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Light Dependant Growth and Nitrogen Fixation Rates in the *Hemiaulus haukii* and *Hemiaulus membranaceus* Diatom-Diazotroph Associations

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Light Dependant Growth and Nitrogen Fixation Rates in the *Hemiaulus haukii* and *Hemiaulus membranaceus* Diatom-Diazotroph Associations

by

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Thesis

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Master of Science in Marine Science

The University of Texas at Austin

December 2011

Dedication

This thesis is dedicated to my parents: Stephen John Pyle III and Mary Ann Pyle. Without their never failing love and support, the work leading up to the culmination of this thesis, and the thesis itself would have been impossible. I would also like to extend a special thanks to my advisor, Dr. Tracy A. Villareal. My acceptance and attendance at the University of Texas Marine Science Institute would have been impossible without him. Thank you.

Acknowledgements

I would like to acknowledge the continued support of my advisor, Dr. Tracy A. Villareal. In addition, I extend a special thanks to my other committee members: Dr. Edward Buskey and Dr. Deana Erdner. I would like to thank past and present Villareal Lab members, especially Lynn Tinnin, Colbi Brown and Katie Swanson. They each spent a considerable amount of time showing me lab techniques and protocols, without which I would not have been able to complete my work. A special thanks is extended to Katie Swanson for her help in the nitrogen source experiment. I would like to thank the University of Texas at Austin and the University of Texas Marine Science Institute in Port Aransas for support and guidance. Special recognition must be paid to Dr. Tracy A. Villareal, Dr. Lee Fuiman, Katherine Quade and Patricia Webb. Their support ultimately allowed me the opportunity to complete this degree. The entire Marine Science Institute consistently showed kindness and support, for which I am grateful for. At this time I would like to thank Patricia Garlough and Sarah Wallace for their compassion and listening abilities. Thank you all.

Abstract

Light Dependant Growth and Nitrogen Fixation Rates in the *Hemiaulus haukii* and *Hemiaulus membranaceus* Diatom-Diazotroph Associations

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Nitrogen-fixation is an essential biochemical reaction involving the reduction of inert, atmospheric dinitrogen (N_2) into biochemically accessible ammonia (NH_3). Organisms that are capable of this process are collectively called "diazotrophs" and are ubiquitous in marine and terrestrial environments. Despite the wide distribution, little is known about the biological nature of the diverse groups of diazotrophs. This study was designed to address the influence of light and nutrients on nitrogen fixation and growth in several marine diazotrophic symbioses collectively termed "Diatom-Diazotroph Associations (DDAs)." The organisms of interest included the diatoms *Hemiaulus haukii* Grunow and *Hemiaulus membranaceus* Cleve, and their diazotrophic endosymbiont *Richelia intracellularis* Schmidt. The study included acetylene reduction assays, growth rate, and nutrient analysis experiments on both associations in order to better understand the similarities and differences within and between the two DDAs. The results indicate distinct differences in nitrogen fixation rates within and between the species. In the nitrogen addition experiment, the "no added nitrogen" treatment had the highest N_2-

fixation rate (N_2-fix_{max} = 7.43 x 10^{-5} nmols N_2 heterocyst^{-1}min^{-1}), followed by the added nitrate treatment (N_2-fix_{max} = 6.49 x 10^{-5} nmols N_2 heterocyst^{-1}min^{-1}) and the added ammonium treatment (N_2-fix_{max} = 3.79 x 10^{-5} nmols N_2 heterocyst^{-1}min^{-1}). The maximum growth rate occurred in the "added ammonium" treatment (0.42 divisions day^{-1}), which had a higher percentage of asymbiotic cells than the two other treatments. The maximum recorded rate of N_2-fixation for *H. haukii* was 7.43 x 10^{-5} nmol N_2 heterocyst^{-1}min^{-1} and the maximum value of N_2-fixation for *H. membranaceus* was 1.88 x 10^{-4} nmol N_2 heterocyst^{-1}min^{-1}. The maximum growth rate for *H. haukii* was 0.99 divisions day^{-1}, and 1.06 divisions day^{-1} for *H. membranaceus.* Growth followed light saturation kinetics in *H. haukii* with a compensation light intensity (I_C) of 10 µmol quanta m^{-2}sec^{-1} and saturation light intensity (I_K) of 100 µmol quanta m^{-2}sec^{-1}. *H. haukii* and *H.membranaceus* expressed light saturation kinetics in N_2-fixation. N_2-fixation was generally limited to the light period, with no evidence of a morning or evening enhancement. The DDAs grew solely on N_2-fixation and did not use nitrate. This study contributes to current knowledge of DDAs and their role in global marine nitrogen fixation.

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Chapter 1

Introduction: Nitrogen Fixation and Diazotrophs

Nitrogen fixation is an essential biochemical reaction involving the fixation of inert, atmospheric dinitrogen (N_2) into biologically accessible ammonia (NH_3). Organisms capable of biological nitrogen fixation are collectively called "diazotrophs," or literally, "eaters of nitrogen ", and are ubiquitous in both terrestrial and marine environments (Paerl 1996). Diazotrophic cyanobacteria are morphologically and physiologically diverse, and include both unicellular and filamentous forms (Carpenter et al.1992). Nitrogen fixation is energetically expensive and requires the metalloenzyme nitrogenase to break the dinitrogen triple bond (Capone et al. 1990). Nitrogenase is deactivated and degraded in the presence of oxygen; therefore, the O_2-evolving PSII photosynthetic reactions must be isolated from the N_2-fixation reaction (Capone et al. 1990; Sherman et al. 1998). Diazotrophs have adapted mechanisms to spatially and or temporally segregate the two reactions. *Richelia intracellularis*, (Fay 1968; Mague 1974), *Anabaena* sp. (Wolk & Shaffer 1976; Ernst 1984), and *Nodularia* sp. (Hubel & Hubel 1974) are diazotrophic species that spatially segregate N_2-fixation via morphologically specialized cells termed "heterocysts." Spatial segregation allows for simultaneous N_2-fixation and photosynthesis during the day. In the absence of structural segregation, non-heterocystous cyanobacteria fix nitrogen in the dark and use stored carbon produced during photosynthesis to fuel the reaction (Reddy et al. 1993; Colon-Lopez 1997; Taniuchi et al. 2008). *Trichodesmium* sp. is a unique and well-studied non-heterocystous cyanobacterium that is capable of N_2-fixation in daylight hours (Dugdale et al. 1961; Goering et al.1966; Taylor et al. 1973; Carpenter & McCarthy 1975; Capone et al. 1997).

Despite extensive studies on heterocystous and non-heterocystous forms, little is known about the mechanisms that regulate growth and nitrogen fixation in diatom-diazotroph associations (DDAs). DDAs include a host diatom and an exo- or endo-

symbiotic cyanobacterial diazotroph. This study will investigate whether there are predictable changes of DDAs in response to light and nutrients, and their relationship to nitrogen fixation and growth. This study provides information on the autocology of two diatom-diazotroph associations (DDAs) isolated from the Gulf of Mexico. It will provide the first information on their growth rates and N_2-fixation rates in lab cultures necessary for understanding more about these globally occurring nitrogen-fixers. An understanding of changes in response to light, nutrients and the effect of ambient nitrogen on nitrogen-fixation is important for understanding the timing and rate of marine nitrogen fixation in specific diazotrophic species and is relevant to measuring the global nitrogen cycle. Because little is known about the biology of these associations, field methodologies may not be addressing or encompassing physical and biological changes which regulate nitrogen fixation and growth. This may lead to potential over- or underestimation of the rate of nitrogen fixation and is important for an understanding of how observed distributions are linked to quantifiable light-rate relationships. Such an understanding is vital because of current recognition of the contribution of DDA nitrogen-fixation to nitrogen supply in ambient waters (Zeev et al. 2008). The significance of marine nitrogen fixation in the global nitrogen budget is becoming increasingly apparent (Mahaffey et al. 2005); as such, field measurements need to be timed reflect temporal variation in rate processes. This study provides information on the autocology and basic biological characteristics of DDAs, which are the least-understood major diazotrophic group. An understanding of the biology of the diazotrophs is required in order to understand how their distribution, growth and N_2-fixation rates are controlled by light and nutrients.

Diazotrophs

There are four diazotrophic functional groups: non-heterocystous unicellular, non-heterocystous filamentous, heterocystous, and the heterocystous forms associated with diatoms (Diatom-Diazotroph Associations). Species specific N_2-fixation rates from the four functional groups are provided in Table 3.

Each group has unique characteristics and it is important to include information on other diazotrophic groups in order to better understand the function of DDAs. However, the focus of this study is the DDA associations including the diatom host *Hemiaulus haukii* and *Hemiaulus membranaceus* and the endosymbiont *Richelia intracellularis.* One reason there is little knowledge about these unique associations has been the inability to maintain them in culture for an extended period of time. This is the first study that examines the *Hemiaulus* spp. DDAs responses to light, growth and nitrogen-fixation.

Non-Heterocystous Diazotrophs

Non-heterocystous, diazotrophic cyanobacteria are ubiquitous in marine and terrestrial environments. Marine, unicellular forms include (but are not limited to) *Crocosphaera watsonii*, *Cyanothece* spp., and *Gloeothece* spp. The marine, filamentous forms include *Trichodesmium* spp. Almost all unicellular and filamentous forms employ diel periodicity to temporally segregate nitrogen fixation at night and photosynthesis during the day. The exception to this is the marine cyanobacteria *Trichodesmium* spp. (Capone et al. 1997), which is well documented to fix nitrogen during the day and will be discussed in detail later. Mohr et al. (2010) presented nitrogen-fixation periodicity in the unicellular diazotroph *Crocosphera watsonii* WH8501. Physiological and gene expression analysis provided clear evidence of temporal separation of N_2-fixation and photosynthesis (Figure 3), with peak N_2-fixation occurring at night, and peak photosynthesis at mid-day.

The genes responsible for the *nif* operon and nitrogenase transcription exhibited peak expression levels during the dark period (Mohr et al. 2010; Figure 4) suggesting that variation in nitrogenase abundance and activity is regulated by endogenous, rather than exogenous environmental factors such as light and temperature (Huang & Chow 1986). In unicellular diazotrophs such as *Crocosphaera watsonii*, nitrogenase is synthesized on a daily basis prior to peak expression at night, and subsequently degraded with the onset of light (Mohr et al. 2010). In *Cyanothece* sp. ATCC 51142, the *nifHDK* operon is

transcribed and activated in the dark and degraded with the onset of light. It was found that 60% of the genes associated with N_2-fixation demonstrated a circadian rhythm independent of the light level (Toepel et al. 2008). When exposed to continuous light, N_2-fixation continued to exhibit the periodicity present in a 12:12 L:D period, albeit with lower intensity (Figure 5). It is possible that these data indicate that N_2-fixation may also proceed in a circadian manner and is not restricted to periods of dark or light; however, it is important to note that nitrogenase gene expression is not equivalent to nitrogen fixation. In *Gloethece* sp. 68DGA there is a daily cycle of synthesis and degradation of nitrogenase, and nitrogenase activity ceases with initiation of the light period (Colon-Lopez et al. 1997; Tanuchi et al. 2008). In addition, photosynthesis, respiration, and the Fe-protein complex of nitrogenase exhibited the same diurnal pattern, which declined and eventually terminated after exposure to continuous light (CL). After 23 days of continuous light, 94% of cells expressed the Fe-protein nitrogenase complex at all times of the day (Figure 6; Tanuchi et al. 2008). This indicates that under CL, diel oscillation in nitrogenase synthesis all but ceases; however, upon return to LD conditions, the pattern returns. The authors concluded that nitrogen fixation in *Gloethece* sp. 68DGA is primarily, but not entirely, controlled by an endogenous, but not circadian, rhythm.

***Trichodesmium* sp.**

Saino and Hattori (1978) documented a 200-fold difference in ethylene evolution between *Trichodesmium thiebautii* colonies collected in the day, and those collected in the night (Table 1). Nitrogenase activity (NA) "turns on" in the early morning, and "turns off" near dusk (Capone et al. 1990). Subsequent work on *T. theibautii* (Wyman et al. 1996; Mullholand et al. 2006) and *T. erythraeum* (Chen et al. 1996, 98; Berman-Frank et al. 2001) shows nitrogen fixation in these diazotrophs is light dependent, and thus exogenously regulated (Zehr et al. 1998, Zehr & Turner 2001; Church et al. 2005). This is in sharp contrast to the trends explored in the studies on non-heterocystous diazotrophs mentioned above, in that nitrogenase activity occurs in the day and ceases at night.

Heterocystous Diazotrophs

Heterocystous cyanobacteria fix nitrogen during the day via maintenance of an anaerobic microenvironment within the specialized heterocyst cells. Heterocyst cells spatially shield nitrogenase from oxygenic degradation (Wolk & Shaffer 1976; Wolk 1999). Simultaneous N_2-fixation and photosynthesis ensures a supply of light to fuel the energetically expensive breakdown of dinitrogen. Ernst et al. (1984) demonstrated a correlation between nitrogenase activity and photosynthesis in *Anabaena variabilis* (Figure 7) and concluded that light mediated the reductant supply in heterocysts to fuel nitrogenase activity. In heterocystous species, the energetically expensive process of nitrogen fixation is tightly coupled to ATP-synthesizing and reducing photosynthesis (Vanderhoef et al. 1975; Paerl 1979; Ernst et al. 1984; Levine & Lewis 1984). Whether there is diurnal variation in the rate of N_2-fixation remains enigmatic. Levine and Lewis (1984) concluded that their data did not indicate temporal variation in the rate of N_2-fixation during the light period. After the initial peak of N_2-fixation following the onset of light, the rate remained relatively constant until the decline at dusk (Figure 8).

Nitrogen fixation rates in *Trichodesmium* and heterocystous diazotrophs are highly light dependent, with exogenous controls on activity working in conjunction with endogenous, circadian rhythms (Capone et al. 1990; Colon-Lopez et al. 1997). Nitrogenase gene expression is temporally decoupled from actual nitrogen fixation, and maximum gene expression occurs prior to maximum fixation rates (Zehr et al. 2007; Foster et al. 2010). It is possible that *nifH* expression is regulated primarily by an endogenous rhythm; whereas, actual N_2-fixation activity is controlled by both a circadian rhythm and exogenous cues (light). Zehr et al. (2007) reports the presence of diel variability in three marine heterocystous phylotypes (het-1, het-2, het-3) and concludes that "culture or environmental conditions (light, nutrients, temperature) affect the daily cycle of nitrogenase gene expression and N_2-fixation. In recent years, N_2-fixation studies on heterocystous diazotrophs have increasingly focused on the temporal expression of nitrogenase and genetic regulation of this enzyme. Zehr et al. (2007) reported on diel

variation in nitrogenase expression from five groups of diazotrophs ubiquitous in the North Pacific Subtropical Gyre (NPSG; Table 2). The authors support the finding of earlier studies in the area (Church et al. 2005) and conclude that "there are different patterns of gene expression in the different cyanobacterial groups." These results do not specifically address diel variability in *Richelia* spp. N_2-fixation rates; rather, they provide patterns of gene expression. The symbiont *Calothrix rhizosoleniae* is morphologically and phylogenetically similar to *R. intracellularis*, and its isolation (from the *Chaetoceros* sp. host) and the subsequent 2010 study are valuable in understanding *R. intracellularis* N_2-fixation dynamics (Foster et. al 2010). Foster et al. (2010) found that *Calothrix rhizosoleniae* displayed clear diel variability in both nitrogenase gene expression and nitrogen fixation. *nifH* expression in both diel experiments (Figure 9) peaked in mid-morning, followed several hours later by a peak in N_2-fixation. Interestingly, in the first diel experiment (Figure 9a), nitrogenase gene expression occurred in the morning at 11:00, but peak N_2-fixation did not occur until 22:00 (just prior to lights out). This is significantly later than that other studies on heterocystous cyanobacteria that report peak N_2-fixation in the early to mid-afternoon (Ernst et. al. 1984; Levine & Lewis, 1984).

Nitrogen Fixation, Light, and Growth Rates

The temporal and spatial distribution of DDAs is of considerable interest because of their role in biogeochemical cycling and potential export of matter to depth (Scharak et al. 1999a,b). Currently, there is limited information on how the biology of the associations affects their temporal and spatial distribution. Laboratory studies on the associations are especially scarce due to the difficulties in isolating and maintaining DDAs in culture (Foster & Zehr 2006). Despite this, there is an increasing amount of literature on the distribution of these diazotrophs. *Hemiaulus* spp. has been described as a "common and ubiquitous" genus which co-dominates warm, oligotrophic seas during stratification periods (Villareal 1991). It is intuitive to assume that nitrogen fixation, fueled by light-limited photosynthesis, occurs at higher rates in surface waters. Montoya

(2004) depicts this in the oligotrophic Pacific Ocean. It is important to note that nitrogen fixation is not exclusively present in surface waters and has been measured at depths up to 60m from bulk water, mixed assemblage samples (Montoya, 2004; Figure 2). Villareal (1991) measured rates of N_2-fixation from *H. haukii, H. membranaceus,* and *H. sinensis* in the southwestern Atlantic and found 21-45 times more N_2-fixation was associated with *Hemiaulus* spp. than with *Rhizosolenia* spp. A combined sample of *H. haukii* and *H. sinensis* had an N_2-fixation rate of 1.25-2.2 x 10^{-6} nmol N_2 $cell^{-1}min^{-1}$. *H. membranaceus* had a rate of 9.58 x 10^{-7} to 1.5 x 10^{-6} nmols N_2 $cell^{-1}min^{-1}$. In a later study, Villareal (1994) confirmed earlier observations of the tropical distribution of *Hemiaulus.* He reported an estimated 98% of observed cells had *Richelia* symbionts, which were located in the host's intracellular space in close proximity to the chloroplasts (Villareal 1991, 1994). It is likely that the proximity to the chloroplasts is a physiological mechanism used to satisfy the high energetic demands of N_2-fixation (Villareal 1992, Wolk et al. 1994, Janson et al. 1999). The high percentage of symbioses (+98%) indicates that the symbiont is an important part of the biology of the host diatom. Despite this, the relationship was non-obligate (Villareal 1994), and accelerated growth rates of a host may lead to an increase in asymbiotic cells because of the un-coupled division cycles. This is similar to observations of *Rhizosolenia-Richelia*, which also display un-coupled division (Villareal 1990).

One fundamental difference between the two DDAs is the high percentage of symbioses observed in *Hemiaulus* spp. The biological difference suggests a potential divergence in N_2-fixation strategies between the two associations (Villareal 1994). Studies in the tropical Atlantic Ocean provide insight into the potential impact of highly symbiotic DDA blooms. Carpenter et al. (1999) observed a *H. haukii* bloom which they estimated contributed 45 mg N/m^2/day to ambient waters. He concluded that N_2-fixation by *Richelia* exceeded the new nitrogen nitrate flux from depth. Scharak et al. (1999a) investigated the temporal and spatial distribution of diatoms in the North Pacific Subtropical Gyre. The primary goal of the investigation was to test the Goldman (1988,

1993) hypothesis that nutrient injection from depth increased diatom abundance in the deep chlorophyll maximum layer (DCML), leading to a subsequent increase in export. The authors found a large abundance of *H. haukii* and *Mastogloia woodiana* in the mixed layer during the summer months of sampling and concluded that diatom assemblages were more abundant here than at the DCML during the summer months. They concluded that the summer conditions favored specific diatom assemblages (including *H. haukii*), and that they became "disproportionately important" during this time and controlled particulate organic matter export to depth (Scharak et al. 1999a, 1999b). It is likely that high iron concentrations and light levels favored the increase in diatom abundance (Sunda & Huntsman 1997). Further, *H. haukii* is considered a lightly silicified diatom, and the lower silicate demands likely favor their growth over other more highly silicified diatom species, where silicate availability may be a limiting factor in their growth (Scharak et al. 1999a). The authors add that physiology, morphology, and life history lead to the selection of certain species during seasonal changes. This further supports the need for a biological understanding of DDAs that are representative in this study in the North Pacific, and others in similarly tropical and subtropical habitats. Though DDA symbioses remain cryptic, a plethora of information was provided in genetic analysis of several associations. Phylogenetic analysis of three DDA associations, including the host diatoms *Hemiaulus haukii*, *Rhizosolenia clevei*, and *Chaetoceros* spp., indicate distinct divergence in lineages of the associated symbionts, suggesting they are different species or strains (Foster & Zehr 2006; Foster et al. 2010). Interestingly, Foster et al. (2010) mentions that it may be possible that *H. haukii* could obtain a *Richelia* spp. strain from a coexisting *Rhizosolenia* cell.

