic communities on longer timescales. It is possible that natural selection will favor certain species, such as *T. erythraeum*, which are able to increase growth rates with elevated $pCO_2$. Future experiments addressing the species-specific responses to $pCO_2$, the environmental regulation of these responses, and the possibility for natural selection over long timescales are needed in order to predict the effect that future OA will have on microbial processes.

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**Table 3-1:** Locations and ambient environmental conditions of seawater from which *Trichodesmium* colonies were collected. Sea surface temperature (SST) and chlorophyll fluorescence at 25 m depth (Chl) are taken for the CTD sensor. SRP values are from discrete samples at 25m taken within one day of the net tow. ND = no data available.

| Date | Location | Collection Depth | SST (°C) | Chl (μg $L^{-1}$) | SRP (nM $PO_4$) | Rate / DNA measurements |
|---|---|---|---|---|---|---|
| 8/22/10 | 22° 44.958° N, 157° 59.866' W | 0-10m | 25.75 | 0.13 | 51 | AR |
| 8/24/10 | 24° 3.666' N 157° 49.824 W | 0-10m | 25.85 | 0.08 | 94 | AR |
| 8/26/10 | 24°40.214' N, 160°52.028' W | 0-10m | 26.19 | 0.30 | 18 | AR |
| 3/14/11 | 22°46.393' N, 158° 1.501' W | 0-10m | 24.55 | 0.13 | 96 | AR |
| 3/15/11 | 22°45.006' N, 158° 0.002' W | 0-10m | 24.5 | 0.10 | 102 | AR |
| 3/16/11 | 22°45.005' N, 157° 57.892' W | 0-10m | 24.42 | 0.13 | ND | AR |
| 3/20/11 | 22°45.015' N, 158° 0.003' W | 0-10m | 24.22 | 0.15 | 72 | AR |
| 8/8/12 | 22° 48.848' N, 158° 1.927' W | 0-10m | 25.46 | ND | ND | $^{13}C$,$^{15}N$ |
| 8/9/12 | 22° 49.022'N, 158° 2.495'W | 0-10m | 25.5 | ND | ND | $^{13}C$,$^{15}N$, DNA |
| 8/10/12 | 22° 48.995'N, 158° 1.710W | 0-10m | 25.57 | ND | ND | $^{13}C$,$^{15}N$, DNA |
| 8/11/12 | 22° 49.003'N, 158° 2.504'W | 0-10m | 25.68 | ND | ND | $^{13}C$,$^{15}N$ |
| 8/12/12 | 22° 49.012'N, 158° 2.508'W | 0-10m | 25.71 | ND | ND | DNA |

**Table 3-2**: Average $pCO_2$ values for all treatments on all cruises. Values are average $pCO_2$ on days in which experiments were run; standard deviation between days are given in parentheses.

| | | Desired $pCO_2$ treatment | | | | | |
|---|---|---|---|---|---|---|---|
| Cruise | Method | 150 | 280 | 400 | 750 | 1000 | 2000 |
| August 2010 | Acid/base addition | - | - | 416 | 728 | 1000 | 1672 |
| March 2011 | Acid/base addition | - | - | 419 (45) | 734 (20) | 1064 (101) | 1465 (172) |
| August 2012 | Gas bubbling | 177 (7) | 272 | 366 (4) | 639 | 812 (0.5) | - |

**Table 3-3**: Summary of primers used for PCR amplification of 16S rRNA, ITS, *hetR*, and *nifH* genes.

| Genetic marker | | Primers | Primer targets | Sequence (5'-3') | References |
|---|---|---|---|---|---|
| 16S | For | CYA-106F | cyanobacteria | CGGACGGGTGAGTAACGCGTGA | Nübel et al. (1997) |
| 16S | Rev | CYA-781R | cyanobacteria | GACTACTGGGGTATCTAATCCCATT | Nübel et al. (1997) |
| ITS | For | tri16S-1247F | *Trichodesmium* | CGTACTACAATGGTTGGG | Orcutt et al. (2002) |
| ITS | Rev | tri-23SR | *Trichodesmium* | TTCGCTCACCGCTACA | Hynes et al. (2012) |
| *hetR* | For | PH1 | heterocyst differentiation gene | TGYGCKATTTAYATGACCTA | Lundgren et al. (2005) |
| *hetR* | Rev | PH2 | heterocyst differentiation gene | ATGAANGGTATKCCCCAAGGA | Lundgren et al. (2005) |
| *nifH* (outer) | For | nifH3 | Nitrogenase reductase gene | ATRTTRTTNGCNGCRTA | Zani et al. (2000) |
| *nifH* (outer) | Rev | nifH4 | Nitrogenase reductase gene | TTYTAYGGNAARGGN | Zani et al. (2000) |
| *nifH* (inner) | For | nifH1 | Nitrogenase reductase gene | TGYGAYCCNAARGCNGA | Zehr and McReynolds (1989) |
| *nifH* (inner) | Rev | nifH2 | Nitrogenase reductase gene | ADNGCC ATCATYTCNCC | Zehr and McReynolds (1989) |

