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Nitrogen fixation by the cyanobacterium *Nostoc punctiforme* in response to variation in nitrogen availability, temperature, and atmospheric CO₂ concentrations

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

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NITROGEN FIXATION BY THE CYANOBACTERIUM *NOSTOC PUNCTIFORME* IN
RESPONSE TO VARIATION IN NITROGEN AVAILABILITY, TEMPERATURE, AND
ATMOSPHERIC CO₂ CONCENTRATIONS

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by

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Graduate Program in Biology

A thesis submitted in partial fulfillment
of the requirements for the degree of
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Abstract

The predominant input of available nitrogen (N) in boreal forest ecosystems originates from moss-associated cyanobacteria, which fix unavailable atmospheric N₂, contribute to the soil N pool, and thereby support forest productivity. Although increases in N availability, temperature, and atmospheric CO₂ concentrations are expected in Canada's boreal region over the next century, little is known about the combined effects of these factors on N₂ fixation by axenic cyanobacteria or the associated mechanisms. I assessed changes in N₂ fixation by *Nostoc punctiforme* under different global environmental change scenarios and examined correlations between the response and changes in growth, heterocyst percentage, and heterocyst activity. With available N present, *N. punctiforme* did not perform N₂ fixation or form heterocysts. Elevated CO₂ stimulated growth and N₂ fixation, but this result was influenced by a temperature-mediated growth cycle. Overall, my findings suggest a decrease in boreal N₂ fixation rates in response to global environmental change.

Keywords

atmospheric CO₂, boreal forest, cyanobacteria, global environmental change, N₂ fixation, nitrogen, temperature

Co-Authorship Statement

This thesis was completed by Danielle Griffith under the supervision of Dr. Zoë Lindo. Experiments were designed jointly by D. Griffith and Z. Lindo, and any manuscripts resulting from this thesis will be co-authored. D. Griffith performed the experiments, analyzed the data, and Z. Lindo provided invaluable assistance with interpretation of these data and the writing of the thesis and manuscript. Parts of this thesis will be submitted as a manuscript for publication.

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1 Introduction

1.1 The Importance of Nitrogen in Boreal Forest Ecosystems

Boreal regions are 17% of Earth's total land area (DeLuca & Boisvenue, 2012), representing a critical carbon (C) sink that facilitates the removal of CO₂ from Earth's atmosphere (Hayes et al., 2011), thereby playing a crucial role in potential climate change mitigation. It is predicted that the ability of these ecosystems to continue sequestering C through increasing net primary productivity will be closely tied to nitrogen (N) availability (Reich et al., 2006), given that plant growth facilitates C sequestration (Nadelhoffer et al., 1999). At the terrestrial ecosystem scale, net primary productivity is strongly limited by available N (Vitousek and Howarth, 1991), because available N is required for plant biochemicals such as hormones (Kende & Zeevart, 1997), pigments (Tanaka et al., 2008), and enzymes (Schneider et al., 1992). In soil systems, available N is present as ammonium (NH₄⁺) and nitrate (NO₃⁻), and soluble organic N, which can be directly taken up by plants for metabolism (Haynes & Goh, 1978; Schimel & Bennett, 2004). However, ecosystem-level increases in N availability, temperature, and atmospheric CO₂ concentrations are expected during the next 100 years due to global environmental change (IPCC, 2007) with ecosystem-level effects anticipated on the processes that supply the soil N pool with biologically available N. Accordingly, further research is required to determine how the processes that generate available N in boreal forest ecosystems will respond to increases in N availability, temperature, and atmospheric CO₂ concentrations.

The largest pool of unavailable N sits in Earth's atmosphere, which is composed of 78% of atmospheric N₂, and unavailable to plants and most soil microorganisms (Galloway et al., 2004). Forms of N that are already fixed are introduced to terrestrial ecosystems and

are subsequently made available to plants by two key processes: N mineralization and N deposition. Nitrogen mineralization, which liberates biologically available N from decaying biomass, is generally low in forest ecosystems at northern latitudes, with rates of $0.02 \text{ g N m}^{-2} \text{ yr}^{-1}$ in a Swedish forest, in contrast to 11 other forest sites across Europe and North America, where N mineralization rates were notably higher (Rustad et al., 2001). Deposition of N via precipitation from industrial processes such as fossil fuel combustion is primarily in the form of NO_x (and minimal inputs of NH_y), which is considered a pollutant form of available N to boreal ecosystems (Vitousek et al., 2002). Nitrogen deposition rates are low in most boreal ecosystems, ranging between 2 and $3 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ for much of Canada's boreal forest (Galloway et al., 2008).