The purpose of this study was to provide information on the biology of the relationship between the host diatoms *Hemiaulus haukii* and *H. membranceus* and their endosymbiont *Richelia intracellularis.* This included analysis of the nitrogen requirements, nitrogen addition effects on N_2-fixation, and the response of N_2-fixation and growth to changes in light. I have examined temporal variation in nitrogen fixation

rates from DDA cells subjected to fixed and fluctuating incident light levels. I contributed to the existing data on DDA species specific growth rates (Table 2). Simultaneous analysis of N_2-fixation and growth rates in these two DDA species has not been performed, and because *Richelia intracellularis* is considered the "most abundant heterocyst forming diazotroph in the marine pelagic realm" (Janson et al. 1999), this work has far-reaching implications for measuring *in situ* N_2-fixation rates, and understanding the biology of the DDA associations.

As the significance of DDA N_2-fixation is becoming increasingly apparent (Heinbokel 1986; Villareal 1991,1994; Subramaniam et al. 2008; Church et al. 2009; Foster et al. 2010) it is vital to understand the mechanisms of cellular N_2-fixation in order to provide the most accurate estimates possible. This work will greatly influence field methodologies for measuring N_2-fixation, and potentially influence current N_2-fixation estimates.

Objectives, Hypotheses, and Methodologies

Objectives:

1. Determine DDA maximum growth rate using media of three different nitrogen sources: no added nitrogen, added nitrate, and added ammonium.

2. Determine if N_2-fixation alone can support growth.

3. Determine the diel pattern of N_2-fixation in two DDA species (*Hemiaulus haukii-Richelia intracellularis*, and *Hemiaulus membranaceus-Richelia intracellularis*) at a fixed light level.

4. Determine the influence of irradiance on nitrogen fixation using an irradiance vs. N_2-fixation curve, and cells adapted to high light or low light (200 µmol quanta $m^{-2}sec^{-1}$, 50 µmol quanta $m^{-2}sec^{-1}$).

5. Quantify the growth vs. irradiance curve response to 7 different light levels at 25°C and a 12:12 L:D photoperiod.

Hypotheses

1. Each DDA species will be able to use N_2, NO_3^-, and NH_4^+
2. N_2-fixation alone can support growth.
3. The maximum growth rate under different N-source conditions will not be the same. NH_4^+ growth rates will be higher than NO_3^-, which will be higher than N_2.
4. The rate of ethylene evolution in heterocystous *Richelia intracellularis* from the two DDA species will vary on a diurnal basis. Maximum rates will be observed in the afternoon and there will be no dark N_2-fixation.
5. N_2-fixation will follow light saturation kinetics, and each DDA species will have a similar response to light and N_2-fixation capacity.
6. Irradiance vs. N_2-fixation cure will follow a PS curve.
7. DDA cell growth can be fit to a Jassby-Platt hyperbolic tangent function curve.
8. The two DDAs will have similar growth rates with increasing light intensity.
9. There will be photoinhibition of growth at high light levels.

Methodologies

Methodologies will follow the order in which the experiments were conducted, beginning with the nitrogen-source experiment and followed by the diurnal N_2-fixation study, N_2 vs. irradiance study and finally, the growth vs. irradiance experiment.

Culturing

All algal culturing was conducted at the University of Texas Marine Science Institute (UTMSI) in Port Aransas, Texas. Cultures of *Hemiaulus membranaceus* and *Hemiaulus haukii* from continental shelf waters of the Gulf of Mexico are available in the Villareal Lab. All *Hemiaulus haukii* strains used were isolated by Colbi Brown, and all *H. membranaceus* strains were isolated by Tracy Villareal. *Hemiaulus haukii* strains #83, #91, and #92 were used for a given experiment, and *Hemiaulus membranaceus* strain #82 was used for all experiments involving this DDA. After isolation, cells were placed in an incubator set at 25 °C under cool white fluorescent illumination of 150-250 µmol quanta $m^{-2}sec^{-1}$ and set on a 12:12 Light:Dark cycle. All cultures were grown as batch cultures and transferred to new medium approximately every 7-10 days. Cultures were maintained in sterile filtered N-free YBCII media with L1 nutrient additions (Ohki et al. 1992; Chen et al. 1996; Guillard & Hargraves 1993) in autoclaved, 50 ml borosilicate tubes in order to prevent compromising tube condition at high temperatures.

Nutrient Collection and Analysis: All Experiments

Samples for nutrient analysis were collected at the beginning of batch culture growth and at the termination of each experiment to verify nutrient additions. 10 ml of sample medium was filtered through 25 mm, 0.22 µm pore-size membrane Millipore filters, and frozen for later analysis. A SEAL Analytical QuAAtro autoanalyzer was used to determine dissolved inorganic phosphate (DIP), nitrate +nitrite (N+N), ammonium (NH_4^+), and silicate (SiO_4^{-2}) concentrations using the manufactures recommended chemistries.

Nitrogen Source Experiment: Nutrient Analysis and Sampling

H. haukii strain # 83 was transferred to 2 liter autoclaved glass Ehrlenmeyer flasks containing YBCII medium and the following nitrogen sources were added: no added nitrogen, added nitrate (40µM) or added ammonium (10µM). Samples were maintained at 25 °C and 35 psu. Nutrients analyses and cell abundance were sampled 10

times throughout the duration of the 20 day experiment. Nutrient and cell counts were done in duplicate. N_2-fixation was measured 4 times over the 20 day period. Chlorophyll *a* was sampled 6 times in duplicate.

Cell Counts and Percent Symbioses

Cell counts were performed using a S52 Sedgewick Microlitre Rafter, on an Olympus BX51 epifluorescent microscope. Excitation/emission wavelengths for the epifluorescent filters were: 450 nm/680nm (chlorophyll *a*), 490 nm/565 (phycoerythrin) and 620 nm/660nm (phycocyanin). Percent symbiosis was calculated as the number of symbiotic hosts distinguished by epifluoresence divided by the total number of potential host cells.

Acetylene Reduction Assay

Acetylene reduction assays (ARA) were performed as described in Capone (1993) for each DDA during the fixed light and light gradient experiments All N_2-fixation calculations were reported as nmol N_2 heterocyst^{-1}min^{-1}. Calculations were corrected using the Bunsen solubility coefficient as described in Breitbarth et al. (2004). Ethylene evolution rates were used to produce N_2-fixation estimates for *Richelia intracellularis* within the two targeted DDA species. Replications were limited to one sample for each given light level and time series, and run on a SRI 8610C 30cm silica gel column gas chromatograph (SRI Instruments, Torrance, CA). Ethylene was converted to nitrogen using a 4:1 molar ratio (Jensen & Cox 1983).

Diel and Light Gradient Assays

H. haukii strain #92 and *H. membranaceus* strain #82 were used for the diel study. *H. haukii* #91 and *H. membranaceus* #82 were used for the light gradient experiments. Each diel study ran for 24 hours, with sampling conducted approximately every 90 minutes. Cultures for the diel study were kept on a 12:12 L:D cycles at 200 µmol quanta m^{-2}sec^{-1}. Cultures used for the light gradient experiment were kept at 7 different light

levels (described below). All cultures were kept at a constant temperature of 25°C and salinity of 35 psu. Ethylene evolution rates were calculated using ethylene peak heights (as determined by gas chromatography) measured at 0, 60 minutes, and each subsequent hour and a half after the initial acetylene injection at time zero.

Growth Rates and Light-Growth Relationship

Growth rates were measured using 7 different light levels (30, 50, 75, 150, 200, 350 and 500 µmol quanta m^{-2}/sec^{-1}) measured by a QSP-170B irradiance meter (Biospherical Instruments). All cultures were preconditioned to light levels for 7 days and remained at the assigned light level through the duration of the experiments. Cultures were maintained at 25°C and 35psu under cool white fluorescent lighting. Cell counts were conducted daily.

Growth rates for all DDAs were calculated following Guillard (1977), as the slope of the log of cell number over the change in time. Rates were fit to the Jassby-Platt hyperbolic tangential function (Jassby & Platt 1976).

Chlorophyll a

Cultures for each experiment were filtered through a 0.4µm pore sized nucleopore filter and extracted in 10 ml of methanol for chlorophyll *a* content using the non-acidification methanol extraction method (Welschmeyer & Naughton 1994). Samples were read on a TD-700 Fluorometer (Turner Designs, CA, USA).

Statistics

Statistics were done using Microsoft Excel software. Delta Graph (Red Rocks Software) was used for graphics of the growth and N_2-irradiance curves.

Results

All cultures were grown in artificial seawater (ASW; see methods) because ASW contains a known composition of elements, as opposed to natural seawater (NSW) which has an inherent amount of variability in its composition. Additionally, ASW yielded more stable cultures than enriched natural seawater.

Nitrogen-Source Experiment: *Hemiaulus haukii*

Cell and Symbiont Numbers

In all three treatments, the average number of cells ml^{-1} increased from 10^2 to 10^3 from day zero to day 20. In the "no added nitrogen" treatment, stationary phase was reached on day 17, but did not decline after that (Figure 11). In contrast, stationary phase in the added nitrate treatment was reached on day 15, and cell numbers declined (Figure 14). In the "added ammonium" treatment, cell numbers began to decline on day 15; however, there followed an increase in cell numbers that lasted through day 20 (Figure 17). Maximum cell growth rates (divisions day^{-1}) were 0.23 (no nitrogen), 0.30 (added nitrate) and 0.42 (added ammonium; Table 5).

In all treatments, the percent symbiotic cells declined after day 10 (Table 4). The added ammonium treatment had the greatest increase in percent asymbiotic cells, and by the termination of the experiment, 49% of the cells were asymbiotic (Figure 17). In contrast, 34% of the cells in the "no-N" treatment were asymbiotic (Figure 11), and 37% in the "added nitrate" treatment (Figure 14). The highest growth rate of asymbiotic cells was in the "added ammonium" treatment, followed by the "added nitrate" treatment, and the "no added N" treatment (Table 6; Figure 17).

Nutrients

In all treatments, DIP (dissolved inorganic phosphorus) and SiO_4^{-2} (Silicate) decreased with the duration of the experiment. In the "no-added nitrogen" medium, DIP decreased from 3.44 μM to 2.19 μM. Silicate decreased from 34.06 μM to 12.95 μM Ammonium declined from 1.08 μM to 0.35 μM, and nitrate levels were undetectable

(Figure 12). In the "added nitrate" medium, DIP decreased from 3.65 μM to 1.88 μM and silicate decreased from 33.65 μM to 8.92 μM. Ammonium declined from 1.01 μM to 0.30 μM, and nitrate declined from 44.87 μM to 38.47 μM (Figure 15). In the "added ammonium" medium, DIP decreased from 3.40 μM to 1.99 μM and silicate decreased from 34.97 μM to 10.83 μM. Ammonium declined from 10.63 μM to 0.82 μM, and nitrate levels were undetectable (Figure 18).

Nitrogen Fixation

An acetylene reduction assay was carried out four times during the duration of the experiment. N_2-fixation was measurable in all three treatments. The highest value of N_2-fixation was measured in the "no added nitrogen" treatment, and the lowest value in the "added ammonium" treatment. In the "no added nitrogen" treatment values ranged from 0 nmol N_2 heterocyst^{-1}min^{-1} to 7.43 x 10^{-5} nmol N_2 heterocyst^{-1}min^{-1} (Figure 13). In the "added nitrate" treatment, the N_2-fixation values ranged from 0 nmol N_2 heterocyst^{-1}min^{-1} to a maximum value of 6.48 x 10^{-5} nmol N_2 heterocyst^{-1}min^{-1} (Figure 16). In the "added ammonium" treatment, N_2-fixation values ranged from 0 nmol N_2 heterocyst^{-1}min^{-1} to 2.93 x 10^{-5} nmol N_2 heterocyst^{-1}min^{-1} (Figure 19). In all treatments, the lowest N_2-fixation rates occurred on day 4, when the cultures were in early exponential phase. Additionally, the highest rates in all treatments occurred on day 8, when cultures were in exponential phase. All rates declined after this peak as cells entered stationary phase.

Diurnal Nitrogen-Fixation Experiments

Hemiaulus haukii

In the first 24 hour assay, nitrogen fixation values were calculated from a *H.haukii* culture adapted to a light level of 200 μmol quanta m^{-2}sec^{-1}, and maintained this light level for the duration of the light portion of the 12:12 L:D photoperiod. Results of the quantity of nitrogen fixation in nmol N_2 heterocyst^{-1} vs. incubation time are in Appendix 1. Diurnal rates of N_2-fixation ranged from -7.42 x 10^{-8} to 8.75 x 10^{-6} nmols N_2 heterocyst^{-1}min^{-1} (Figure 20). The data is described as a curve including a variety of individual points (samples). When considering the individual points, there is a large

amount of variability between assays; however, the curve itself indicates symmetry in the rates of nitrogen fixation, and there is no evidence of a peak in the rate of N_2-fixation at a specific point. Low rates of N_2-fixation occurred during the dark and early morning. Negative values resulted from a higher blank N_2-fixation value than the sample value. Nitrogen fixation continued to occur in the early portion of the dark photoperiod for 2 hours.

Hemiaulus membranaceus

In the second 24 hour assay, nitrogen fixation values were calculated from a *H.membranaceus* culture pre-adapted to a light level of 200 µmol quanta $m^{-2}sec^{-1}$, and maintained this light level for the duration of the light portion of the 12:12 L:D photoperiod. Results of the quantity of nitrogen fixation in nmol N_2 $heterocyst^{-1}$ vs. incubation time (mins) are in Appendix 2 and show there was a linear increase in the amount of nitrogen fixed during the day, before N_2-fixation ceased with the commencement of the dark period.

The range of N_2-fixation was from a minimum of 3.83 $x10^{-7}$ nmol N_2 $heterocyst^{-1}min^{-1}$ to a maximum of 1.88 x 10^{-4} nmol N_2 $heterocyst^{-1}min^{-1}$ (Figure 21). There is variability in the individual points; however, the pooled data results in a symmetrical curve that indicates no period of accelerated N_2-fixation rates. Low rates of N_2-fixation occurred in the early morning, and there was no evidence of N_2-fixation occurring during the dark period.

Nitrogen Fixation- Irradiance Curves: Light Gradient Experiments

Hemiaulus haukii

N_2-fixation followed light saturation kinetics when fit to the Jassby-Platt hyperbolic tangential function (Figure 22; Jassby & Platt 1976). The maximum value of N_2-fixation for the low light level adapted curve (50 µmol quanta $m^{-2}sec^{-1}$) was 3.17 x 10^{-6} (nmol N_2 $heterocyst^{-1}min^{-1}$) and occurred at an irradiance of 150 µmol quanta $m^{-2}sec^{-1}$. The saturation light intensity (I_K) was 107 µmol quanta $m^{-2}sec^{-1}$ (R^2=0.76). The alpha value was 2.01 x 10^{-8} nmols N_2 $heterocyst^{-1}min^{-1}$/µmol quanta $m^{-2}sec^{-1}$, and N_2-fixation

max (N_2-fix$_{max}$) was 2.14 x 10^{-6} nmols N_2 heterocyst^{-1}min^{-1}. There was no observed photoinhibition of N_2-fixation.

The maximum value of N_2-fixation for cells pre-adapted to a high light level (200 µmol quanta m^{-2}sec^{-1}) was 2.40 x 10^{-6} nmol N_2 heterocyst^{-1}min^{-1} and occurred at 500 µmol quanta m^{-2}sec^{-1}. I_K was 226 µmol quanta m^{-2}sec^{-1} (R^2 = 0.95), α was 1.03 x 10^{-8} nmols N_2 heterocyst^{-1}min^{-1}/µmol quanta m^{-2}sec^{-1} and N_2-fix$_{max}$ was 2.33 x 10^{-6} nmols N_2 heterocyst^{-1}min^{-1} (Table 11). I_C could not be calculated because the curves did not intersect the x-axis. There was no observed photoinhibition of N_2-fixation.

Hemiaulus membranaceus

The maximum value of N_2-fixation for cells adapted to low light (50 µmol) was 3.49 x 10^{-6} (nmol N_2 heterocyst^{-1}min^{-1}) and occurred at 150 µmol quanta m^{-2}sec^{-1}. The saturation light intensity was (I_K) was 112 µmol quanta m^{-2}sec^{-1} (R^2 = 0.91). The alpha value was 2.47 x 10^{-8} nmols N_2 heterocyst^{-1}min^{-1}/µmol quanta m^{-2}sec^{-1}, and the N_2-fix $_{max}$ was 2.77 x 10^{-6} nmols N_2 heterocyst^{-1}min^{-1}. The maximum value of N_2-fixation for cells adapted to high light (200 µmol) was 6.17 x 10^{-6} (nmol N_2 heterocyst^{-1}min^{-1}) and occurred at 500 µmol quanta m^{-2}sec^{-1}. The I_K was 144 µmol quanta m^{-2}sec^{-1} (R^2 = 0.95). Alpha was 4.23 x 10^{-8} nmols N_2 heterocyst^{-1}min^{-1}/µmol quanta m^{-2}sec^{-1}, and N_2-fix$_{max}$ was 6.09 x 10^{-6} nmols N_2 heterocyst^{-1}min^{-1} (Table 11; Figure 23). I_C could not be calculated because the curves did not intersect the x-axis due to dark fixation potential.

Growth-Irradiance Curves

Hemiaulus haukii

The growth-irradiance curve followed saturation kinetics (Figure 24). Maximum growth occurred at 500 µmol quanta m^{-2}sec^{-1} and was recorded as 0.99 divisions day^{-1}. Minimum growth occurred at 30 µmol quanta m^{-2}sec^{-1}, and was recorded as 0.19 divisions day^{-1} (Table 9; Figure 24). When fit to the Jassby-Platt curve, growth saturated (I_K) at 100 µmol quanta m^{-2}sec^{-1} (R^2 = 0.99), with a μ_{max} of 9.96 x 10^{-1} divisions day^{-1} and alpha of 9.98 x 10^{-3} divisions day^{-1}/ µmol quanta m^{-2}sec^{-1} (Table 10, Figure 24). I_C was 10 µmol quanta m^{-2}sec^{-1}.

Hemiaulus membranaceus

Maximum growth occurred at 350 µmol quanta $m^{-2}sec^{-1}$ and was recorded as 1.06 divisions day^{-1} (Figure 25). Minimum growth occurred at 500 µmol quanta $m^{-2}sec^{-1}$ and was recorded as -1.10 divisions day^{-1} (Table 9). Negative growth rates resulted from a steady decline in cell numbers over the course of the experiment. Data from this experiment could not be fit to a hyperbolic tangent function, and µ max, α, I_C, I_K and R^2 values could not be calculated. The culture strain was lost, and the experiment could not be repeated.

Discussion

Nitrogen-Source Experiment: Hemiaulus haukii

The nitrogen source experiment addressed the influence of environmental conditions (nutrients) on cell growth, percent symbioses and N_2-fixation capabilities. In all diatom cultures, there was exponential growth; despite this similarity, there were marked differences in the growth dynamics of batch cultures. Unlike the "added nitrate" and "added ammonium" treatments, the "no added nitrogen" treatment reached stationary phase but did not decline after stationary phase was reached. In the "added nitrate" treatment, the cells reached stationary phase and began to decline after day 15. In the "added ammonium" treatment, cells began to decrease after the exponential phase; however, they began to increase again after the initial declination. It is possible that this is due to sampling error, as there was a dramatic decline after Day 10 (Figure 17).

In all treatments, the concentrations of silicate and phosphorus declined steadily throughout the duration of the experiment. This is to be expected, as diatom cells require both nutrients for growth and survival. What is significant is that in the "added nitrate" treatment, the cells did not use the added nitrate. This finding is surprising, as there was no added ammonium in this treatment. It can be hypothesized from these results that the cells were using the nitrogen supplied by nitrogen fixation (Villareal 1992; Bar-Zeev 2008). Supply of combined nitrogen in the euphotic zone, and its role in biogeochemical cycling, has attracted a significant amount of interest (Dugdale & Goering 1967; Eppley

& Peterson 1979; Mague et al. 1977). In the context of these studies, the fact that the experimental DDA did not use nitrate is of considerable note because it indicates that in open ocean, oligotrophic regions where nitrogen is a limiting nutrient, DDAs have an adaptive advantage in that they may not use nitrate. From the results of this study, it can be concluded that phosphate and silicate are more likely to be limiting to DDAs than nitrogen.