**Table 3-4:** *Trichodesmium* colony morphology and cruise-averaged $N_2$ fixation rates and coefficients of variation. Rates and coefficients of variation are means of ambient $pCO_2$, ambient nutrient condition treatments from each day an experiment was conducted. Standard deviations are given in parentheses.

| Cruise | *Trichodesmium* morphology | $^{15}N_2$ assimilation (nmol N μmol $C^{-1}$ $d^{-1}$) | Acetylene reduction (nmol $C_2H_4$ μg chl $a^{-1}$ $hr^{-1}$) | Daily coefficient of variation |
|---|---|---|---|---|
| August 2010 | Colonial | - | 42.5 (52.8) | 17.4% |
| March 2011 | Tufts only | - | 30.2 (1.0) | 1.7% |
| August 2012 | Small puffs and loose tufts | 1.53 (1.04) | - | 76.2% |

**Table 3-5:** Summary of results from sequencing *hetR*, 16S, ITS, and *nifH* gene clone libraries. The total number of sequenced clones are given by "n"; these clones are further segregated into the four *Trichodesmium* clades (see Fig. 3-6) and other organisms associated with *Trichodesmium* colonies.

| Gene or region | n | Total *Trichodesmium* | *Trichodesmium* Clade I | *Trichodesmium* Clade III | *Trichodesmium* Clade IV | Associated organisms |
|---|---|---|---|---|---|---|
| *hetR* | 250 | 181 | 132 | 49 | 3 | 66 |
| 16S | 93 | 42 | 27 | 15 | 0 | 51 |
| ITS | 117 | 114 | 99 | 15 | 0 | 3 |
| *nifH* | 93 | 16 | 16 | 0 | 0 | 77 |

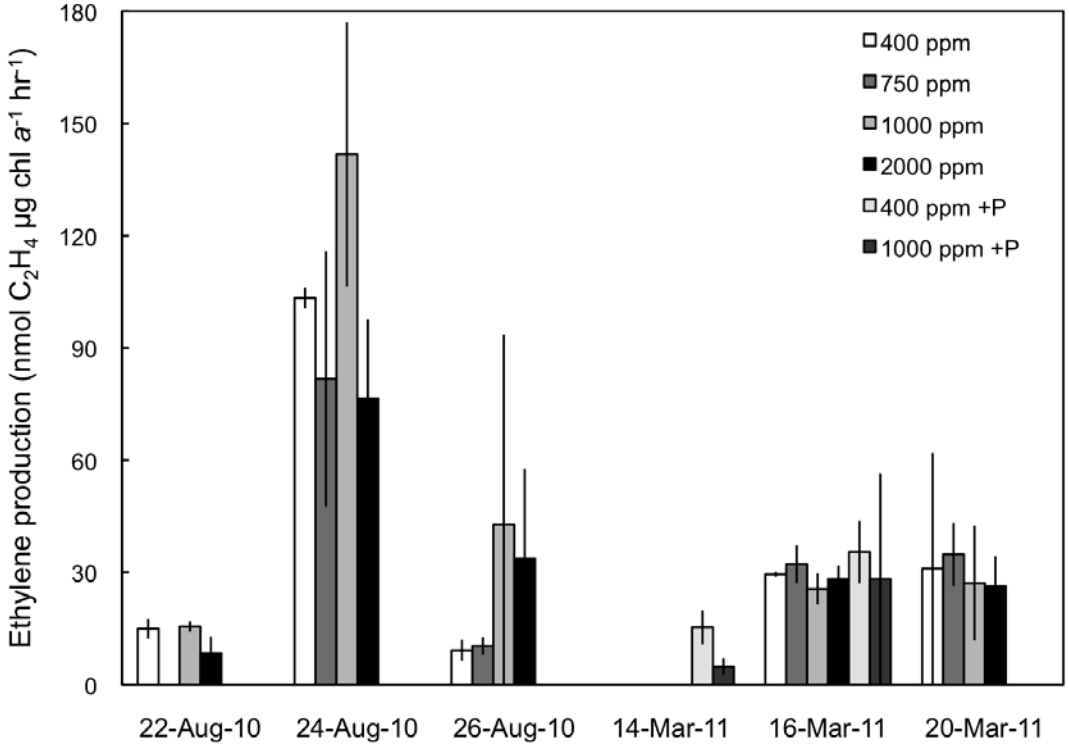


**Figure 3-1:** Acetylene reduction rates (as a proxy for $N_2$ fixation) by *Trichodesmium* colonies in six independent incubation experiments from August 2010 and March 2011 cruises. Error bars represent standard deviations of duplicate or triplicate bottles.