It is predicted that N availability in boreal forest ecosystems will increase by the year 2100 due to rising temperatures driving increases in decomposition and N mineralization rates, in addition to increased pollution from anthropogenic sources (IPCC, 2007). Even though an increased N input from these sources is predicted, the N produced by these processes alone is likely not enough to sustain primary productivity in the boreal forest, given that these ecosystems are expected to have stored an estimated 41 Tg C yr^{-1} during recent decades (Hayes et al., 2011). Since this C sequestration activity is directly linked to N availability, it is critical to determine how available N will be impacted by global environmental change.

1.2 Nitrogen Fixation Pathways in Boreal Forest Ecosystems

N_2 fixation is the only process by which unavailable atmospheric N_2 can enter the soil N pool. In terrestrial ecosystems, there are three main processes by which N_2 fixation can occur: the Haber–Bosch process (anthropogenic), lightning strikes (abiotic), and biological N_2 fixation (biotic). In agricultural settings, the industrial Haber-Bosch process uses a

reaction between N_2 and H_2 to create ammonia (NH_3), which is used as a fertilizer for crops (Galloway et al., 2004; Erisman et al., 2008; Galloway et al., 2008). However, influxes of NH_3 are more common in ecosystems that border on agriculture, in contrast to boreal forests at high northern latitudes that experience fewer anthropogenic N inputs (IPCC, 2007; Vitousek et al., 2002). In these forests, N_2 fixed by lightning strikes transforms N_2 to nitrite (NO_2^-) at an estimated rate of 10^{26} molecules per strike (Noxon, 1976). Available N from N_2 fixation from the Haber-Bosch process and lightning strikes are minimal in boreal ecosystems, where most of the N input stems from biological N_2 fixation.

Diazotrophs convert atmospheric N_2 to NH_3 and thereby supplement the soil N pool with available N (Dixon & Kahn, 2004; Haynes & Goh, 1978). Many different types of prokaryotes can perform N_2 fixation, including archaea (Cabello et al., 2004) and various genera and types of bacteria including *Frankia* (Reddell & Bowen, 1985), *Rhizobium* (Pagan et al., 1975), and cyanobacteria (Dixon & Kahn, 2004) are also capable of this critical ecosystem-level process. These organisms are distributed widely across Earth's ecosystems, with cyanobacteria being the predominant group of diazotrophs in terrestrial and marine environments (Vitousek et al., 2002; Bergman et al., 2012). Cyanobacteria are photosynthetic and may be unicellular (Fu et al., 2007) or multicellular (Campbell et al., 2007). Multicellular forms can be heterocystous, bearing specialized cells that perform N_2 fixation (Meeks & Elhai, 2002; Dixon & Kahn, 2004; Gentili et al., 2005), or non-heterocystous (Levitan et al., 2007), and both are capable of fixing N_2 (Dixon & Kahn, 2004). Because the ability to fix both C and N is not commonly found in the same organism, the capability of cyanobacteria to fix both CO_2 (Meeks & Elhai, 2002) and N_2 (Rippka et al., 1979; Dixon & Kahn, 2004) positions them at the interface between C and N cycling in boreal forests. In boreal forests, cyanobacteria are dominantly of the heterocystous and filamentous genus *Nostoc* (Dodds et

al., 1995). Although cyanobacteria from the *Nostoc* genus occur as a free-living species in soils, particularly as *N. commune* (Potts et al., 1987), cyanobacteria from this genus are also found in symbiosis. The discussion of cyanobacterial N₂ fixation in this thesis focuses largely upon symbiotic species.