In the "no added nitrogen" treatment, the average cells and symbionts per milliliter increased throughout the experiment. This is in contrast to the "added nitrate" and "added ammonium" treatments, in which both the cell and symbiont numbers declined after a peak on day 15. The results that cells and symbiont numbers declined in the "added ammonium" treatment are not surprising because of the cellular use of ammonium as a sole nitrogen source. Ammonium was not replenished during the experiments; therefore, the supply declined with time, until the concentration could no longer support growth. Despite this, the "added ammonium" treatment had the highest growth rate (0.42 div day $^{-1}$) calculated from total cell numbers, and also the highest growth rate of asymbiotic cells (0.37 divisions day^{-1}). It is possible that the high grow rate indicated parent cells dividing faster than their symbionts; therefore, vertical transfer of the symbionts to the daughter cell declined. This un-coupling of host and symbiont is similar to what Villareal (1990) found in the DDA *Rhizosolenia-Richelia* relationship. The percent asymbiotic cells also increased in the "added nitrate" and "no added nitrogen" treatments. In the "no added nitrogen" treatment, the increase in asymbiotic cells indicates that there was some supply of nitrogen. There are two possible explanations for this: the cells were receiving nitrogen from symbiotic cell's N_2-fixation or there was cell lysis that provided nutrients. The later is much less likely, considering that the total cell numbers in the "no added nitrogen" treatment did not decline throughout the experiment; therefore, it is unlikely there was a large amount of cell lysis. The results that the cell and symbiont numbers declined in the "added nitrate" treatment are harder to explain, considering that the cells were not using nitrate. A possible explanation is that nitrate is not a preferred form of nitrogen for *H.haukii*.

There was approximately a 2-fold amount of variation in N_2-fixation between the three treatments, based on the maximum values of N_2-fixation rates calculated. The "no added nitrogen" treatment had the highest value of N_2-fixation (7.43×10^{-5} nmols N_2 heterocyst^{-1}min^{-1}; Figure 13). It is possible that in the absence of an added nitrogen source, cells increase the capacity for nitrogen fixation in order to meet cellular physiological needs (Villareal 1992; Janson et al. 1995; Foster et al. 2010). The "added ammonium" treatment had the lowest maximum rate of N_2-fixation (3.79×10^{-5} nmol N_2 heterocyst^{-1}min^{-1}; Figure 19). A study on the heterocystous species *Nodularia spumigena* found that N_2-fixation was inhibited by the presence of ammonium (Lehtimaki et al 1997; Vintila & El-Shehawy 2007); however, the frequency of heterocysts was not influenced. The authors found that ammonium suppresses *nifH* expression thus, suppressing nitrogenase activity (Vintila & El-Shehawy 2007). This could explain the lower rate of N_2-fixation found in the ammonium treatment of this study. The influence of nitrate on nitrogen-fixation has been studied extensively in *Anabaena* spp., with conflicting results and conclusions. Early studies on *Anabaena cylindrica* found that combined nitrogen inhibited heterocyst formation and N_2-fixation; however, cells were able to take up nitrate for growth (Fogg 1949). In the same species, Ohmori and Hattori (1972) found that nitrate neither inhibited nor repressed nitrogenase activity and N_2-fixation. The authors also found that cells grew equally well in nitrate, ammonium and dinitrogen media. Several studies have reported that *Anabaena* sp. strains ATCC 33047 and ATCC 29414 failed to form heterocysts in the presence of nitrate, though vegetative cells were capable of growth in nitrate medium (Bottomley et al. 1979; Wolk et al. 1994). Ramaswamy et al. (1996) found that within *Anabaena* sp. strain PCC7120 there were cells which could differentiate and form heterocysts, while other cells were incapable of heterocyst formation in the presence of nitrate. In summary, nitrate appears to be less effective at suppressing nitrogen-fixation (via heterocyst inhibition) than ammonium; however, this conclusion is highly species and even strain-dependent (Meeks et al. 1983; Vintila & El-Shehawy 2007).

Diurnal Nitrogen Fixation

One goal of this study was to determine whether there was diurnal variation in nitrogen fixation rates. Though previous studies have addressed this question in other species (Saino & Hattori 1978; Capone et al. 1990; Taniuchi et al. 2008), none have addressed it in *Hemiaulus haukii* and *H. membranaceus.* The results indicate that there is no evidence for diurnal N_2-fixation variation in either species. Both *H. haukii* and *H. membranaceus* display N_2-fixation behavior consistent with other light-dependent heterocystous species (Levine & Lewis 1984; Stal & Krumbein 1985; Haselkorn et al. 1992; Church et al. 2005; Zehr et al. 2011). *H. haukii* and *H. membranaceus* both displayed a slow initiation of N_2-fixation with the onset of the light period; however, after this rates of N_2-fixation leveled out throughout the day. There was no indication that the rate of nitrogen fixation increased at a specific time during the photoperiod after the initial commencement of N_2-fixation; however, there was a certain amount of experiment to experiment variability.

There was evidence in both DDAs of nitrogen-fixation occurring immediately after the photoperiod in the dark; however, this was considerably more evident in *H. haukii* (Figures 20 & 21; Appendix 1 & 2). There have been literature reports of a similar phenomenon in other heterocystous species. Fay (1976) describes dark N_2-fixation in *Anabaenopsis circularis* and notes that the ability to fix nitrogen in the dark is a function of the length and intensity of light exposure during the day. He explains that the organic reserves acquired during light N_2-fixation can be suitable to fuel dark N_2-fixation. This was also found in studies of *Nostoc muscorum* (Allison 1937) and *Chlorogloea fritschii* (Fay 1965). Dark fixation was shown to occur in the DDA *Chaetoceros-Calothrix* (Foster et al. 2010). In a study on the isolated exosymbiont *Calothrix rhizosoleniae* SC01, the authors found N_2-fixation persisted into dark period, and accounted for 3-20% of the total N_2-fixation activity. The conclusion from the above studies is that nitrogen fixation occurring in the dark is likely due to an intracellular stored supply of energy required to fuel the reaction (Wolk 1994). Further studies are needed to investigate this hypothesis in

other DDAs. The fact that dark N_2-fixation was more evident in *H. haukii* cultures suggests different strategies between the two DDAs.

Nitrogen Fixation-Irradiance Curves

The relationship between *H. haukii* high and low light adapted cells is surprising. The data indicates that the low light adapted cells have higher rates of N_2-fixation than the high light adapted cells. This remained consistent throughout the 12 hour assay. To ensure that these results were accurate, the experiment was run twice, and in both experiments the results were the same: *H. haukii* cells adapted to a high light level have lower capacity for N_2-fixation than those cells adapted to a low light level (Figure 22; Table 10). The steeper slope in the low light treatment (α = 2.01 x 10^{-8} nmols N_2 heterocyst^{-1}min^{-1}/μmol quanta m^{-2}sec^{-1}) indicates a faster response to initial N_2-fixation, and the higher I_K value indicates it saturates at a higher light level. N_2-fixation rates remain comparable to, and in some cases higher than, literature values of N_2-fixation (Mague et al. 1974; Villareal 1991; Bar-Zeev et al. 2008; Foster et al. 2010; Table 3). The maximum N_2-fixation rate calculated by Villareal (1990) from a field study by Mague (1974) on *Rhizosolenia-Richelia* was 7.91 x 10^{-6} (nmols N_2 trichome^{-1}min^{-1}), comparable to a laboratory rate (Villareal 1990) of 4.16 x 10^{-6} to 5.83 x 10^{-5} nmol N_2 trichome^{-1}min^{-1} on the same association. In his study, ethylene evolution rate was linear below 100-150 μmol quanta m^{-2}sec^{-1}, and the initial slope (α) was 3.6 x 10^{-5} nmol N_2 trichome^{-1}min^{-1}. All samples were incubated at 300 μmol quanta m^{-2}sec^{-1} and did not display photoinhibition at highlight (780 μmol quanta m^{-2}sec^{-1}). Foster et al. (2010) analyzed the effect of irradiance on N_2-fixation in a lab study on the DDA endosymbiont *Calothrix rhizosoleniae*. Her data shows that N_2-fixation rates are a function of both light and biomass. All samples showed light dependence and displayed saturation kinetics. The N_2-fixation light saturation (I_K) values ranged from 27.52 to 301.3 μmol quanta m^{-2}sec^{-1}. The reported alpha values were 1.1 x 10^{-3} to 1.4 x 10^{-1} mmol C_2H_4 mg chl *a*$^{-1}$hr^{-1}. N_2-fixation rates ranged from 0.2 to +5.0 μmol C_2H_4 mg chl *a*$^{-1}$l^{-1}. These values are important to include because they are among the scarce amount of information available on the light-

N_2-fixation relationship in DDAs. Despite this, there are some limitations in the application of this data. Foster (2010) normalized all data to chl *a* content, which is not directly comparable to rates normalized to individual trichomes or heterocysts. Further, the study is on the isolated exosymbiont of *Chaetocerous* spp., and can not be directly compared to the DDAs in this study.

Data from Villareal (1990) was used to calculate Jassby-Platt parameters for the DDA *Rhizosolenia-Richelia.* In this association, the initial slope was higher than that seen for *H.haukii* and *H.membranaceus* (α=2.55 x 10^{-7}nmols N_2 heterocyst^{-1}min^{-1}/μmol quanta m^{-2}sec^{-1}). The N_2-fixation light saturation level (I_K) was 134 μmol quanta m^{-2}sec^{-1}, which is comparable to the values found in this study. Similar to this study, the I_C value for *Rhizosolenia-Richelia* could not be calculated, due to dark fixation potential. Villareal (1991) provides data on nitrogen-fixation in a field study of three *Hemiaulus* spp. DDA associations. All samples were incubated at 306 μmol quanta m^{-2}sec^{-1}. Rates for the combined sample of *H. haukii*/*H.sinensis* were 1.25-2.2 x 10^{-6} nmol N_2 cell^{-1}min^{-1}. These are lower than the maximum rate for *H. haukii* found in this study which was 3.35 x 10^{-5} nmol heterocyst^{-1}min^{-1}. Caution should be used when comparing the two studies, as Villareal (1991) used a combination of *H. haukii* and *H. sinensis*; therefore, it is difficult to extrapolate the amount of N_2-fixation associated with *H. haukii* alone. In the same 1991 study, Villareal reported rates for the *H.membranaceus* association as 9.58 x 10^{-7} to 1.5 x 10^{-6} nmol N_2 cell^{-1}min^{-1}. These are lower than the maximum N_2-fixation rate for *H. membranaceus* found in this study, which was 6.17 x 10^{-6} nmol N_2 heterocyst^{-1}min^{-1}.

Unlike *H.haukii*, the *H. membranaceus* light-gradient experiments indicate cells that are adapted to low light have a lower capacity for N_2-fixation than cells that were adapted to a high light level. The I_K for the high light adapted treatment was 144 μmol quanta m^{-2}sec^{-1}, and the initial slope (α) was 4.23 x 10^{-8} nmols N_2 heterocyst^{-1}min^{-1}/μmol quanta m^{-2}sec^{-1}. The I_K for the low light adapted treatment was 112 μmol quanta m^{-2}sec^{-1}, and the initial slope was 2.47 x 10^{-8} nmols N_2 heterocyst^{-1}min^{-1}/μmol quanta m^{-2}sec^{-1}. It is important to note that there appeared to be no photoinhibition of N_2-fixation at higher

light levels (500 µmol quanta $m^{-2}sec^{-1}$). The lack of photo-inhibition in both DDAs suggests tolerance to high light for both growth and N_2-fixation, which is consistent with Venrick's (1974) characterization of them as part the shallow community. The significance of these results can be applied to N_2-fixation sampling in diverse geographical regions where the light intensity and photoperiod may vary considerably. The lack of photo-inhibition does not preclude the existence of diazotrophy at depth. For example, the compensation light intensity (I_C) for both species could not be calculated because the N_2-Irradiance curves did not intersect the x-axis, indicating the potential for N_2-fixation at low light levels. These results are supported by Crombet et al. (2011) who describes a "deep glass forest" composed of a diatom assemblage that includes *H. haukii* and *Rhizosolenia stylyformis* in the Mediterranean Sea. He notes that *Richelia intracellularis* was present at nearly all sites. He found that in some cases, the deep silica maximum (DSM) was as deep as 175m. Deep diatom assemblages up to 60m composed of DDAs (*Hemiaulus* spp., *Rhizosolenia* spp.) have also been reported in the North Pacific Subtropical Gyre (Dore et al. 2008), indicating that light may not be the primary limiting factor on N_2-fixation. The literature suggests this is not unique to DDAs, but occurs in other diazotrophic organisms as well. Montoya et al. (2004) reports N_2-fixation occurring at depths below the subsurface pigment maximum in the oligotrophic Pacific (Figure 2). In this case, unicellular diazotrophs were the organisms of interest; however, it should be noted that N_2-fixation measurements from this study came from whole water samples, and not isolated organisms.

Growth-Irradiance Curves

Hemiaulus haukii

The purpose of the growth-irradiance curves was to determine the influence of irradiance on cell growth rates. Cell growth rates increased with increasing light intensity to a maximum of 0.99 divisions day^{-1} at a light level of 500 µmol quanta $m^{-2}sec^{-1}$ (Table 8; Figure 24). There did not appear to be any inhibition of growth at higher light levels. This is significant because it indicates that *Hemiaulus haukii* growth is not inhibited by higher light levels. Further, though growth rates were lower at lower light levels (30-75

μmol quanta $m^{-2}sec^{-1}$), growth did occur, which could potentially allow for further vertical distribution on the basis of light intensity. Venrick (1974) found aggregates of the DDA *Rhizosolenia-Richelia* as deep as 200m in the North Pacific. This data suggests that *Richelia intracellularis* is not limited to the upper water column, but can maintain a range of vertical distributions. A limitation to this interpretation is the absence of *H. haukii* at that depth; however, the study was focused on the distribution of *Richelia intracellularis*, and it was not until 1991 that *R. intracellularis* was known to be an endosymbiont of *Hemiaulus* spp. (Heinbokel 1986; Villareal 1991). When fit to the Jassby-Platt hyperbolic tangential function, the compensation light intensity at which there would be no growth (I_C) was 10 μmol quanta $m^{-2}sec^{-1}$. The light-limited initial slope (α) was 9.98 x 10^{-3} divisions day^{-1}/μmol quanta $m^{-2}sec^{-1}$. The I_K at which α intersected μmax was 100 μmol quanta $m^{-2}sec^{-1}$ (Table 10; Figure 24). The data suggests that *H.haukii* is capable of sustaining growth at a wide range of light intensities.

Growth rates for both DDAs in this study were comparable to other laboratory rate measurements; however, they were significantly lower than growth rates calculated in the field (Table 2). Brown & Villareal (unpublished, personal communication) found *H. haukii* maximum growth rates of 0.40-0.41 divisions day^{-1}. Johnson & Villareal (unpublished, personal communication) found higher rates of 0.61-0.68 divisions day^{-1} for *H. haukii*, and 0.30-0.32 for *H. membranaceus.*. These are both lower than the maximum rate of 0.99 divisions day^{-1} (*H. haukii*) and 1.06 divisions day^{-1} (*H. membranaceus*) found in this study. Conversely, field maximum growth rates for *H. haukii* were measured at 4.3 divisions day^{-1} (Vargo 1983) and 2.2 divisions day^{-1} for *Hemiaulus* spp. (Furnas 1991). Discrepancies in the growth rates in the laboratory measurements are likely a result of the different media used when culturing. For example, Brown (unpublished, personal communication) used natural seawater with added L1 medium additions, including nitrate and ammonium. Similarly, Johnson used natural seawater with added nitrate and standard L1 medium additions. A second reason is the ambient light level at which the cultures were incubated. Maximum growth rates for both DDAs in this study were recorded at high light intensities (350 μmol quanta $m^{-2}sec^{-1}$, and

500 µmol quanta $m^{-2}sec^{-1}$). It is possible the cells with lower growth rates were incubated at lower light intensities. The third, and possibly most important reason for growth rate discrepancies lies in the location of study, and whether it was conducted in the field or in the lab. The much higher growth rates recorded from the field studies (Vargo 1983; Furnas 1991) indicate there is a large amount of inherent variability between natural and lab populations.

Hemiaulus membranaceus

The growth-irradiance curve experiment for *H.membranaceus* provided vastly different results than that for *H. haukii*. The experiment had unforeseen problems which led to uninterpretable data. What can be taken away from this experiment is that the maximum growth rate was ~ 1 division day^{-1}, and occurred at a high light level (350 µmol quanta $m^{-2}sec^{-1}$; Table 10; Figure 25).

Ecological Context and Conclusions

Blooms of DDAs, including those DDAs addressed in this study, have been reported in globally diverse locations. These include, but are not limited to, the North Pacific (Heinbokel 1986; Dore et al. 2008), Southwest North Atlantic (Villareal 1994; Carpenter et al. 1999), Gulf of California (White et al. 2007), tropical waters of the North Atlantic beyond the Amazon River plume (Subramaniam et al. 2008), and the Mediterranean Sea (Bar-Zeev et al. 2008). All of these studies have involved field analysis of bloom dynamics and their potential influence on biogeochemical cycles. This study provides essential information on the autocology of the relationship between the host and symbiont. Villareal (1994) and Heinbokel (1986) both report high percent symbioses, and this study confirms that over 80% of *Hemiaulus haukii* and *H. membranaceus* are found in association with *Richelia-intracellularis.* Field (Heinbokel 1986) and lab (Villareal 1994) studies both found high percent symbioses, which has positive implications for the application of this lab study to field observations. Both studies imply that in regions where DDAs area found in high abundance, it is likely N_2-fixation is taking place. N_2-fixation measurements in this study were comparable to, in

some cases, and considerably higher than in others, those reported in the literature for other diazotrophic associations. Foster et al. (2010) provided nitrogen-fixation rates for the DDA *Chaetocerous-Calothrix* that were 10 fold lower than those reported in this study. N_2-fixation rates reported in Bar-Zeev et al. (2008) were comparable, though lower than those found in this study. That analysis included several DDA associations, among those *Rhizosolenia-Richelia* and *Hemiaulus haukii.* Samples from that study also included rates of N_2-fixation from the non-heterocystous *Trichodesmium* spp. All rates were depth-integrated, and not species specific; thus, it is hard to draw conclusions about N_2-fixation from the individual DDAs. This study provides DDA-specific rates of nitrogen-fixation in a controlled laboratory environment. In doing so, it removes a variety of potential constraints involved in field analysis. Other literature values of N_2-fixation include those by Mague et al. (1974), who reported on the DDA *Rhizosolenia-Richelia.* Rates found in this study for *H. haukii* and *H. membranaceus* were higher than for the *Rhizosolenia-Richelia* association. This is consistent with findings by Villareal (1991), who found that the total contribution of N_2-fixation associated with *Hemiaulus haukii, H. membranaceus* and *H. sinensis* was 21-45 times greater than that of *Rhizosolenia-Richelia* in the southwest Atlantic.

This study has confirmed that both associations are able to grow in no nitrogen medium, suggesting that the nitrogen source sustaining growth originates from the N_2-fixation of *R.intracellularis*. It has confirmed that N_2-fixation in both associations follows light-saturation kinetics, and that *H.haukii* cells adapted to low light have a higher capacity for N_2-fixation. It has shown that there is no diurnal variation in N_2-fixation rates, and that *H. haukii* is capable of limited nitrogen-fixation in the dark, after exposure to a period of light. Further, it has confirmed an earlier observation by Johnson & Villareal (unpublished, personal communication) that *H. haukii* did not use nitrate. DDAs in this study have been observed as blooms associated with river plumes from the Amazon (Foster et al. 2007; Subramaniam et al. 2008) and Mekong Rivers (Gross et al. 2010; Bombar et al. 2011). In light of the knowledge that they are not using nitrate, it is likely that they have an adaptive advantage over other phytoplankton in that they are not

competing for nitrogen. This is very different from the *Rhizosolenia-Richelia* association, which will actively take up nitrate and grow aymbiotically, which clearly indicates they are very different DDAs. The nitrogen source experiment needs to be repeated with *H. membranaceus* in order to determine if it takes up nitrate in medium. In conclusion, this study has contributed to the current knowledge of the "cryptic" (Villareal 1990) symbiotic relationship of *H.haukii, H.membranceus* and their endosymbiont *Richelia intracellularis.* Finally, the presence of *H. haukii* and *H. membranaceus* in the Gulf of Mexico suggests that there is a considerable amount of N_2-fixation taking place in areas where these DDAs proliferate. Laboratory analysis of these associations has provided a unique opportunity to study these globally important organisms, and the results can be applied to further investigation of their role in nutrient cycling and biogeochemical processes.

Tables

Table 1. Literature summary of diurnal nitrogenase activity in several diazotroph genera. "NA" = Nitrogenase Activity.