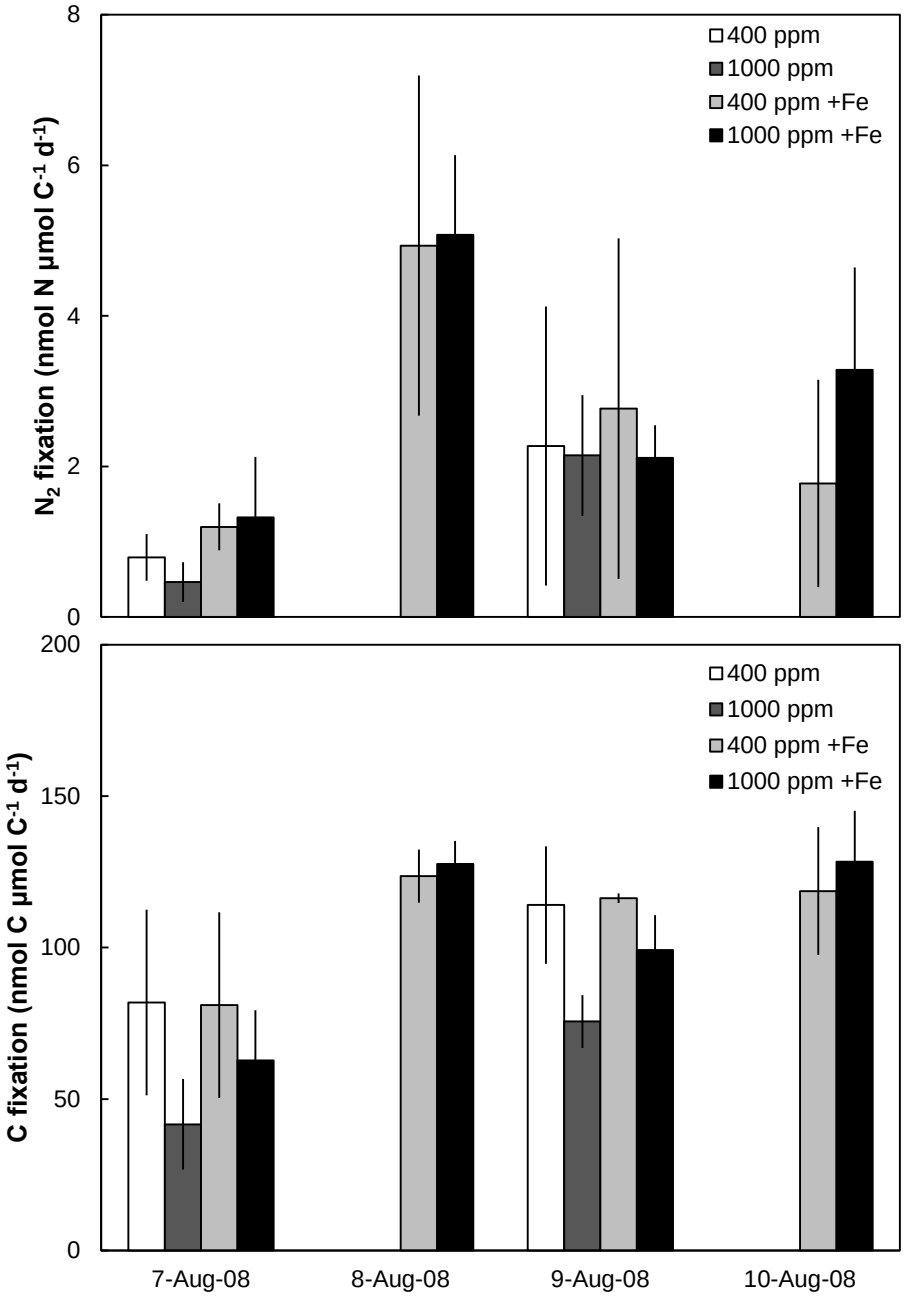


**Figure 3-2:** Rates of $N_2$ (top) and C fixation (bottom) by *Trichodesmium* colonies under ambient and elevated $pCO_2$ conditions with and without Fe amendments. Rates are from $^{15}N$ and $^{13}C$ assimilation on the August 2012 cruise. Error bars represent standard deviations of triplicate incubation bottles.