Cyanobacteria participate in symbioses with fungi, non-vascular plants, and vascular plants (Dodds et al., 1995; Meeks & Elhai, 2002). However, these associations are non-ubiquitous, with spatial differences in the types of associations present between different ecosystems. It was previously thought that cyanobacteria symbioses with plants were scarce at high latitudes (Vitousek & Howarth, 1991), in contrast to tropical ecosystems where N₂ fixation rates by bacteria symbiotically associated with tree root systems are much higher, ranging between 20 and 60 kg N ha⁻¹ yr⁻¹ (Houlton et al., 2008). N₂ fixation by below-ground systems such as these appears to be minimal in Northern ecosystems such as boreal forests (Vitousek et al., 2002), where N₂ fixing root systems in plants are generally absent (Crews, 1999). As a result, it was previously challenging to understand the missing source of available N in the boreal N budget (Vitousek et al., 2002). Recently, it has been demonstrated that the predominant N input in these ecosystems comes from cyanobacteria-moss associations (DeLuca et al., 2002; Zackrisson et al., 2004; Zackrisson et al., 2009; Gundale et al., 2011; Gundale et al., 2012). Studies currently estimate the N input from this association at 2.7 kg N h⁻¹ yr⁻¹ for boreal forest ecosystems (DeLuca et al., 2002), although lower values of 0.4 to 1.6 kg N h⁻¹ yr⁻¹ have been reported (Zackrisson et al., 2004; Zackrisson et al., 2009). Much of the research to date has focused extensively on N₂ fixation by intact cyanobacteria-moss associations (Smith et al., 1984; DeLuca et al., 2002; Zackrisson et al., 2004; Gundale et al., 2009; Zackrisson et al., 2009; Jean et al., 2012; Gundale et al., 2012; Rousk et al., 2013). However, the actual N₂ fixation process is exclusively carried out by the

cyanobacterium, and many terrestrial studies fail to observe N₂ fixation in cyanobacteria isolates from the moss association (but see Gentili et al., 2005). Moreover, it is unknown how the quantity of N produced from the associated cyanobacteria will be altered by global environmental change.

Different species of mosses and their associated cyanobacteria are known to participate in symbiosis. Feather mosses, including *Pleurozium schreberi* (Zackrisson et al., 2004; Zackrisson et al., 2009), *Hylocomium splendens* (Zackrisson et al., 2009; Gundale et al., 2012), and *Anomodon attenuatus* (Jean et al., 2012) have cyanobacteria that fix N₂. In feather mosses such as *Hylocomium splendens*, the majority of N₂ fixation activity is in the middle region of the moss shoots, with fewer cells on the capitula (Gavazov et al., 2010), although cyanobacterial cells can be scattered across any region of the moss plant. Studies on *Sphagnum* mosses have also suggested that cyanobacteria are not normally present on the capitula, and tend to colonize the middle region of the moss shoots (Berg et al., 2013). In contrast to liverworts and hornworts, which often have endophytic associations (Costa et al., 2001), cyanobacteria tend to attach to form epiphytic associations with mosses (Smith, 1984; Zackrisson et al., 2004; Zackrisson et al., 2009); the exception to this is *Sphagnum*, where the association tends to be endophytic (Berg et al., 2013). Although the underlying ecological benefits to the moss remain somewhat elusive (Lindo et al., 2013), cyanobacteria often supply the moss partner in the association with fixed N₂ in the form of NH₄⁺ (Berg et al., 2013), thereby reducing the extent of N limitation. However, the benefits for the cyanobacterium remain more enigmatic. The moss may provide the cyanobacteria with fixed C (Meeks & Elhai, 2002), thereby providing a specific benefit to the cyanobacterium by associating with the moss. In addition, the moss may provide the cyanobacterium with an environment full of nutrient rich litter (Wardle et al., 2003), where the cyanobacterium is

protected from desiccation and predation (Dodds et al., 1995). Cyanobacteria such as *Nostoc* (DeLuca et al., 2002; Gentili et al., 2005; Jean et al., 2012), *Calothrix* (Jean et al., 2012; Gentili et al., 2005), *Scytonema* (Gentili et al., 2005; Lindo & Whiteley, 2011), and *Stigonema* (Gentili et al., 2005) participate in symbioses with mosses, and fix N₂. Nevertheless, most of the studies on the cyanobacteria-moss association fail to examine the constituent cyanobacteria. Further, there is a dearth of studies on these axenic cyanobacteria and global environmental change (but see Gentili et al., 2005).