Organism	Description	N2-Fix (Day)	N2-Fix (night)	Reference
Trichodesmium thiebautii	non-heterocystous cyanobacterium	NA "turned-on" in early morning(nitrogenase produced de novo each day); peak at noon	NA "turns-off" at dusk	Capone et al. (1990); Wyman et al (1996)
Trichodesmium thiebautii	non-heterocystous cyanobacterium	increase in morning, with peak at noon	low, but not zero: cyclic phosphorylation fueling?	Saino & Hattori (1978)
Trichodesmium thiebautii	non-heterocystous cyanobacterium	peak times all in afternoon	none	Mullholand et al (2006)
Trichodesmium erythraeum	non-heterocystous cyanobacterium	increase in morning, with peak at noon	none	Chen et al. (1996, 98); Berman-Frank et al. (2001)
Oscillatoria sp. Strain 23 (Odenburg)	non-heterocystous cyanobacterium; benthic (associated with microbial mats)	aerobic, no NA. anaeobic, high peak when light turned on; zero at noon	aerobic, NA present. Anaerobic, two NA peaks visible; zero at midnight	Stal & Heyer (1987)
General non heterocystous Cyanobacteria	mixed species	none	NA present	Stal & Krumbein (1985); Huang & Chow (1986); Mullineaux et al (1981)
Crocosphaera watsonii WH8501	unicellular cyanobacterium; group B diazotroph	none	NA present; max reached after 6hrs in dark	Mohr et al (2010); Tuit et al (2004)
Cyanothece sp.	unicelluar	none	NA present (fueled by photosynthetic carbon stored in granules)	Reddy et al. (1993); Colon-Lopez (1997)
Cyanothece sp ATCC 51142	unicelluar	none; incubated at continuous light, and maintained circadian (light independent) rhythm	60% n2 fix genes associated were independent of light period.	Toepel et al (2008)
Gloeothece sp.CCAP	unicelluar	none	exclusive N2-fix at night	Griffiths et al. (1987)
Gloeothece sp. 68DGA	unicelluar	none	*nifH* expression & NA activity exclusively at night	Taniuchi et al. (2008)
Anabaena PCC 7120	Heterocytous cyanobacteria	NA preset	(anaerobic) NA absent	Stal & Krumbein (1985); Haselkorn et al. (1992)
Anabaena variabilis (ATCC 29413)	Heterocystous	NA present & at max ("light controls nitrogenase activity via reductant supply" Ernst et al._1984)	NA absent	Ernst et al. (1984); Wolk & Shaffer (1976).
Nodularia sp.	Heterocystous microbenthos	NA present	NA absent	Hubel & Hubel (1974)
Heterocystous (Het-1; Het-2; Het-3)	pelagic NPSG study	*nifH* expression high	*nifH* expression low	Church et al. (2010); Zehr et al. (2009)

Table 2. Previously reported growth rates for three diatom-diazotroph associations with the following diatoms: *Rhizosolenia clevei*, *Hemiaulus haukii*, and *Hemiaulus membranaceus.* "NSW" = natural seawater; "ASW" = artificial seawater.

Diazotroph Taxon	Growth Rate (divisions day^{1})	Comment	Reference
Rhizosolenia clevei	0.72	Laboratory: N-replete media; light = 83μE m^{-2} s^{-1}; NSW	Villareal (1990)
Rhizosolenia clevei	0.44	Laboratory: N-deplete media; light = 83μE m^{-2} s^{-1}; NSW	Villareal (1990)
Hemiaulus haukii	3.8	Laboratory Fluorescence	Brand & Guillard (1981)
Hemiaulus haukii	4.3	*in situ*: Florida	Vargo (1983)
Hemiaulus haukii	0.40-0.41	Laboratory; NSW + L1 medium	Brown & Villareal (unpublished)
Hemiaulus sinensis	1.99	Laboratory Fluorescence; ASW	Brand et al. (1983)
Hemiaulus spp.	2.2	*in situ:* Great Barrier Reef	Furnas (1991)
Hemiaulus membranaceus	0.30-0.32	Laboratory; NSW + L1 medium	Brown & Villareal (unpublished)
H. haukii	0.61-0.68	Laboratory; NSW + L1 medium (modified, no NH_4)	Johnson & Villareal (unpublished)
H. haukii	0.43-0.63	Laboratory; NSW + L1 medium (modified, No added Nitrogen)	Johnson & Villareal (unpublished)

Table 3. Literature rates of N_2-fixation. "fw" = freshwater; "ASW" = artificial seawater; "NPSG" = North Pacific Subtropical Gyre;" "DDA" = Diatom-Diazotroph Association.

Diazotroph Taxon	Morphological Group	N2-Fixation Rate	Reference
Hemiaulus haukii-Richelia intracellularis	Heterocystous DDA	8.1 x 10^5 fmolN L^{-1} day^{-1}	Foster et al. 2007
Hemiaulus sp.-*Richelia intracelluris*; *Rhizosolenia sp-R.intracellularis*	Heterocystous DDAs	0.16-1.24 nmolN L^{-1} day^{-1}	Bar-Zeev et al. 2008
Rhizosolenia-Richelia intracellularis	Heterocystous DDA	1.9 x 10^{-12} moles ethylene x $trichome^{-1}hr^{-1}$	Mague et al. 1974
Rhizosolenia-Richelia intracellularis	Heterocystous DDA	0.001-0.014 nmol ethylene x $trichome^{-1}hr^{-1}$	Villareal 1990
Hemiaulis haukii, H. sinensis-Richelia intracellularis	Heterocystous DDA	3.0 & 5.3 x 10^{-13} mol ethylene $cell^{l-1}hr^{-1}$	Villareal 1991
Hemiaulus membranaceus	Heterocystous DDA	2.3 & 3.6 x 10^{-13} mol ethylene $cell^{-1}hr^{-1}$	Villareal 1991
Anabaena sp.	Heterocystous cyanobacterium	1.1 x 10^{-14}-7.7 x 10^{-11} mole ethylene*$heterocyst^{-1}$*hr^{-1}	Horne & Goldman 1972; Horne et al. 1972
Anabaena sp.	Heterocystous cyanobacterium	4.7 x10^{-10} mol ethylene*(µg Chl.-*a*)hr^{-1}	Villareal 1987
Oscillatoria erythraea	Heterocystous cyanobacterium	1.4-8.3 x 10^{-11} mol ethylene*(µg Chl.-a)hr^{-1}	Villareal 1987
Azolla caroliniana	Heterocystous DDA	49.6- (~) 95.0 µmol*$g^{-1}h^{-1}$	Hechler & Dawson 1995
Mixed Species: whole water sample (NPSG)	Heterocystous; non-heterocystous filamentous; unicellular	0.03-0.05 nmol N $L^{-1}hr^{-1}$	Zehr et al. 2007
>100µm size fraction (Arafura Sea)	Unicellular	20.0-62.0-0.05 nmol N $L^{-1}hr^{-1}$	Montoya et al. 2004
Mixed Species: whole water sample **(fw)**	Heterocystous; non-heterocystous; unicellular	0.01-5.1 µmol ethylene L^{-1} hr^{-1}	Storch et al. 1990
Rhizosolenia-Richelia intracellularis; R.intracellularis; Trichodesmium sp.	Heterocystous DDA; heterocystous cyanobacteria; non-heterocystous filamentous	0.01-1.0 nmol N $L^{-1}hr^{-1}$	White et al. 2007
Mixed heterocyst species: *Anabaena spiroides, Cylindrospermapsis stagnale, Anabaenopsis circularis* **(fw)**	Heterocystous	31-37 nmol N*10^6 $heterocysts^{-1}hr^{-1}$	Levine & Lewis 1984
Crocosphaera watsonii	Unicellular	0.1-1.5 fmol ethylene*$cell^{-1}hr^{-1}$	Mohr et al. 2010
Gloeothece sp. 68DGA	Unicellular	1.0-2.6 fmol ethylene*$cell^{-1}$*hr^{-1}	Taniuchi et al. 2008
Cyanothece sp. ATCC 51142	Unicellular	196 nmol N*$10^8$$cells^{-1}hr^{-1}$	Toepel et al. 2008
Trichodesmium sp.	Non-heterocystous filamentous	0.44-2.41 nmol N*$col^{-1}hr^{-1}$	Mulholland et al. 2006
Trichodesmium thiebautii	Non-heterocystous filamentous	60 nmol ethylene*(µg Chl.-*a*)$^{-1}h^{-1}$	Saino & Hattori 1978
Trichodesmium thiebautii	Non-heterocystous filamentous	1.5 nmol N *$col^{-1}hr^{-1}$	Capone et al. 1990
Trichodesmium thiebautii	Non-heterocystous filamentous	~4-9 nmol N*$col^{-1}day^{-1}$	Wyman et al. 1996

Table 4. Nitrogen-source Experiment: Percent Symbioses in the three treatments over the course of the experiment (no added nitrogen, added nitrate, and added ammonium).

Percent Symbioses (Symbiotic *Hemiaulus haukii*)					
Treatment	Day 0	Day 6	Day 10	Day 15	Day 20
No-N	77	82	83	74	66
+ NO_3	76	86	88	81	63
+ NH_4	64	71	67	52	51

Table 5. Growth rates for the Nitrogen-source experiment. Table includes divisions per day, and the standard error and R^2 values are reported around this value.

Treatment Medium	Divisions day^{-1}	R^2 (div day^{-1})
No Nitrogen	0.23 ± 0.09	0.90
Nitrate (40 µM)	0.30 ± 0.08	0.95
Ammonium (10 µM)	0.42 ± 0.09	0.97

Table 6. Growth rates for asymbiotic cells in the N-source experiment. Table includes divisions per day, and the standard error and R^2 values are reported around this value.

Treatment	Divisions/day^{-1}	R^2 (div day^{-1})
No-N	0.20 ± 0.18	0.60
+NO_3	0.27 ± 0.19	0.77
+NH_4	0.37 ± 0.27	0.69

Table 7. Growth rates for fixed light level diurnal studies and light gradient studies. Table includes divisions/day for *H. haukii* and *H.membranaceus.* The standard error and R^2 values are reported around this value.

Species	Experiment	Maximum Divisions day^{-1}	R^2 (div day^{-1})
H. haukii	Fixed Light (200 µmol quanta $m^{-2}sec^{-1}$)	0.35 ± 0.05	0.94
H. haukii	Light Gradient High Light (200 µmol quanta $m^{-2}sec^{-1}$)	0.44 ± 0.08	0.82
H. haukii	Light Gradient Low Light (50 µmol quanta $m^{-2}sec^{-1}$)	0.43 ± 0.09	0.95
H. membranaceus	Fixed Light (200 µmol quanta $m^{-2}sec^{-1}$)	0.56 ± 0.10	0.98
H. membranaceus	Light Gradient High Light (200 µmol quanta $m^{-2}sec^{-1}$)	0.53 ± 0.05	0.96
H. membranaceus	Light Gradient Low Light (50 µmol quanta $m^{-2}sec^{-1}$)	0.40 ± 0.06	0.98

Table 8. Summary of growth rates for the *H. haukii* irradiance curve. Table includes divisions per day, and the standard error and R^2 values are reported around this value.

Light Level (μmol)	Divisions day^{-1}	R^2 (div day^{-1})
30	0.19 ± 0.16	0.73
50	0.37 ± 0.09	0.88
75	0.52 ± 0.07	0.98
150	0.82 ± 0.05	0.99
200	0.85 ± 0.06	0.98
350	0.87 ± 0.08	0.96
500	0.99 ± 0.10	0.96

Table 9. Summary of growth rates for the *H. membranaceus* growth-irradiance curve. Table includes divisions per day, and the standard error and R^2 values are reported around this value.

Light Level (µmol quanta $m^{-2}sec^{-1}$)	Divisions day^{-1}	R^2 (div day^{-1})
30	-0.47 ± 0.20	0.43
50	0.82 ± 0.23	0.66
75	0.15 ± 0.09	0.25
150	-1.29 ± 0.40	0.61
200	0.65 ± 0.09	0.88
350	1.06 ± 0.19	0.83
500	-1.30 ± 0.46	0.64

Table 10: μ_{max}, α, I_C, I_K and R^2 for *Hemiaulus haukii* and *Hemiaulus membranaceus* Growth-Irradiance curves. "NA" = Not Applicable.

Experimental Curve	μ max (div day^{-1})	α (div day^{-1}/μmol quanta $m^{-2}sec^{-1}$)	I_c (μmol)	I_k (μmol)	R^2
H. haukii growth-irradiance	9.96×10^{-1}	9.98×10^{-3}	10	100	0.99
H. membranaceus growth-irradiance	NA	NA	NA	NA	NA

Table 11: N_2-fixation$_{max}$, α, I_C, I_K and R^2 for *Hemiaulus haukii* and *Hemiaulus membranaceus* N_2-Irradiance curves. NA = Not Applicable.

Experimental Curve	**N_2-Fix$_{max}$** (nmol N_2 Heterocyst^{-1}min^{-1})	**α** (nmol N_2 heterocyst^{-1}min^{-1}/µmol quanta m^{-2}sec^{-1})	**I_C** (µmol quanta m^{-2}sec^{-1})	**I_K** (µmol quanta m^{-2}sec^{-1})	R^2
H. haukii N_2-irradiance (High Light, 200 µmol quanta m^{-2}sec^{-1})	2.33 x 10^{-6}	1.03 x 10^{-8}	NA	226	0.95
H. haukii N_2-irradiance (Low Light, 50 µmol quanta m^{-2}sec^{-1})	2.14 x 10^{-6}	2.01 x 10^{-8}	NA	107	0.76
H. membranaceus N_2-irradiance (High Light, 200 µmol quanta m^{-2}sec^{-1})	6.09 x 10^{-6}	4.23 x 10^{-8}	NA	144	0.95
H. membranaceus N_2-irradiance (Low Light, 50 µmol quanta m^{-2}sec^{-1})	2.77 x 10^{-6}	2.47 x 10^{-8}	NA	112	0.91

Figures

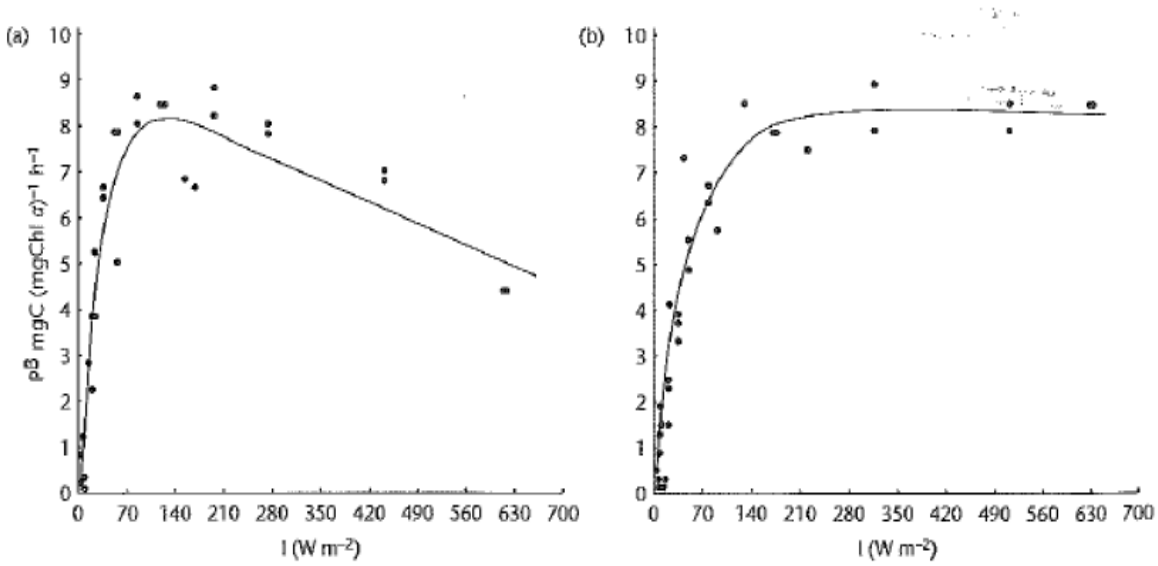


Figure 1. General photosynthesis vs. irradiance curve. a.) diatom assemblage exhibiting photoinhibition and b.) a dinoflagellate assemblage without photoinhibition (Platt et al. 1980)

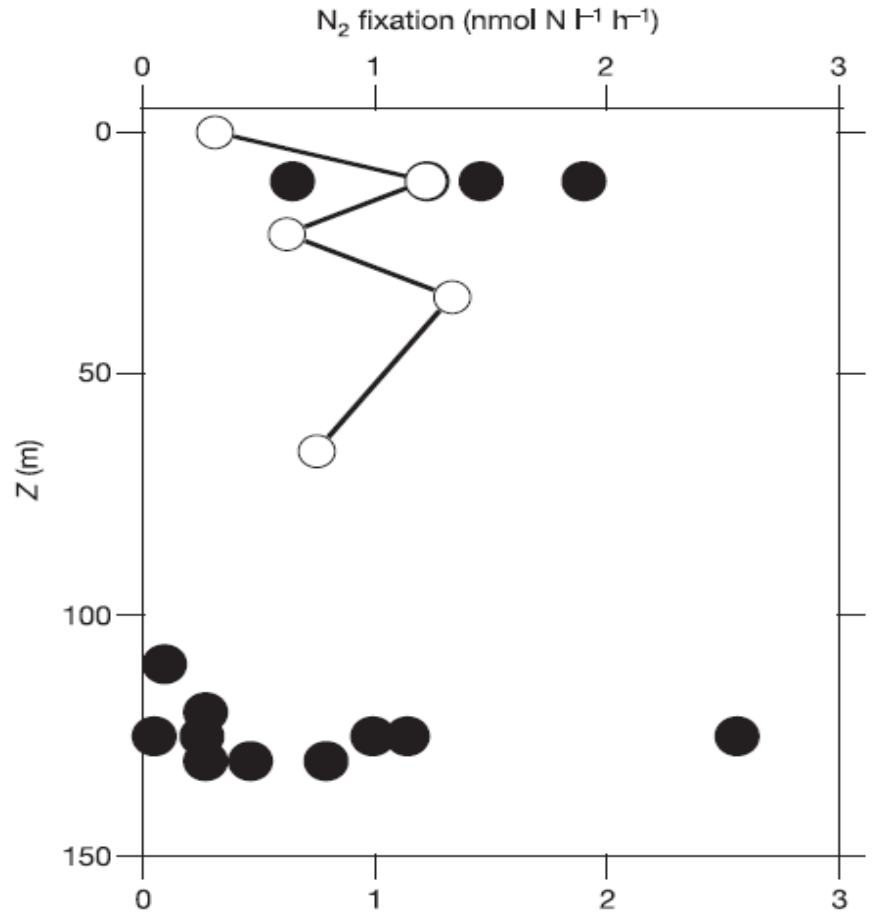


Figure 2. Nitrogen fixation vs. depth. Open circles= N_2-fixation measured at 5 depths in the mixed layer. Closed circles = N_2-fixation measured from samples collected below the subsurface pigment maximum (Montoya et al. 2004).

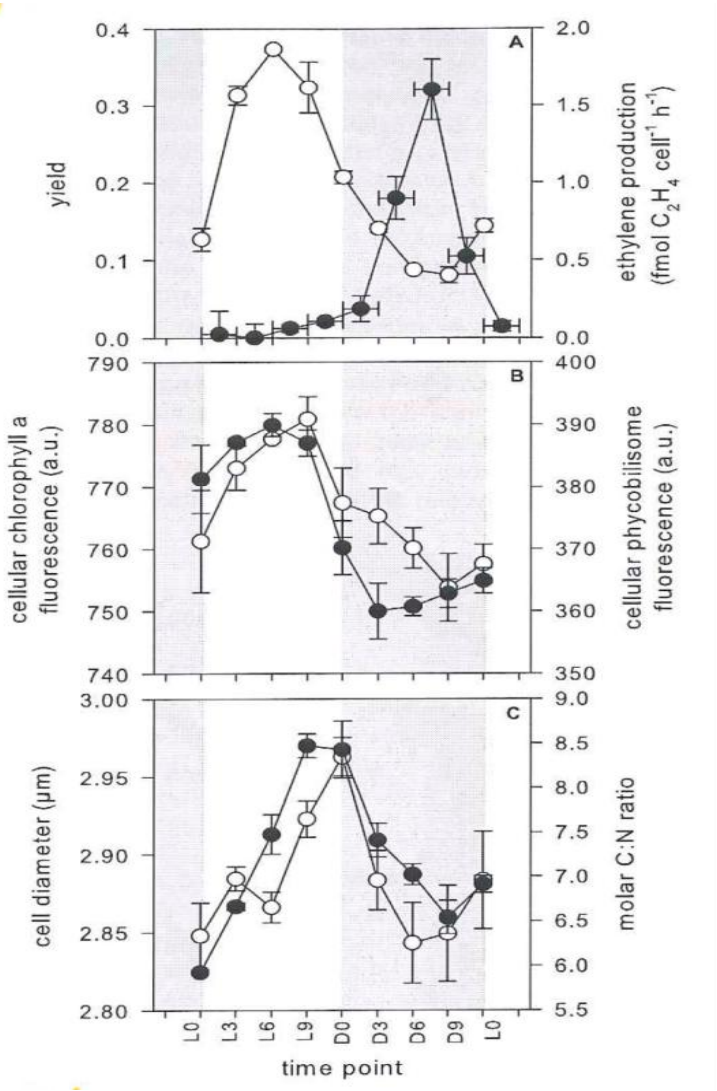


Figure 3. Physiological properties in *C. watsonii* WH8501 in light-dark conditions. Shaded areas correspond to dark period. A. Photosynthesis quantum yield (open circle) and N2 fixation (filled circle). B. cellular chlorophyll *a* (open circle) and phycobilisome (filled circle) fluorescence as measured through flow cytometry. C. Cell size (open circle) and molar C:N ratio (filled circle) as measured with a Coulter counter and an elemental analyzer, respectively (Mohr et al. 2010).