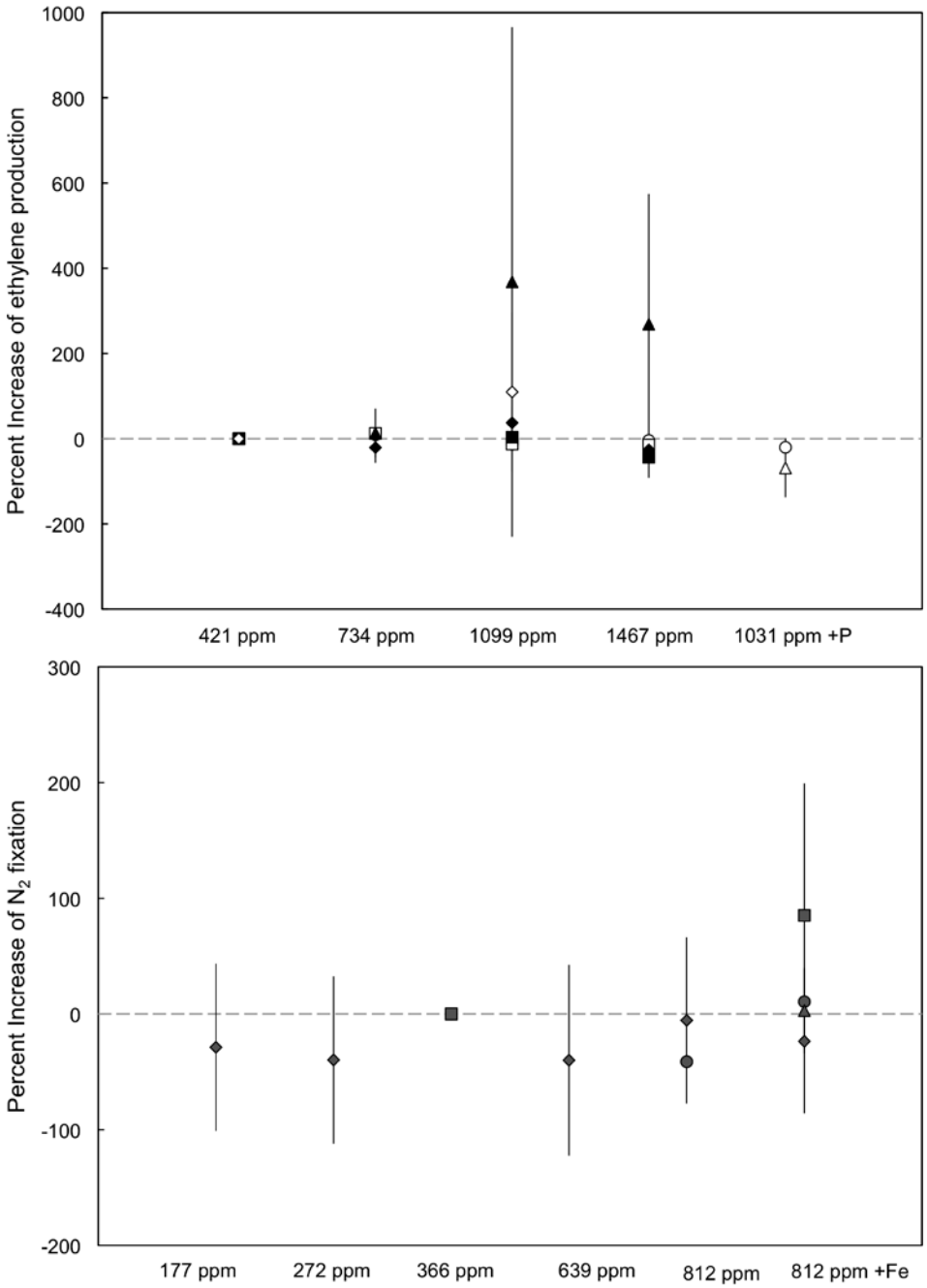


**Figure 3-3:** Effect of $CO_2$ and nutrient manipulations on *Trichodesmium* $N_2$ fixation rates (acetylene reduction, top; $^{15}N_2$ assimilation bottom). Top: August 2010 = white, March 2011 = dark, different shapes represent different days within cruises. Bottom: different shapes represent different days in March 2012. Rates are expressed as percentage increase compared to ambient $pCO_2$ (366 pm or 421 µatm). Fe and P replete, elevated $pCO_2$ treatments are compared to ambient $pCO_2$, Fe or P replete treatments. Error bars represent propagated standard deviations of duplicate or triplicate incubation bottles.

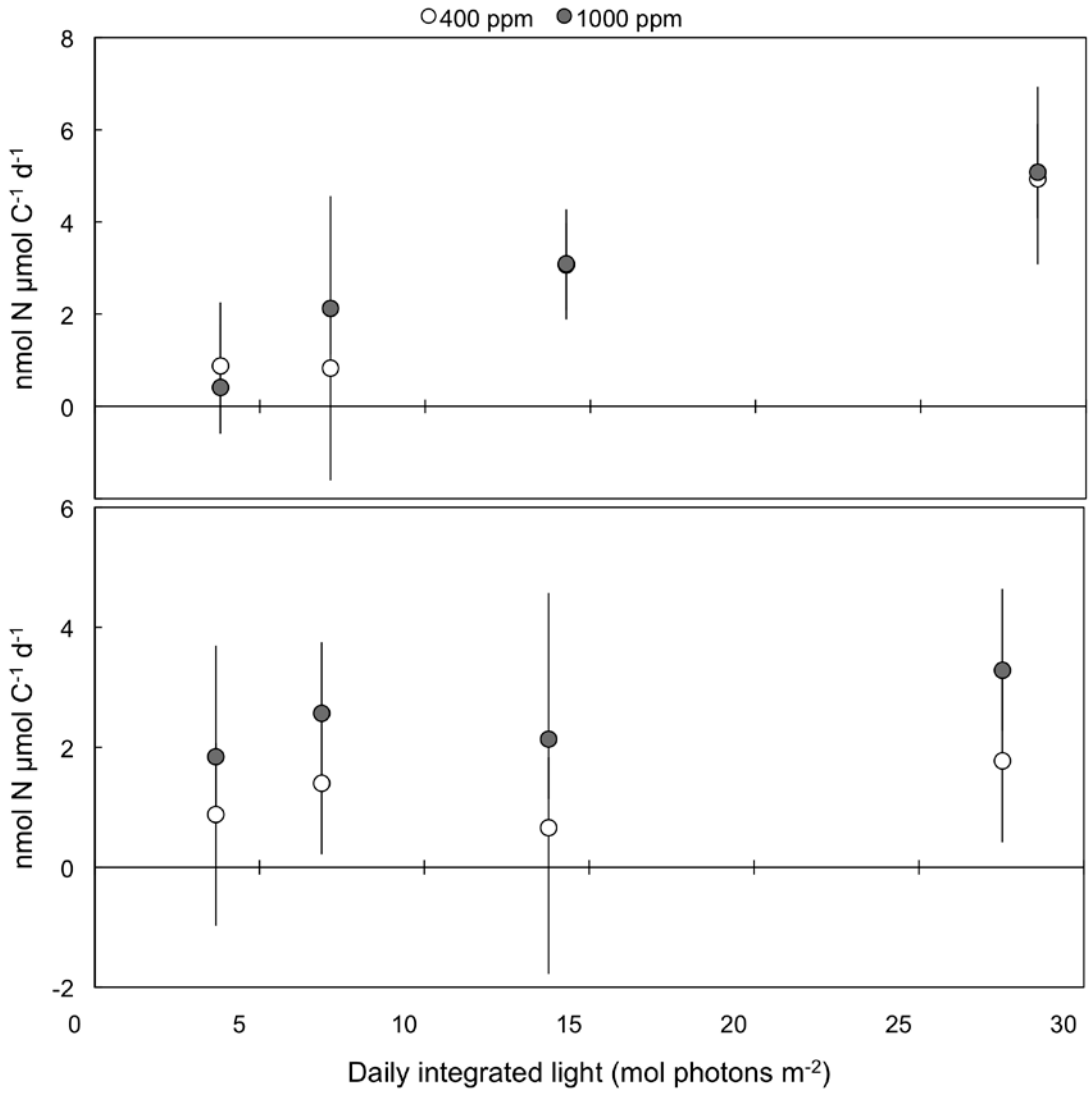


**Figure 3-4:** *Trichodesmium* $N_2$ fixation rates at ambient (366 µatm, light circles) and elevated (812 µatm, dark circles) $pCO_2$. All treatments were P and Fe replete. Top and bottom show two independent trials on August 9 and August 11, respectively. Error bars represent standard deviations of triplicate incubation bottles.