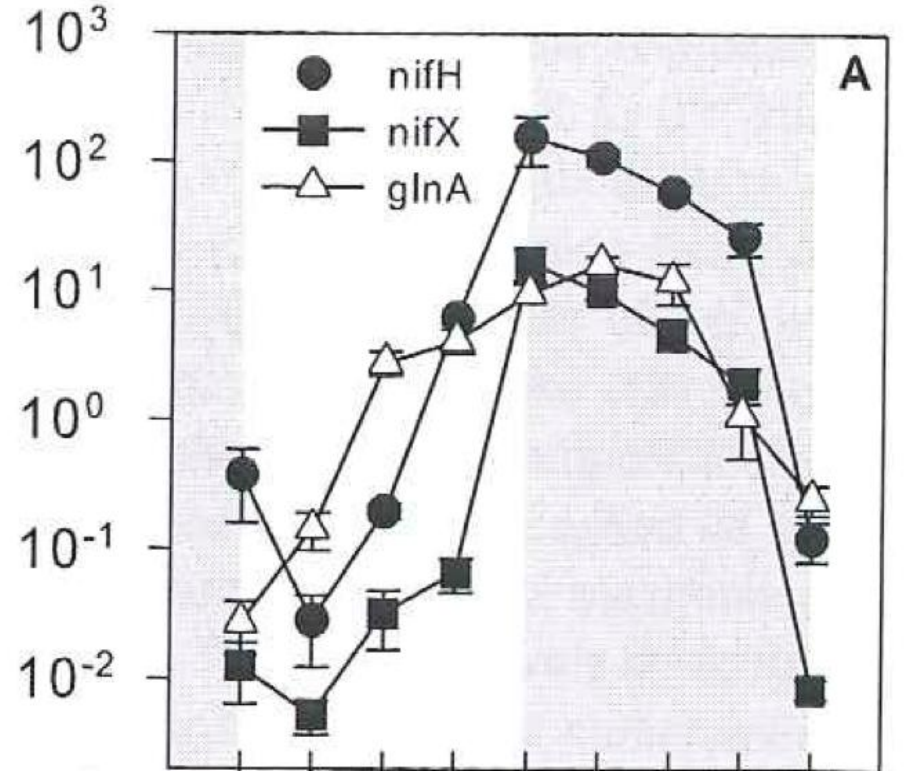


Figure 4. Relative enrichment in *nifH* : Enrichment factor delta Ct rpb1 (filled circle, y-axis), *nifX* (filled square) and *glnA* (open triangle) transcripts. Time point of sampling displayed on x-axis. Shaded region represents dark period (Mohr et al. 2010).

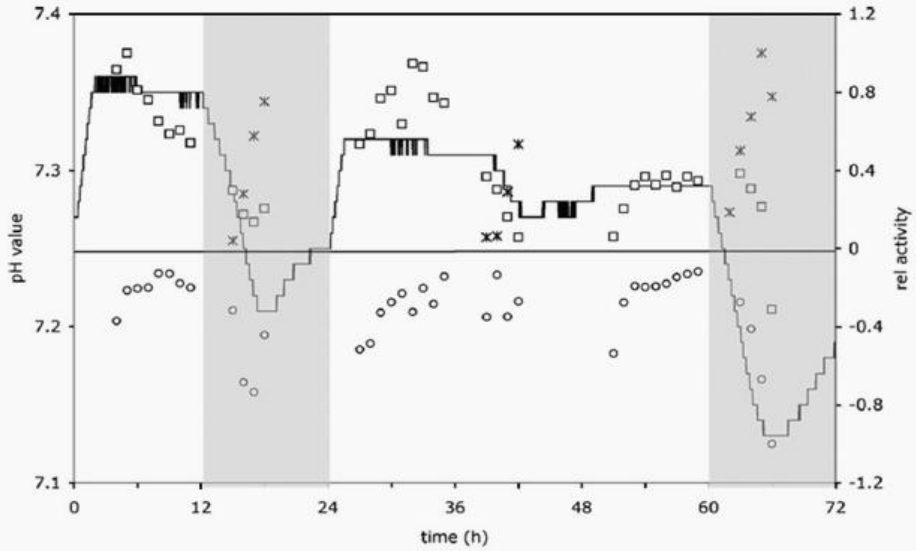


Figure 5. Physiological parameters (pH = solid line; N_2-Fixation = star; respiration = open circle; photosynthesis = open square) of *Cyanothece* sp. strain ATCC 51142 during a 12:12 L:D photoperiod, followed by a 36hr light period and 12 hr dark period (Toepel et al. 2008).

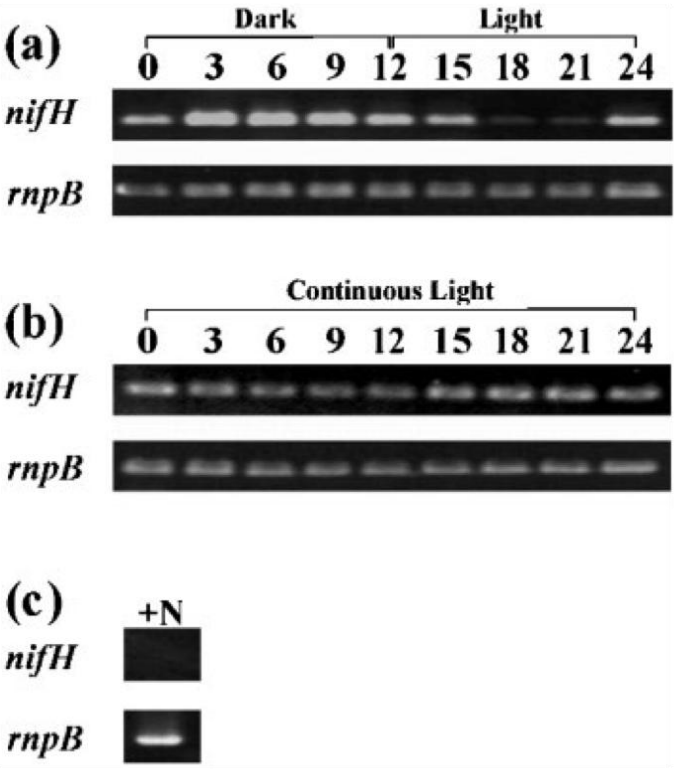


Figure 6. RT-PCR analysis of the *nifH* gene transcript. (a.) Cells from a culture grown under LD conditions. (b.) cells from a culture grown under CL conditions. (c.) negative control: *nifH* expression under added N conditions ($NaNO_3$). *rnpB* represents the control gene (Taniuchi et al. 2008).

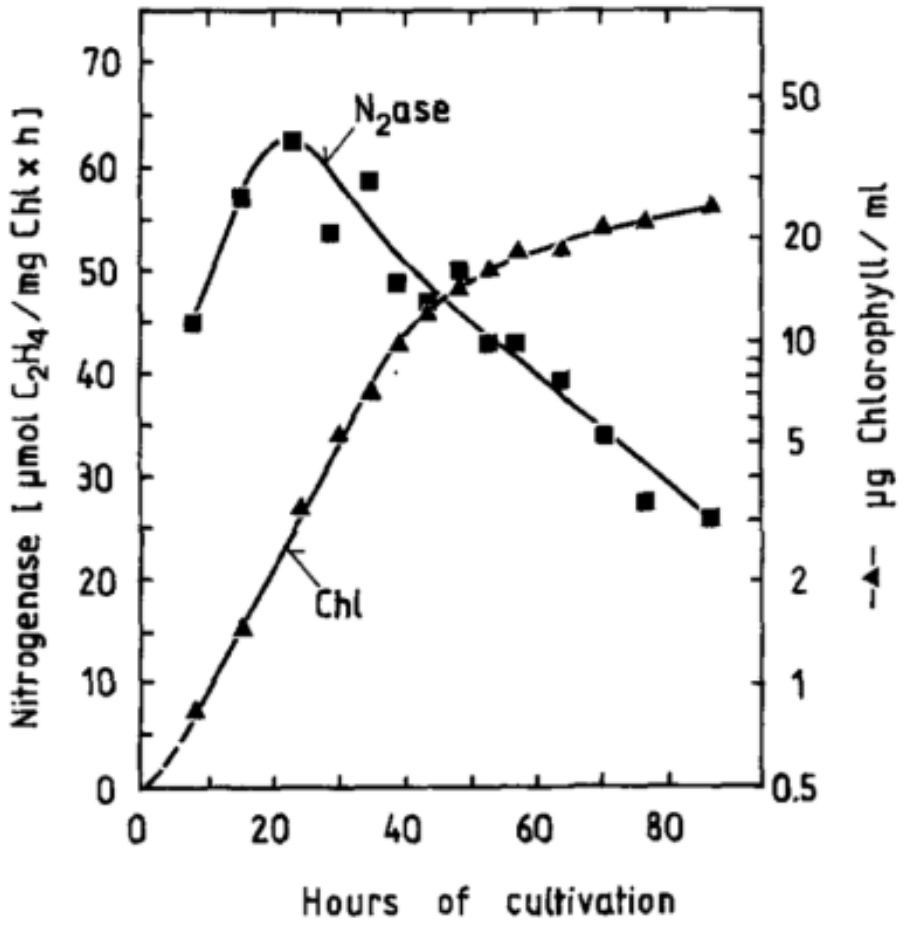


Figure 7. Growth of *Anabaena variabilis* in batch culture. Chlorophyll is given as a parameter to be compared with nitrogenase activity (ARA). Ernst et al. 1984.

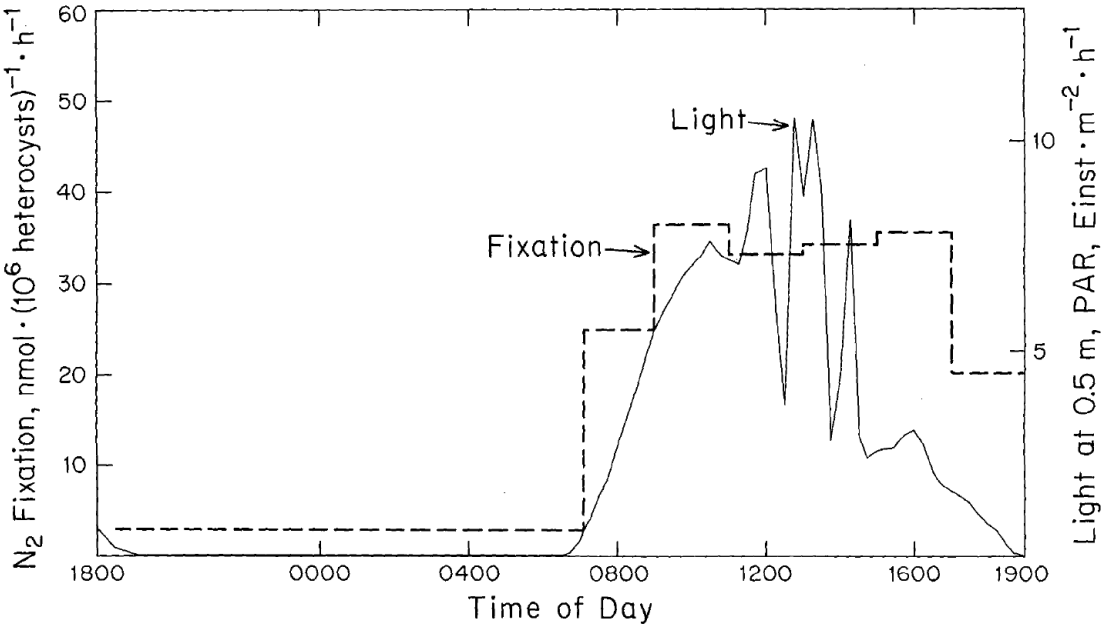


Figure 8. Diurnal variation of heterocyst-normalized N_2-fixation. Taken from Levine& Lewis 1984.

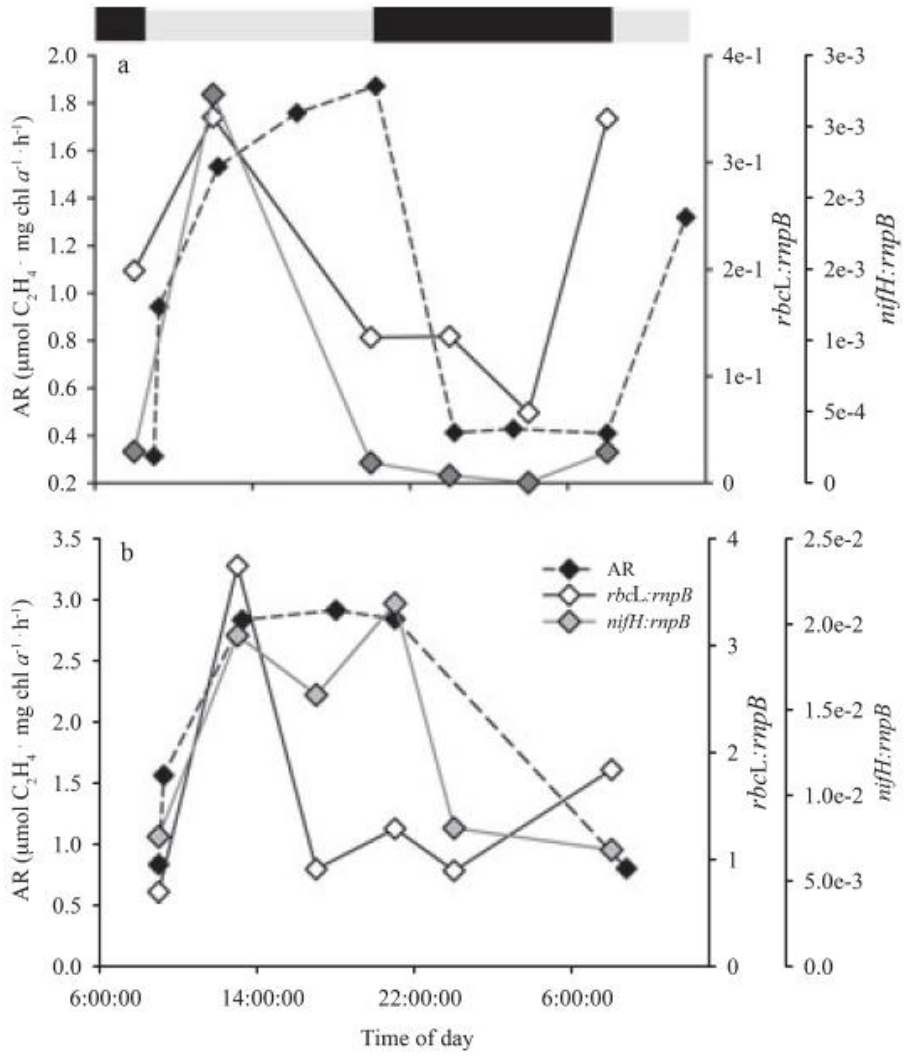


Figure 9. Temporal variability in N_2-fixation by *Calothrix rhizosoleniae* (Foster et. al. 2010).

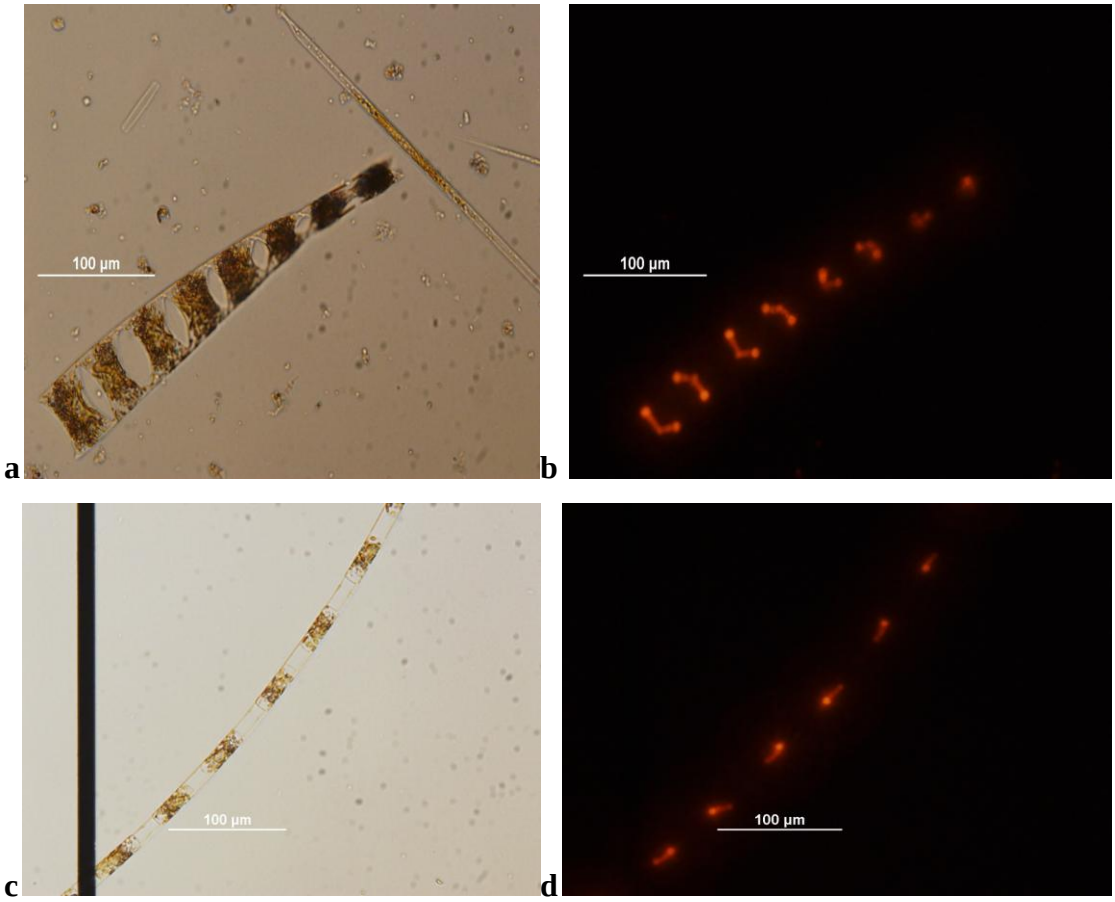


Figure 10. Photographs of *Hemiaulus haukii* and *Hemiaulus membranaceus.* **a**. *Hemiaulus membranaceus* under brightfield microscopy (BF). **b**. *Hemiaulus membranaceus* with symbionts visible under epifluorescent microscopy (EPI). **c**. *Hemiaulus haukii* under brightfield microscopy. **d**. *Hemiaulus haukii* with symbionts visible under epifluorescent microscopy. Photos by Amy E. Pyle (2011).