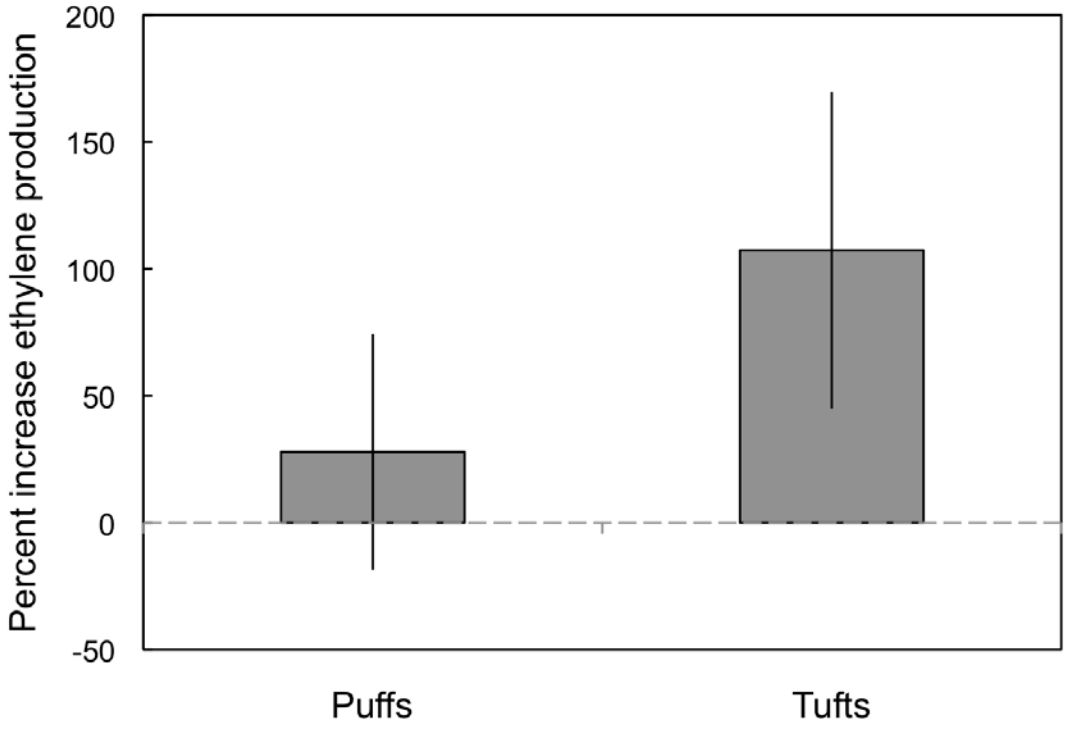


**Figure 3-5:** Effect of elevated $pCO_2$ (1099 μatm) on rates of ethylene production from acetylene reduction (proxy for $N_2$ fixation) by *Trichodesmium* colonies with "puff" and "tuft" morphologies. Rates are shown as percentage increase compared to ambient $pCO_2$. Error bars represent propagated error from linear regression. Duplicate incubation bottles were used for all except the 1099 μatm puffs treatment, which had a single bottle.