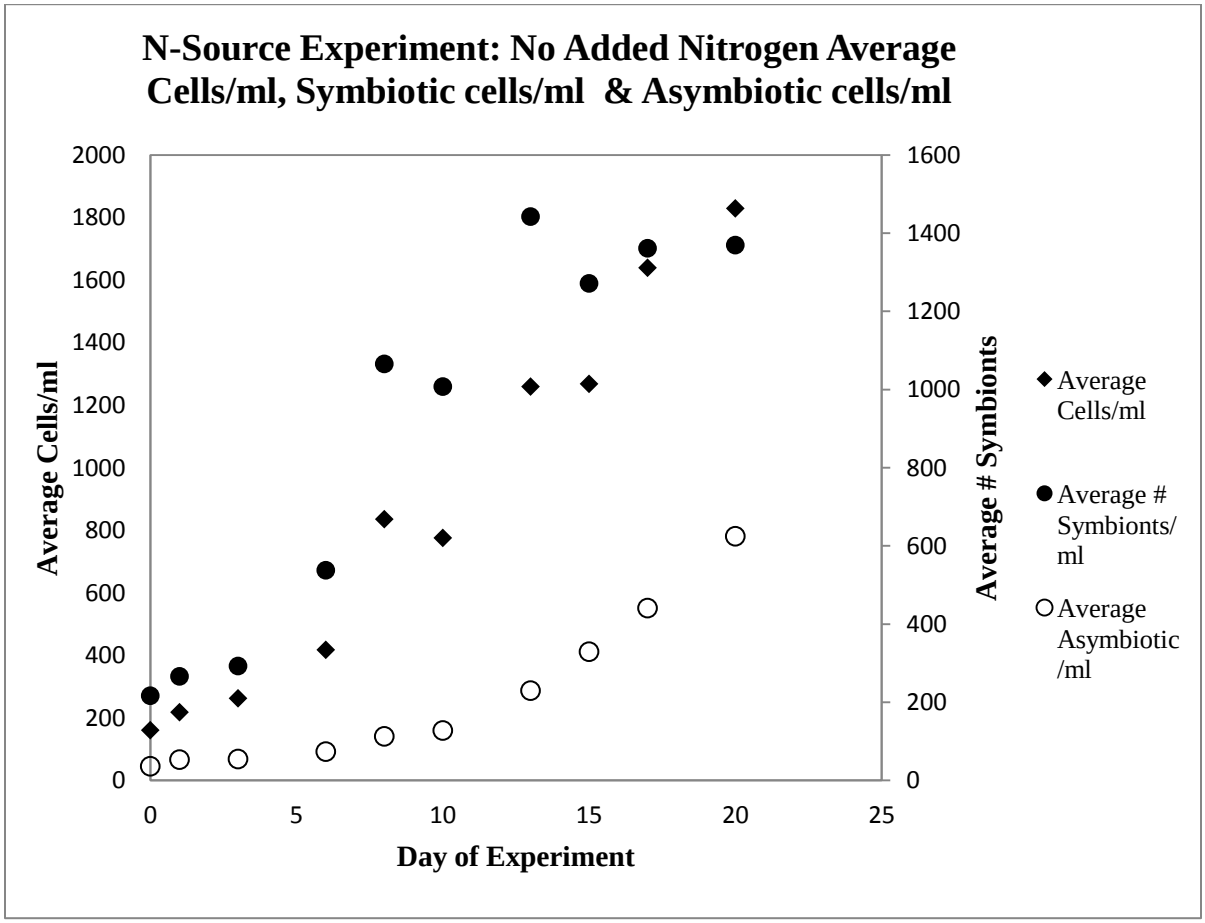


Figure 11: N-source experiment: $-NO_3^-$, $-NH_4^+$ medium average number of cells, symbiotic, and asymbiotic cells ml^{-1}.

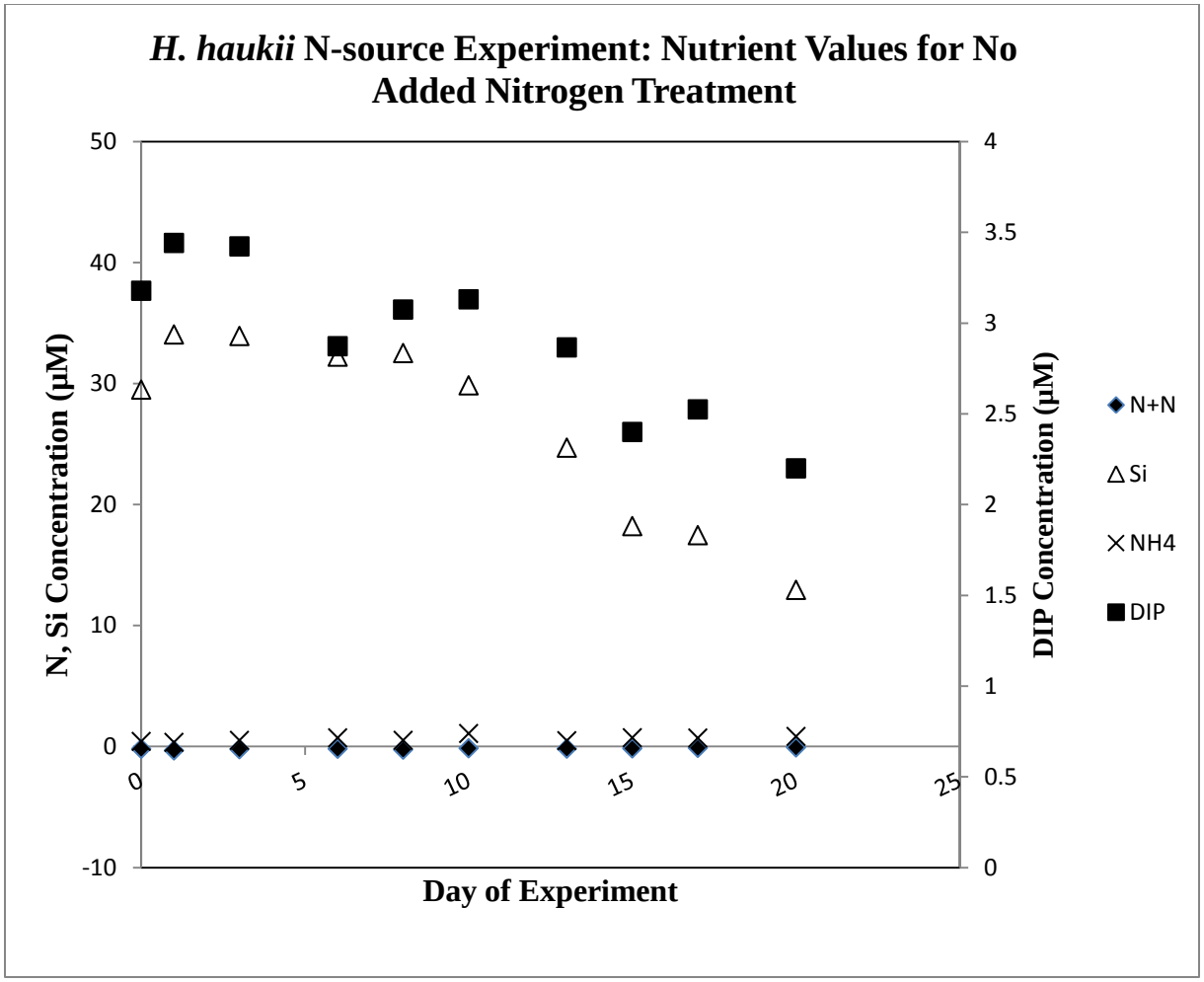


Figure 12. Nitrogen-source Experiment: $-NO_3^-$, $-NH_4^+$ medium nutrient concentrations. N+N = Nitrite + Nitrate; SiO_4^{-2} = Silicate; NH_4^+ = Ammonium; DIP = Dissolved Inorganic Phosphorus.

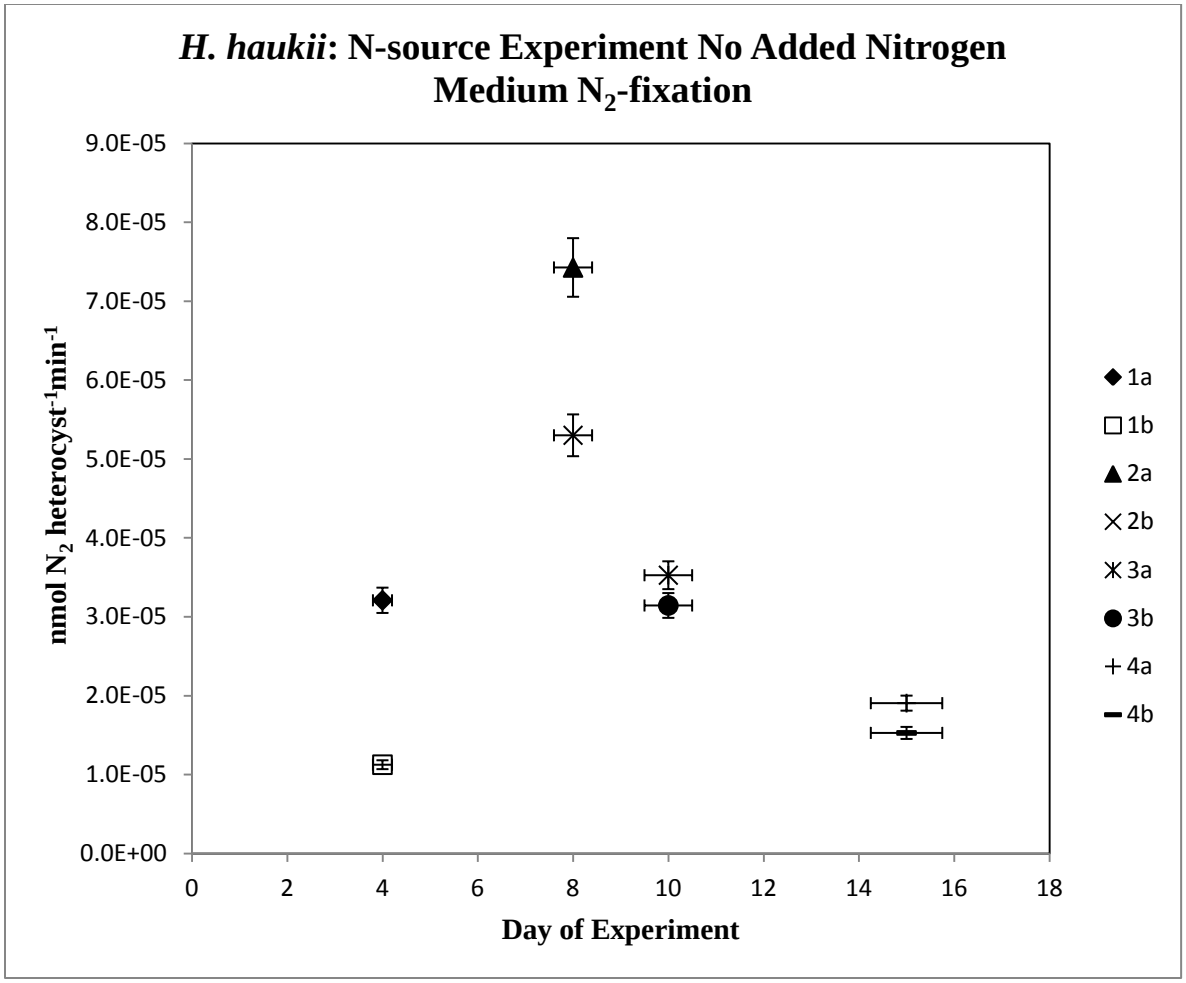


Figure 13. Nitrogen-source Experiment: $-NO_3^-$, $-NH_4^+$ medium N_2-fixation (nmol N_2 heterocyst^{-1}min^{-1}). The legend represents a given sample on each day.

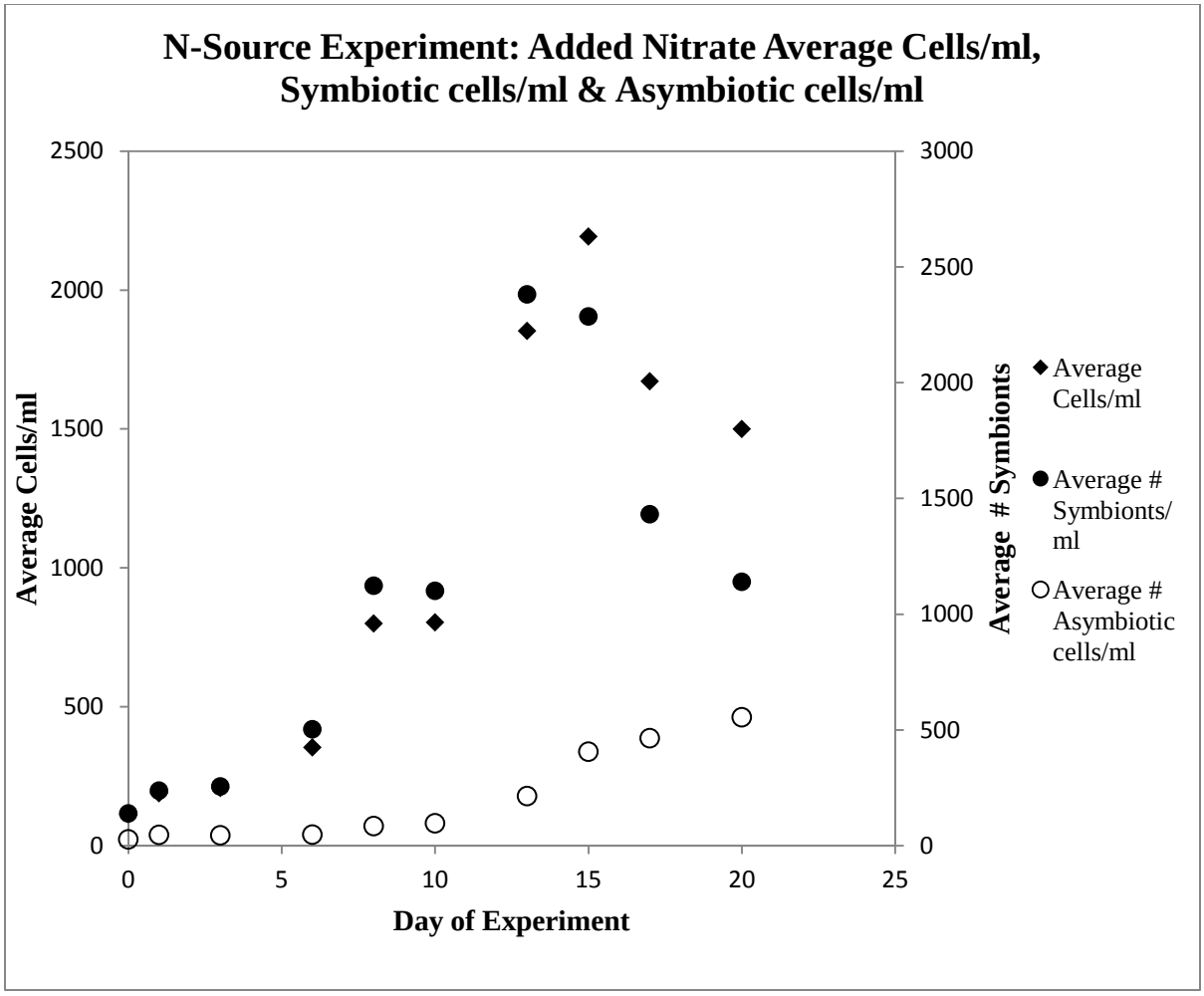


Figure 14: N-source experiment: $+NO_3^-$ medium average number of cells, symbiotic, and asymbiotic cells ml^{-1}.

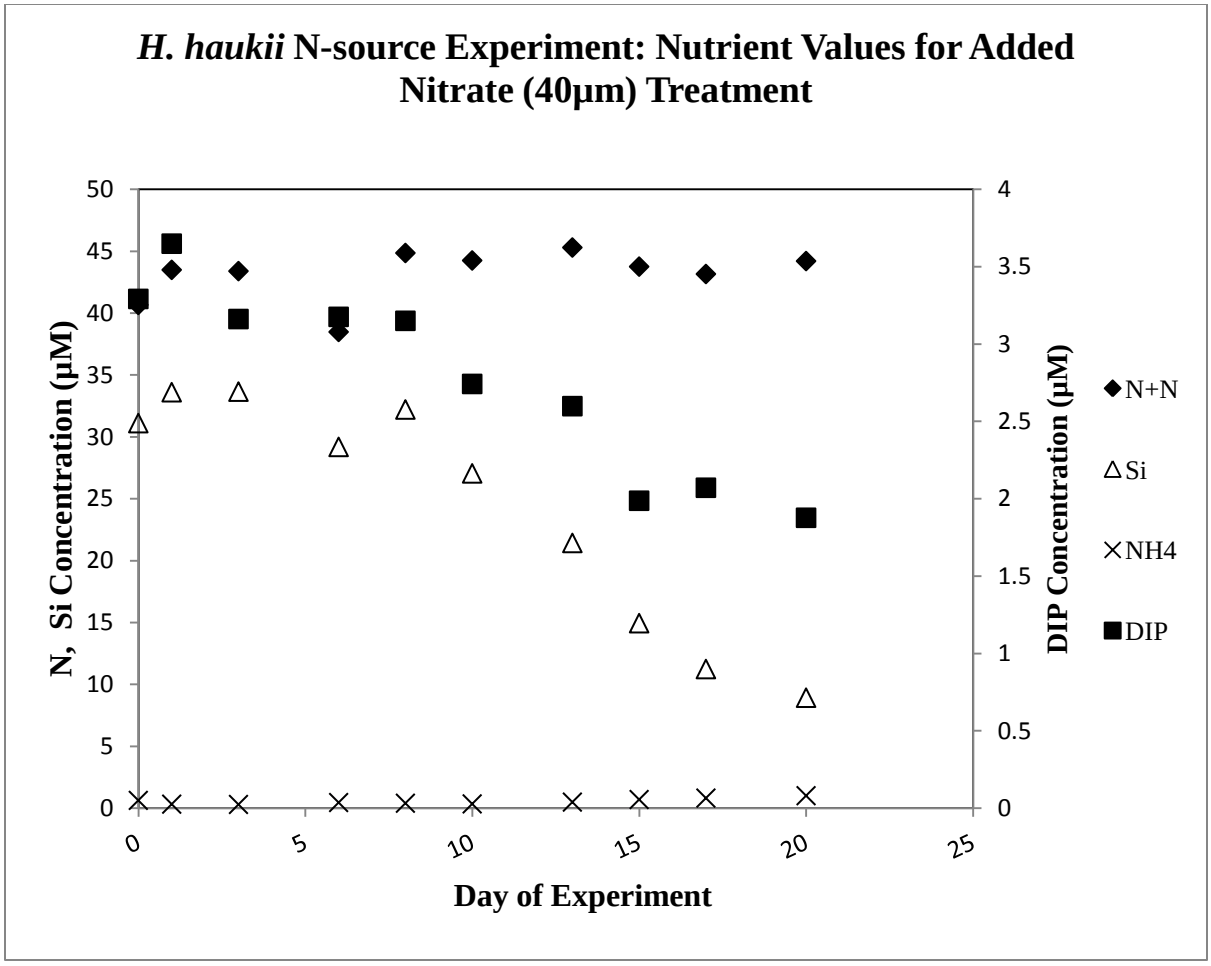


Figure 15: Nitrogen-source Experiment: + NO_3^- medium nutrient concentrations. N+N = Nitrite + Nitrate; SiO_4^{-2}= Silicate; NH_4^+ = Ammonium; DIP = Dissolved Inorganic Phosphorus.

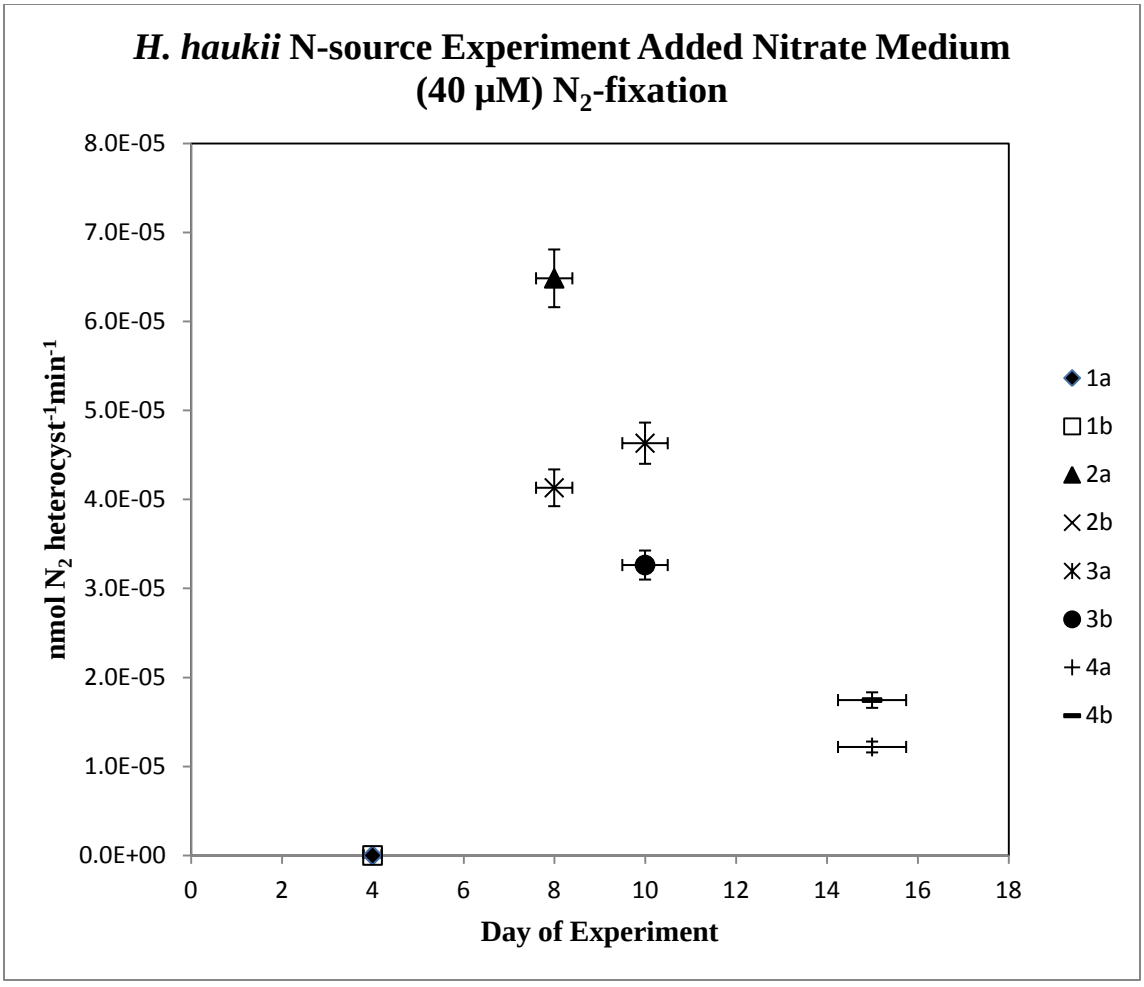


Figure 16: N-source experiment**:** $+NO_3^-$ medium N_2-fixation (nmol N_2 heterocyst^{-1} min^{-1}). The legend represents a given sample on each day.