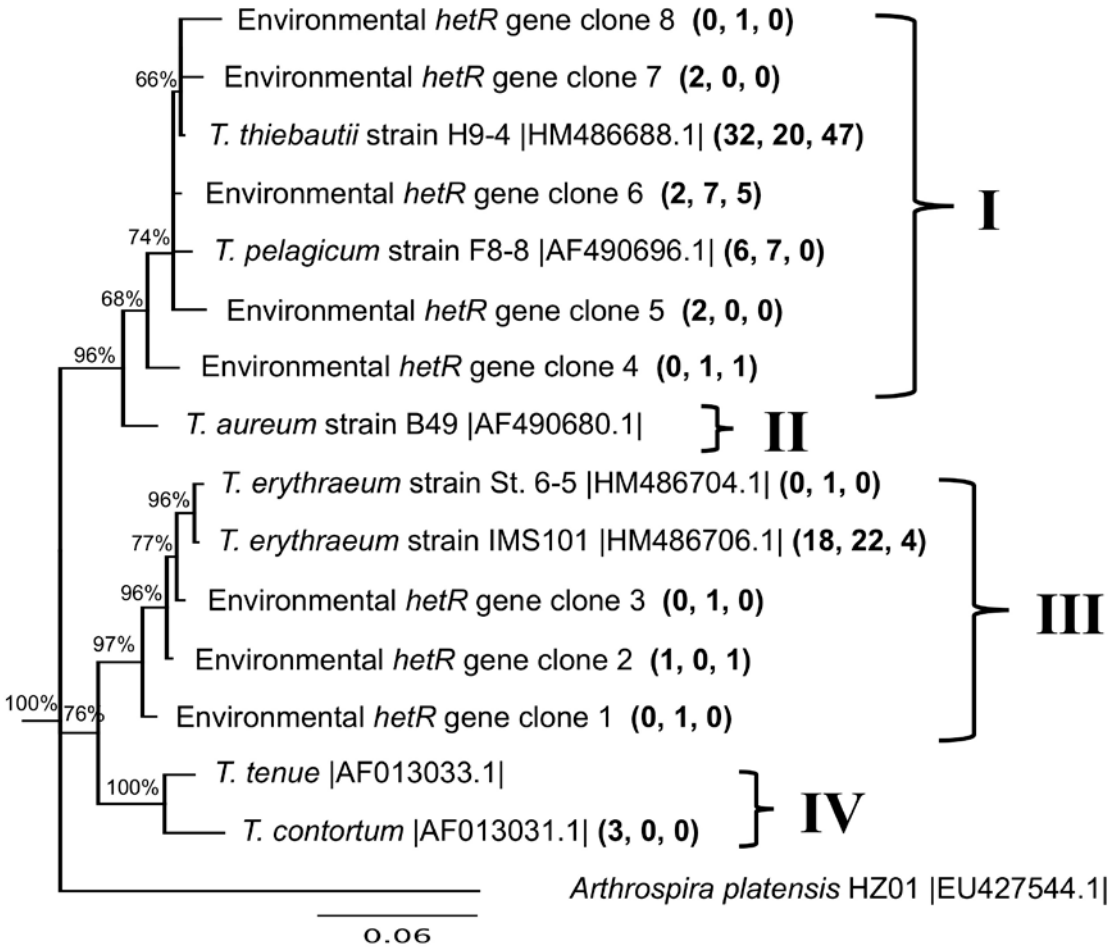


**Figure 3-6:** Neighbor-joining phylogenetic tree depicting the relationships between *Trichodesmium hetR* gene sequences from our environmental clones and cultured representatives from GenBank (accession numbers in brackets). Sequences >99% identical DNA were grouped together. Phylogenic relationships were bootstrapped 1000 times, and bootstrap values >50% are shown. Numbers in parentheses indicate the number of clones from 8/9/12, 8/10/12, and 8/12/12, respectively. The four major clades of *Trichodesmium* phylotypes (Lundgren et al. 2005) are shown in roman numerals.

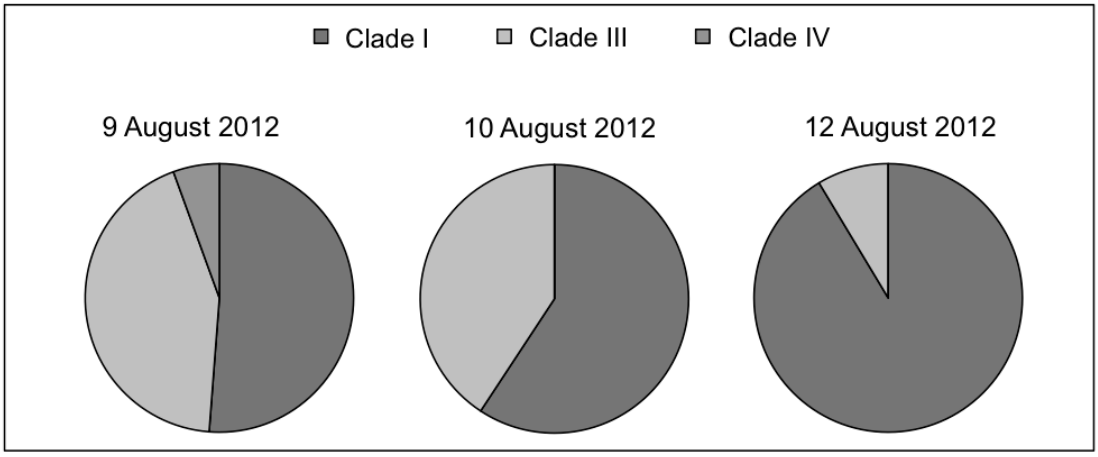


**Figure 3-7:** Major clades of *Trichodesmium* field colonies over a four-day period. Clade designations are based on *hetR* gene clone library sequences compared to sequences from GenBank. n=184 total *hetR* clone sequences.