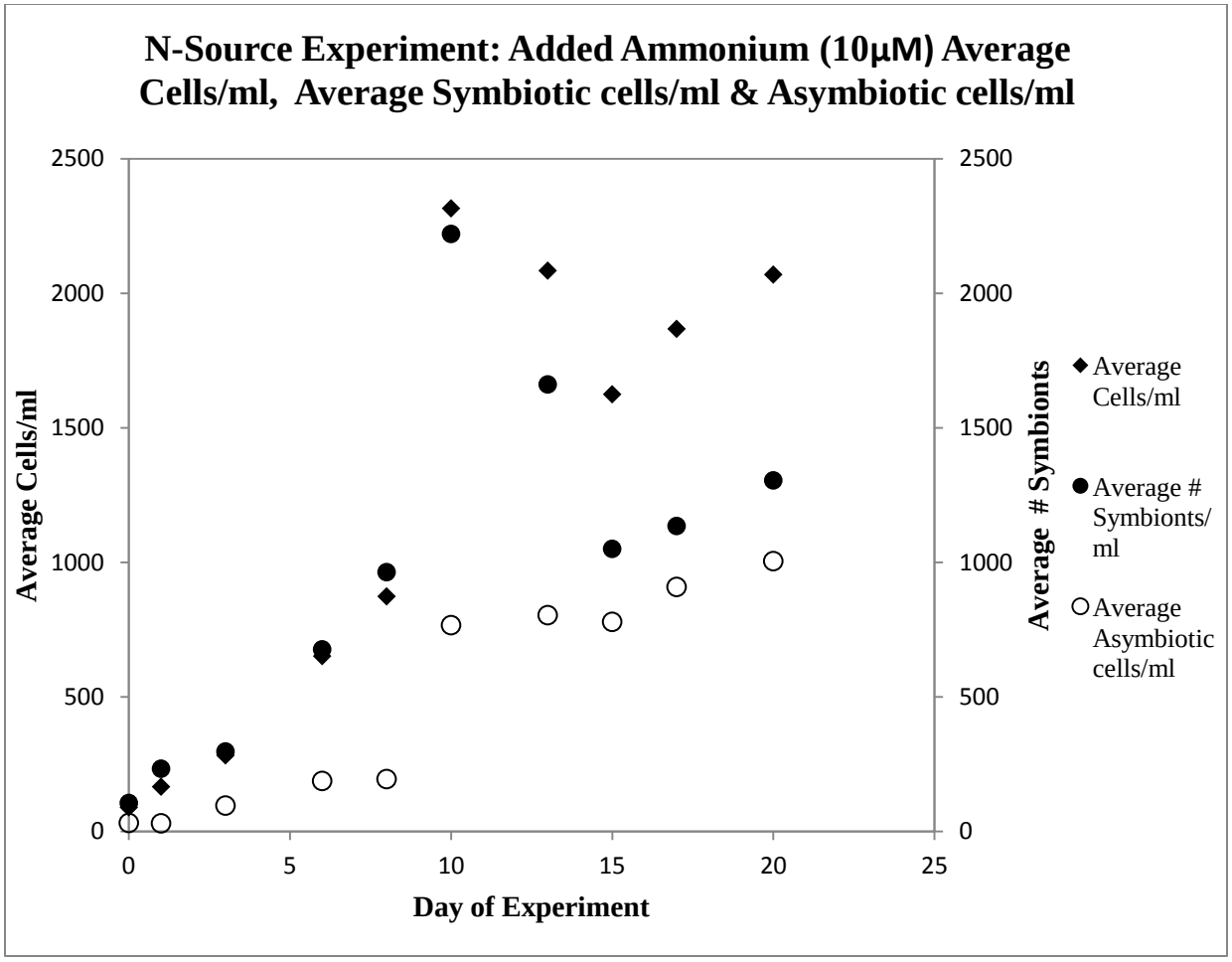


Figure 17: N-source experiment: $+NH_4^+$ average number of cells, symbiotic, and asymbiotic cells ml^{-1}.

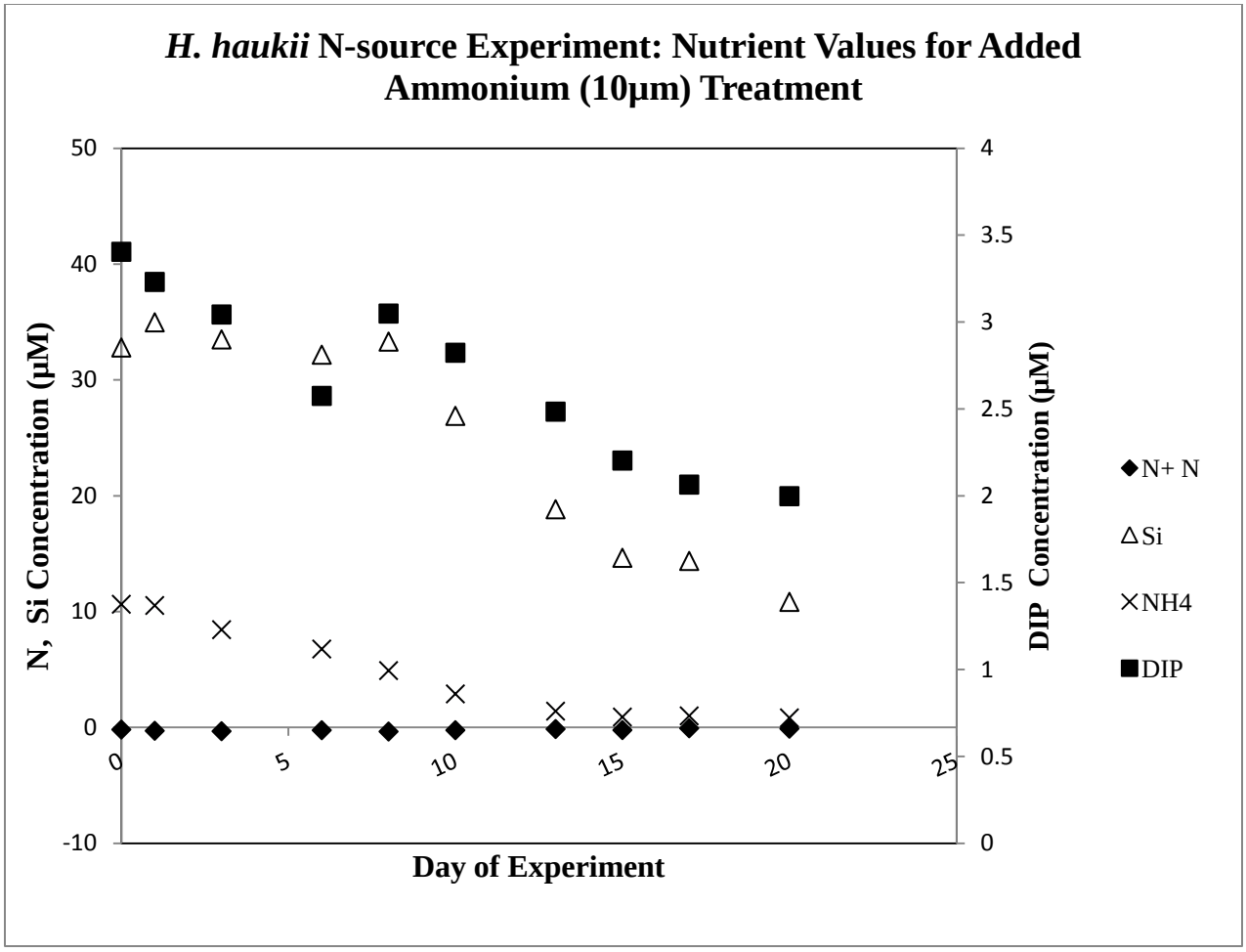


Figure 18: Nitrogen-source Experiment: $+NH_4^+$ medium nutrient concentrations.

N+N = Nitrite + Nitrate; SiO_4^{-2} = Silicate; NH_4^+ = Ammonium; DIP = Dissolved Inorganic Phosphorus.

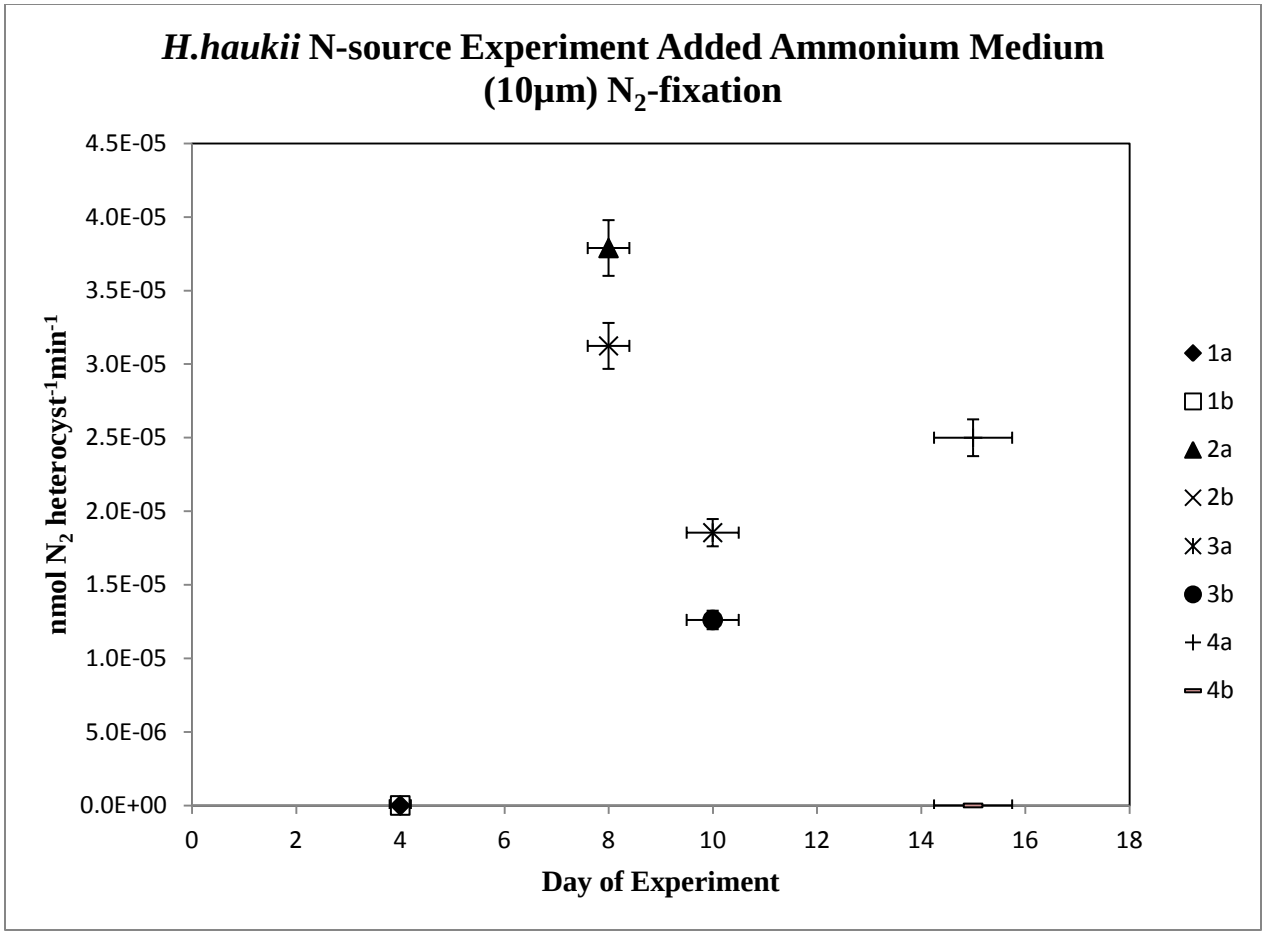


Figure 19: N-source experiment $+NH_4^+$ medium N_2-fixation (nmol N_2 heterocyst^{-1}min^{-1}). The legend represents a given sample on each day.

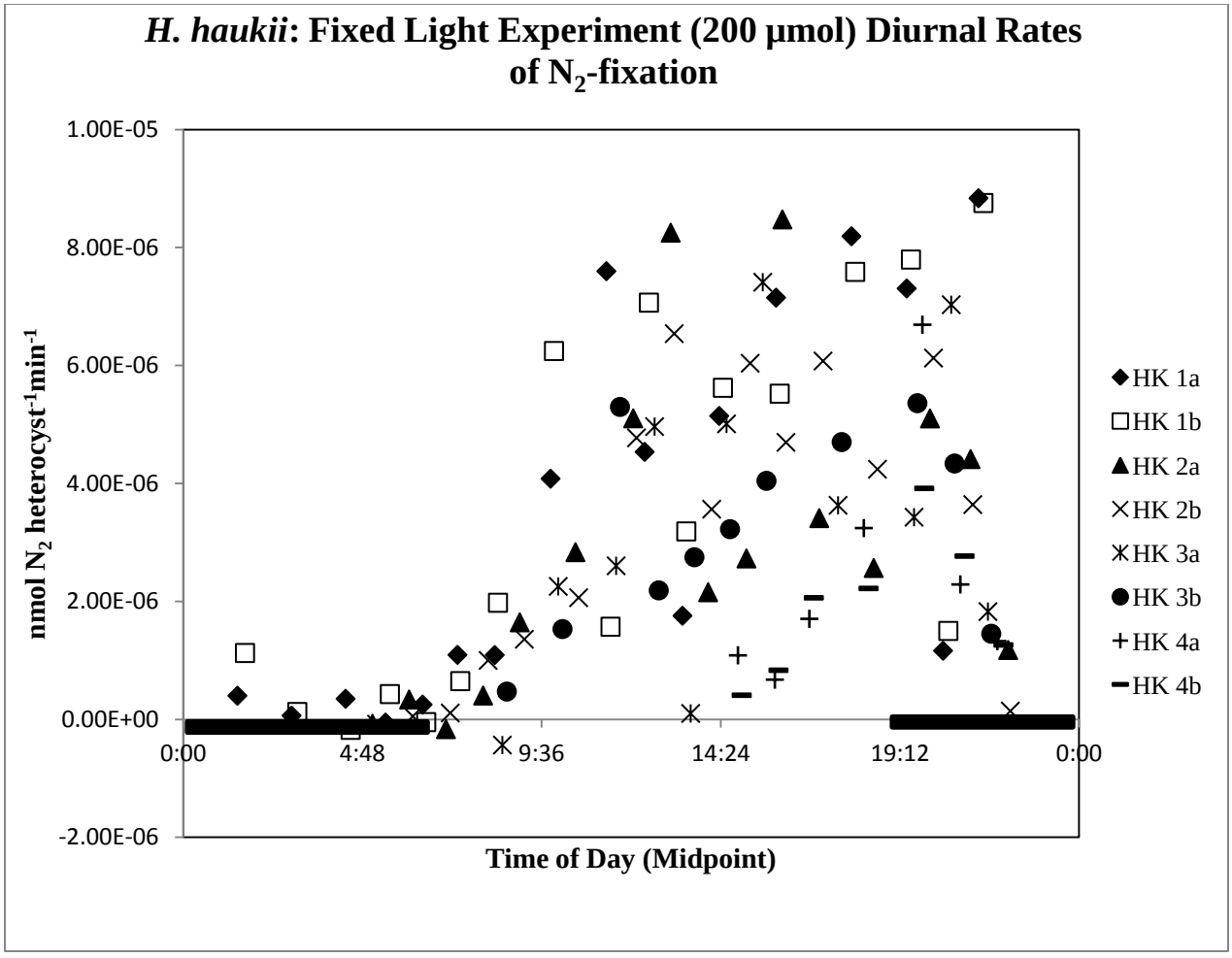


Figure 20. *H. haukii* diurnal rates of N_2-fixation (nmol N_2 heterocyst^{-1}min^{-1}) vs. time of day (represented as the midpoint of two consecutive sampling points). Dark bars indicate the dark portion of the photoperiod. "HK" = *Hemiaulus haukii.* Negative rates resulted from a control value higher than the sample value.

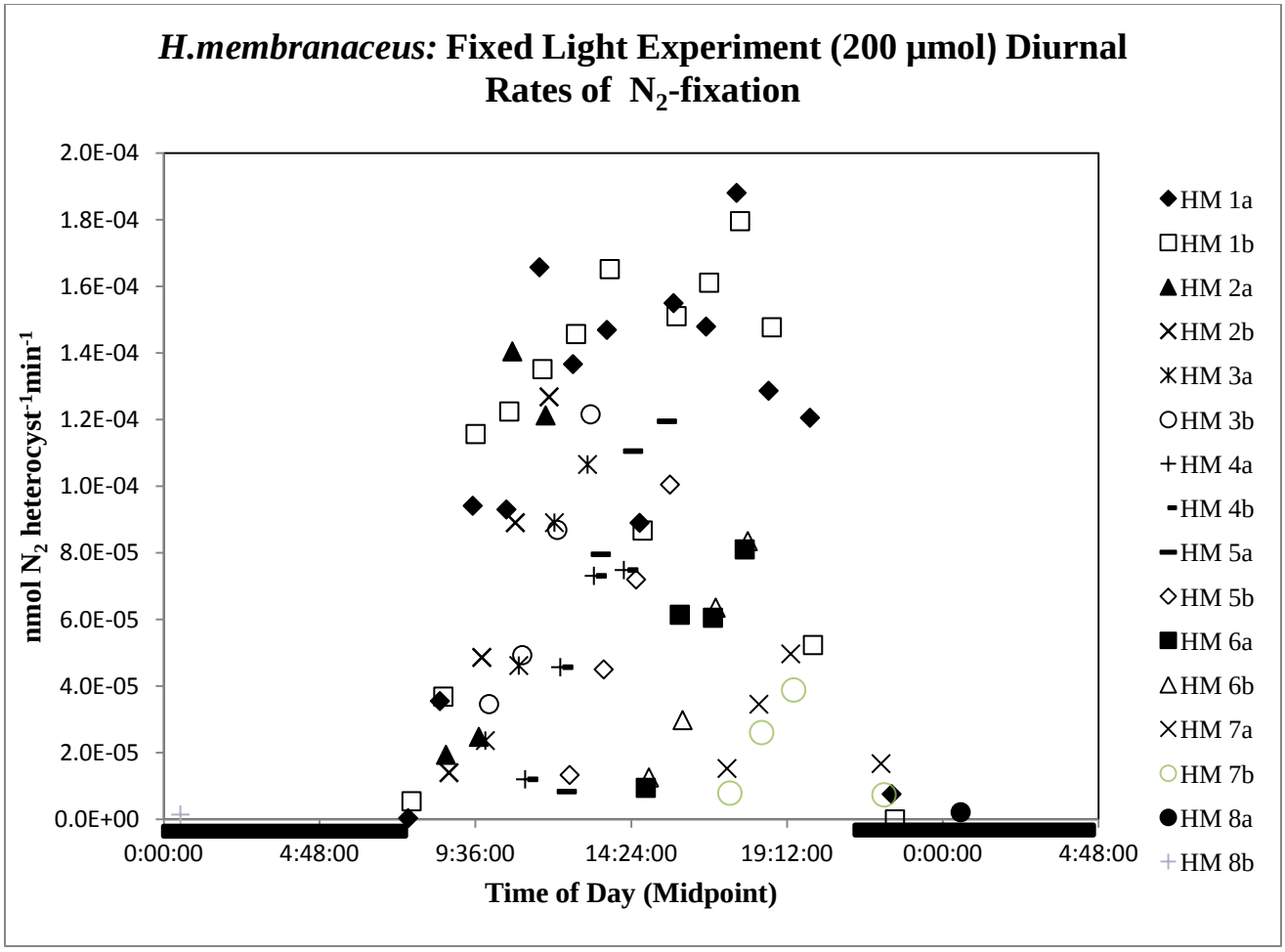


Figure 21. *H. membranaceus* diurnal rates of N_2-fixation (nmol N_2 heterocyst^{-1}min^{-1}) vs. time of day (represented as the midpoint of two consecutive sampling points). Dark bars indicate the dark portion of the photoperiod. "HM" = *Hemiaulus membranaceus*

H. haukii High (200 µmol) and Low (50 µmol) Adapted Cells N_2-fixation-Irradiance Curves

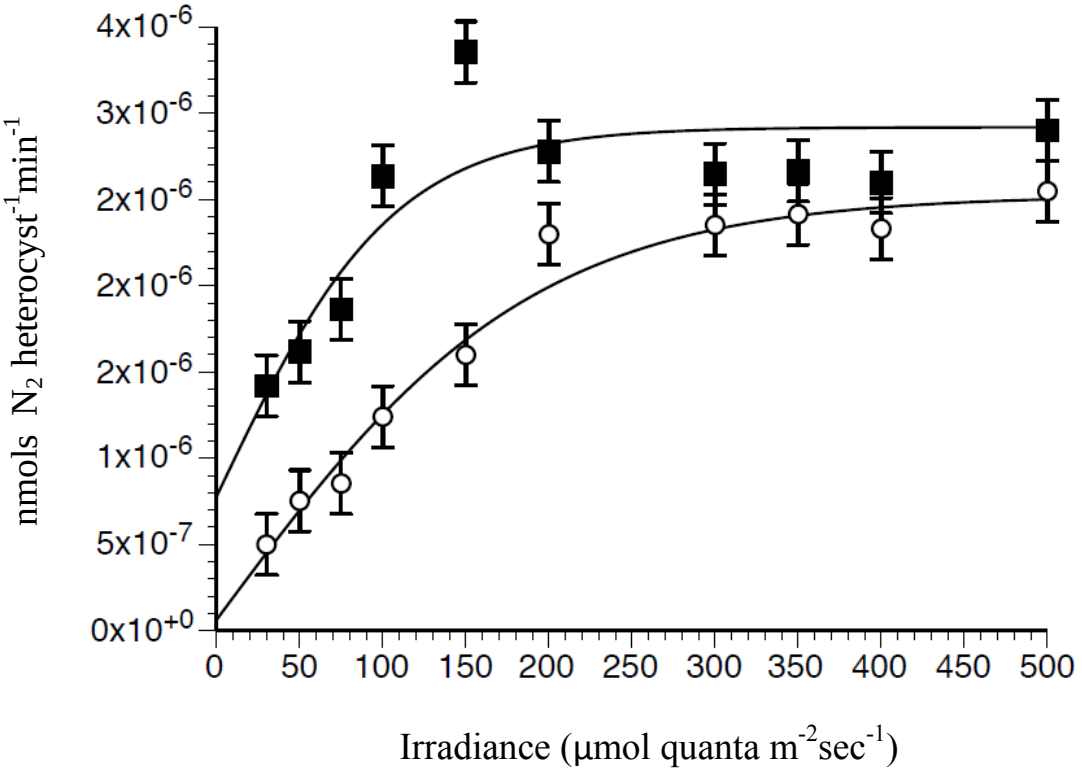


Figure 22. *H. haukii* N_2-fixation-irradiance curve. Curves represent comparison between *H. haukii* high (200 µmol, open circles) and low (50 µmol, closed squares) adapted cells. Both curves are fit to the Jassby-Platt hyperbolic tangent function.

H. membranaceus High (200 µmol) and Low (50µmol) light N_2-fixation-Irradiance Curves

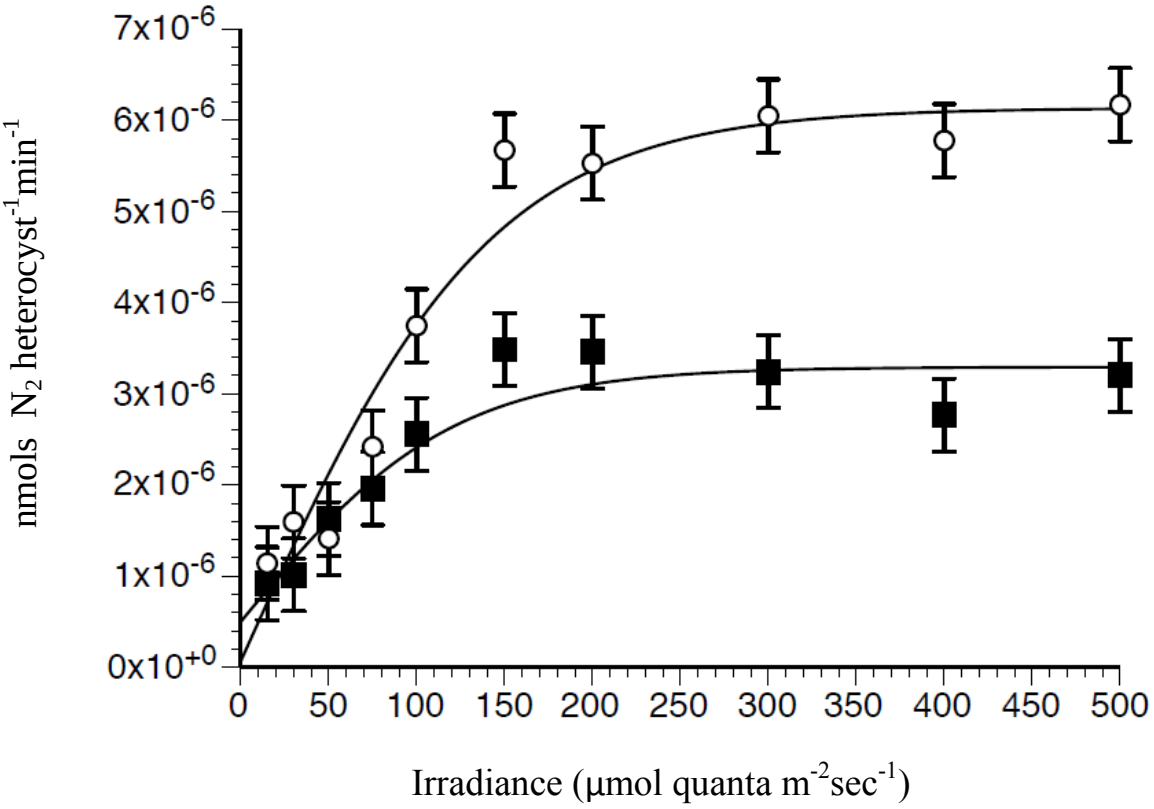


Figure 23. *H.membranaceus* N_2-fixation-irradiance curve. Curves represent comparison between *H. membranaceus* high (200 µmol, open circles) and low (50 µmol, closed squares) adapted cells. Both curves are fit to the Jassby-Platt hyperbolic tangent function.

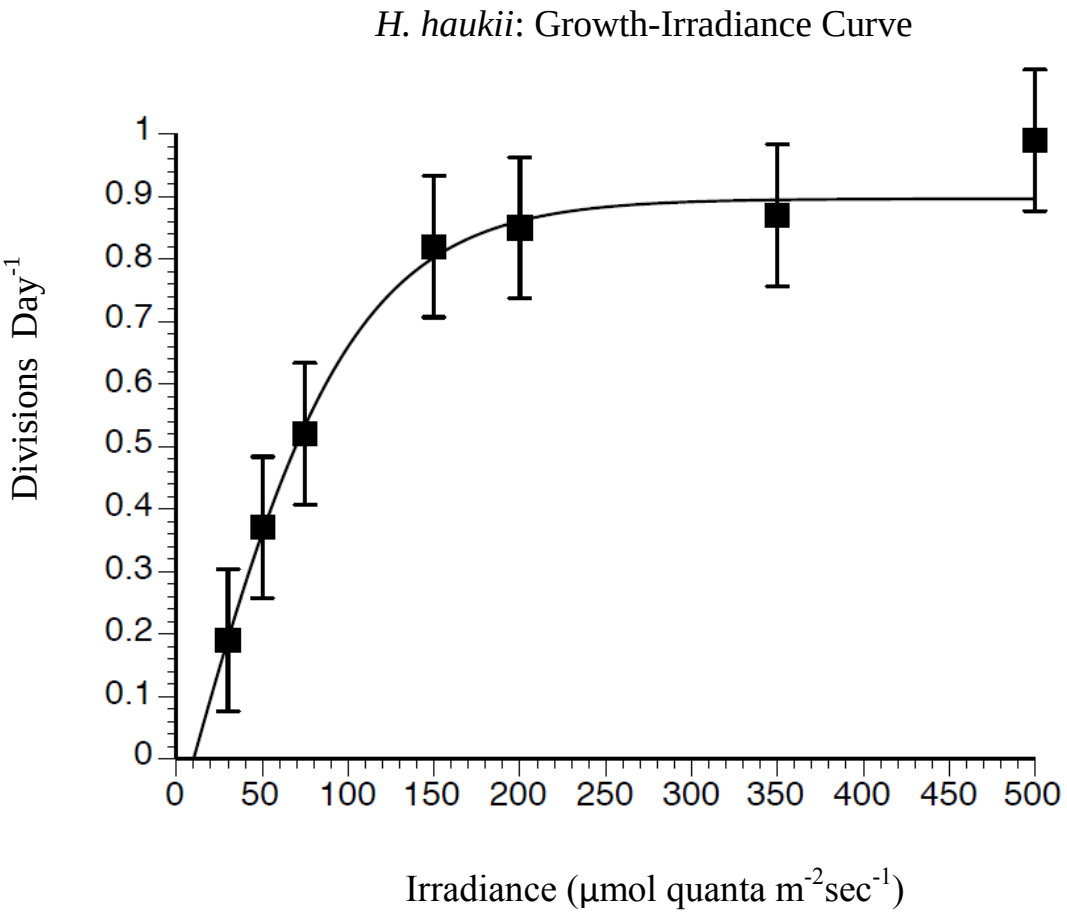


Figure 24: *H. haukii* growth-irradiance curve. Curve is fit to the Jassby-Platt hyperbolic tangential function (Jassby & Platt 1976).

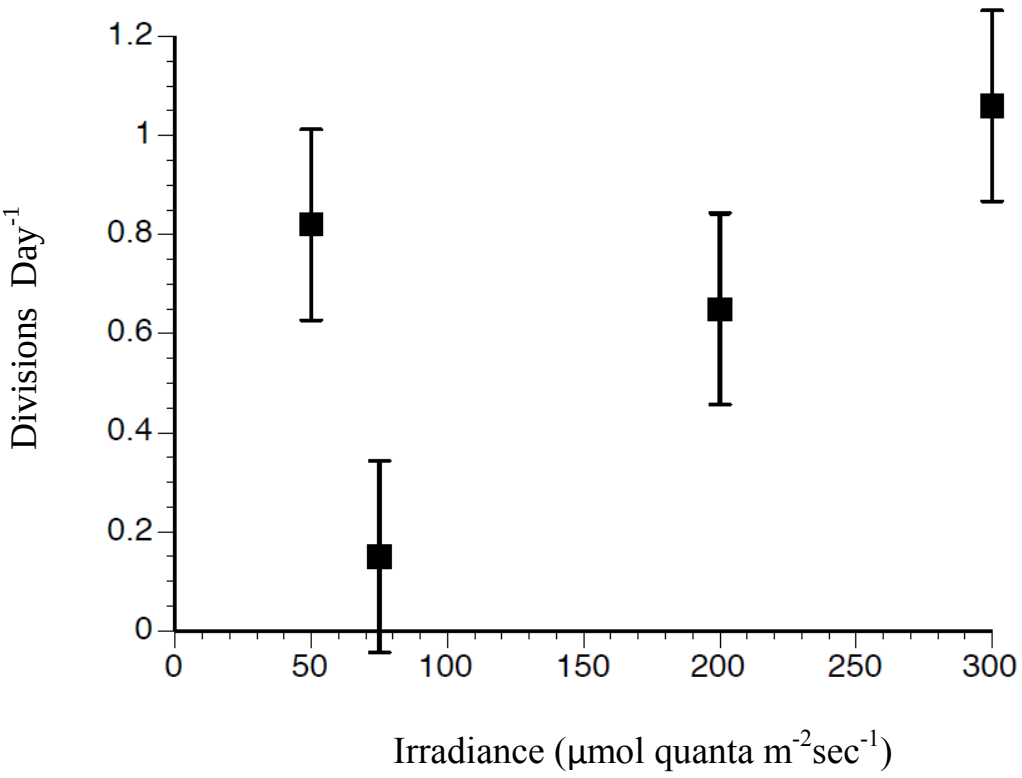
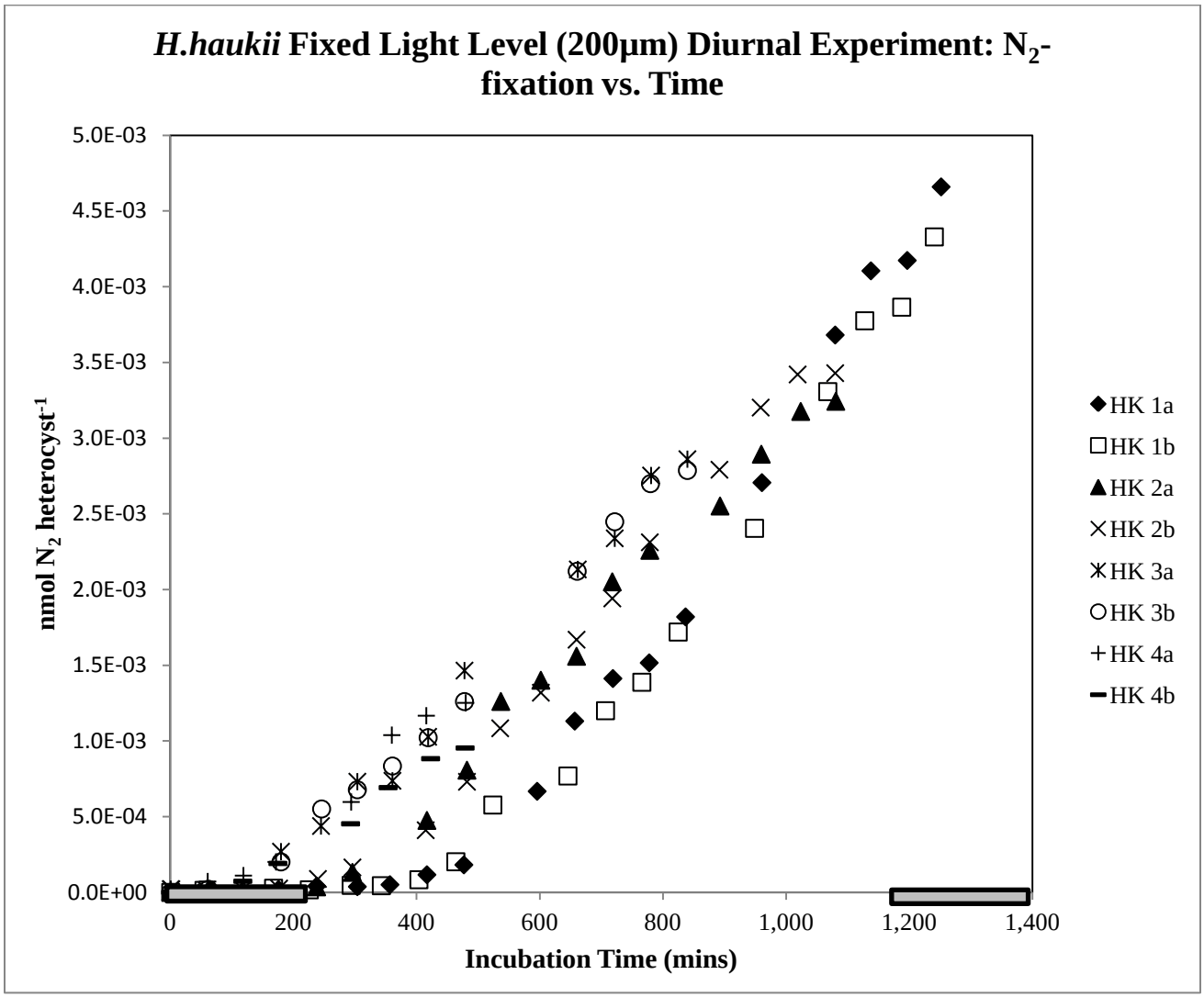
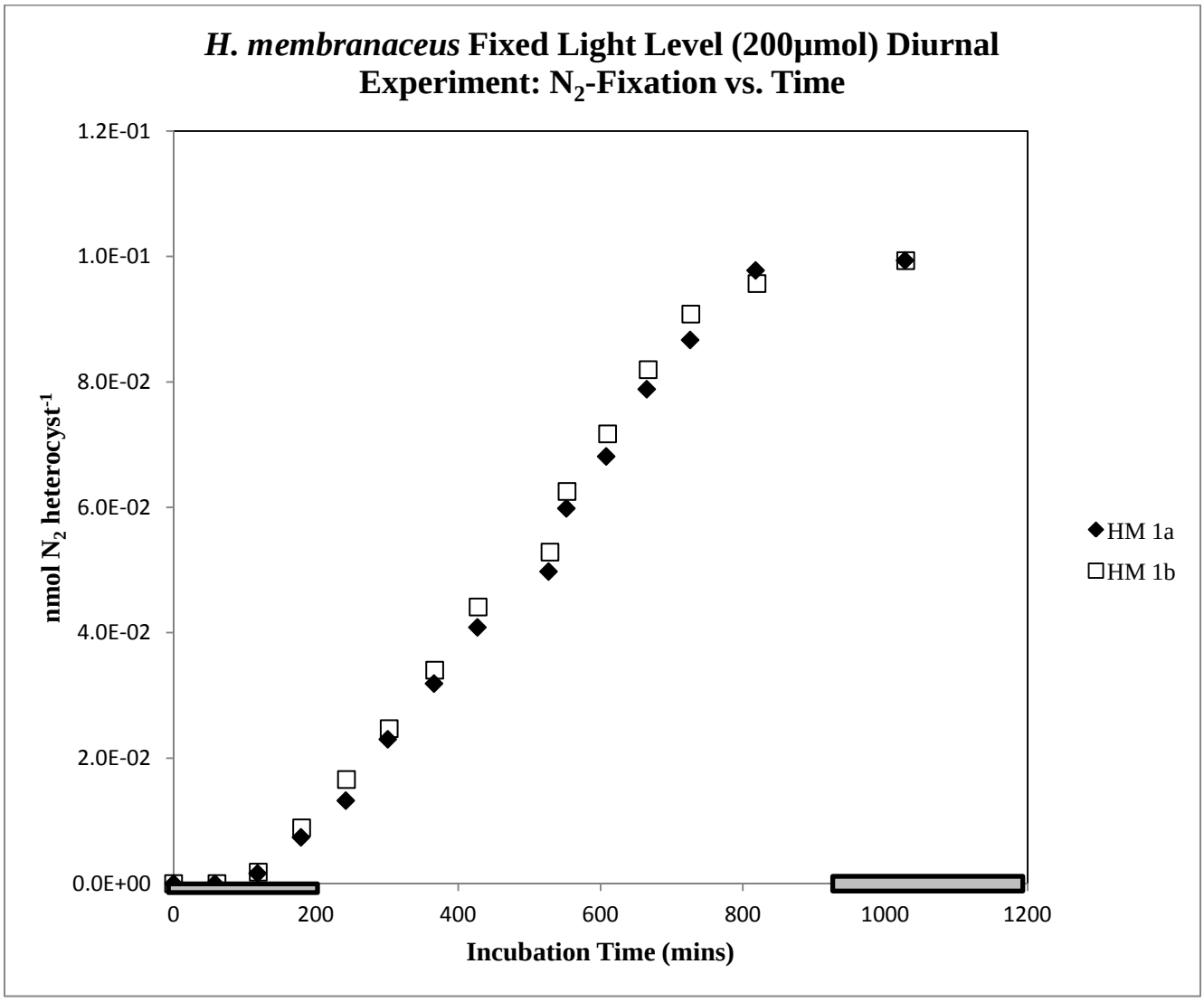


Figure 25: *H. membranaceus* growth-irradiance curve.

Appendix 1: *H. haukii* N_2-fixation vs. Time (mins), Diurnal Fixed Light Experiment



Appendix 2: *H.membranaceus* N_2-fixation vs. Time (mins), Diurnal Fixed Light Experiment



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