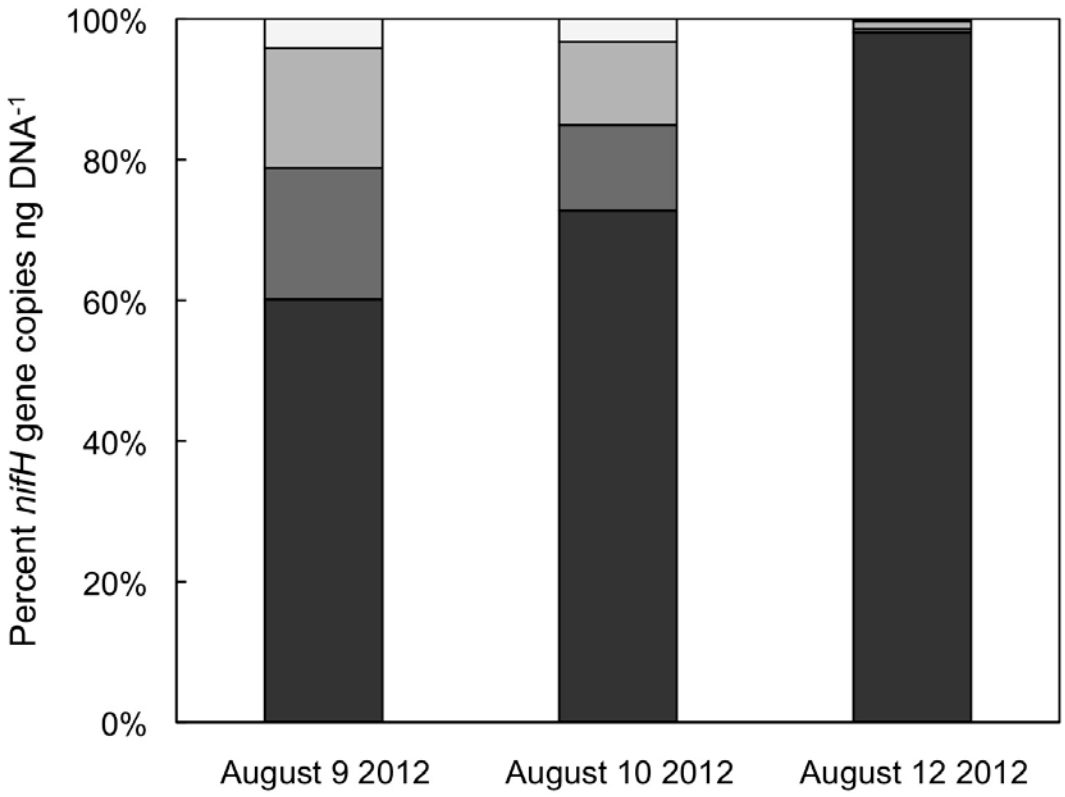


**Figure 3-8:** *nifH* gene copies from phylotypes corresponding to, in order from bottom to top: *Trichodesmium* (bottom), Group B, Het 1, and a combination of Group A, Het 2, Het 3, and Cluster III (top). Values are normalized to the total DNA extracted from each filter and presented as percentages of the total combination of all phylotypes.

# 4. GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

$N_2$ fixation is a keystone process in open ocean ecosystems, where it can provide half of the new N supporting primary productivity and C export. Some recent studies suggest that certain species of $N_2$-fixing cyanobacteria may be limited by the availability of $CO_2$ at current atmospheric levels. If future OA significantly increases community rates of $N_2$ fixation, the extra input of fixed N could fertilize open ocean ecosystems and lead to increased C drawdown by phytoplankton. Yet at this point, the overall effect of $pCO_2$ on marine community $N_2$ fixation remains unclear. This problem is challenging to address experimentally because of species-specific responses in laboratory studies and the variable environmental conditions, genetics, morphology, and physiology of natural open-ocean diazotrophic communities. The aim of this thesis was to expand our knowledge of how OA affects diazotrophs and how this response might be modulated by environmental conditions through a combination of laboratory and field experiments.

Several conclusions can be drawn from this research. Chapter two showed that enhanced $pCO_2$ increased the growth, N accumulation, and C accumulation by a *Crocosphaera* isolate under laboratory conditions. Monitoring cultures over the diurnal cycle showed that *Crocosphaera* has a distinct daily pattern of C and N accumulation. $pCO_2$ appears to change the diurnal pattern of N accumulation in *Crocosphaera*, with a greater portion of N accumulation occurring earlier in the dark period under elevated $pCO_2$ conditions. Additionally, active growth under the large daily $pCO_2$ fluctuations within treatments speaks to the physiological tolerance of this organism. Yet overall, it is not known how well the response of *Crocosphaera* isolate WH8501 to enhanced $pCO_2$ reflects the future response of natural diazotrophic communities to OA.

In chapter three, we presented data from experiments performed during three cruises at Station ALOHA. During these cruises, we observed no evidence that $pCO_2$ enhanced $N_2$ or C fixation by the community of *Trichodesmium* and associated organisms. This response did not change when seawater was amended with P or Fe, or

when incubations were carried out at lowered light levels. Sequences of *Trichodesmium* colonies from August 2012 provide convincing evidence that the diversity within our samples is the underlying reason for the lack of an observed $pCO_2$ enhancement of C and $N_2$ fixation rates. Our samples contained a diverse community of *Trichodesmium* and other associated organisms, and the community was not dominated by the *Trichodesmium* species that has shown a positive response to $pCO_2$ in laboratory studies. Ultimately, our results suggest that diversity trumps acidification: in our experimental setup, and perhaps in some oceanic regions, the variability resulting from high biological diversity masked any possible $pCO_2$ enhancement of certain species within the diazotrophic assemblage.

The overall results from this thesis suggest that while enhanced $pCO_2$ stimulates elevated $N_2$ and C fixation by certain species under laboratory conditions, the effect of OA on natural diazotrophic communities is more nuanced. If there is a net enhancement of community $N_2$ fixation rates, it will likely be much smaller in magnitude than the enhancement observed in monocultures of *Trichodesmium* and *Crocosphaera*. Another possible consequence of OA is the selection for species that are able to take advantage of elevated $pCO_2$, such as *T. erythraeum*. Future studies should include long term laboratory experiments testing for the potential of natural selection, laboratory experiments testing the effects of $pCO_2$ on previously untested strains and groups of diazotrophs, and field experiments with larger sample sizes to decrease variability. Together, these approaches will help us understand how $N_2$ fixation in the open ocean may change with OA.

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