1.3 Regulation of N₂ Fixation in Heterocystous Cyanobacteria

Cyanobacteria exhibit two different growth strategies in terrestrial ecosystems: the vegetative cell cycle, and the heterocyst cell cycle. Vegetative cells are the photosynthetic cells that fix C (Meeks & Elhai, 2002), while heterocysts are specialized cells that fix N₂. In conditions where nutrients are non-limiting, namely where available N is present, cyanobacteria persist in a vegetative cell cycle (Meeks et al., 2002). This cycle is characterized by cyanobacteria filaments that are solely composed of vegetative cells, without heterocysts, akinetes, or hormogonia present (Campbell et al., 2007). Akinetes are thick-walled cells usually produced in response to cold or environmental stress, whereas hormogonia are short gliding filaments that act as a dispersal mechanism (Meeks et al., 2002). Heterocyst cells contain the enzyme nitrogenase, which converts N₂ and to NH₃ (Dixon & Kahn, 2004), but cyanobacteria do not produce active heterocysts in conditions where available N, such as ammonium (NH₄⁺) and nitrate (NO₃⁻), is a non-limiting resource (Meeks et al., 2002; Campbell et al., 2007); rather, N₂ fixation is stimulated when N is limiting in the environment. At the ecosystem scale, the presence of cyanobacteria in vegetative cell cycles versus heterocyst cell cycles is dependent upon environmental

conditions, and will dictate the amount of available N entering the soil N pool from N₂ fixation.

Most of the known information about the collection of signals that facilitate N₂ fixation comes from studies on multicellular cyanobacteria such as *Nostoc punctiforme* (Meeks et al., 2001). Under N limitation, cyanobacteria receive a signal to fix N₂, and produce heterocyst cells with active nitrogenase. The formation of active heterocysts occurs 24 to 36 h after N limitation begins (Wong & Meeks, 2001), with partially formed heterocysts appearing as early as 6-12 h (Adams, 2000). Ultimately, heterocysts reach a frequency around 8% in the cyanobacterial filament for *N. punctiforme* (Meeks et al., 2001; Meeks et al., 2002; Meeks & Elhai, 2002), with N free culture experiments confirming a frequency of heterocysts at 8.7% (Campbell et al., 2007). However, the nitrogenase structural genes *nifHDK* are not normally expressed until 13-16 h after nitrogen limitation (Adams, 2000), suggesting that heterocyst production and nitrogenase synthesis experience temporal separation. Once heterocysts containing nitrogenase enzymes are produced, they cannot return to vegetative cells (Böhme, 1998; Meeks et al., 2001), and simply become inactive in response to environmental cues of enough available N being present in the system. This regulation occurs at a genetic level, as NH₄⁺ inhibits N₂ fixation in *Nodularia spumigena* due to decreased expression of N₂ fixation (*nif*) genes (Vintila & El-Shehawey, 2007). Although several studies focus on heterocyst production from a genetic basis surrounding *nif* genes (Meeks et al., 2001; Meeks et al., 2002; Campbell et al., 2007), there is an ecological basis for the regulation of N₂ fixation as well. The environmental signal arising from N limitation is commonly referred to as a 'nitro-stat' (Menge & Hedin, 2009), meaning cyanobacteria can sense when the extracellular environment lacks available N. Given that N₂ fixation is an energetically costly process (Vitousek et al., 2002; Turetsky, 2003; Houlton et al., 2008;

Dixon & Kahn, 2004), the signals that give rise to heterocysts with active nitrogenase are tightly regulated by the cyanobacterium to prevent unnecessary N₂ fixation (Campbell et al., 2007). However, little is known about how changes in global environmental change factors such as N availability, temperature, and CO₂ will influence the timing and amount of N₂ fixation in these conditions.

There are three key mechanisms by which cyanobacteria can alter N₂ fixation in response to global environmental change: growth, heterocyst percentage, and heterocyst activity. Firstly, it is possible that cyanobacterial abundance increases in response to N limitation, with more cells present overall, ultimately colonizing more moss and fixing more N₂. Although cyanobacteria can use a variety of N sources, it is shown that cyanobacteria preferentially assimilate ammonium (NH₄⁺) (Meeks et al., 2001), suggesting that they may grow more in cultures where NH₄⁺ is not the nitrogen source. Increased cyanobacterial growth and biomass can result in a net increase in N₂ fixation as shown during the exponential growth stage under elevated CO₂ conditions in *Trichodesmium* (Levitan et al., 2007). Studies on the unicellular cyanobacterium *Synechococcus* also report increased growth rates in response to elevated CO₂ and increased temperature (Fu et al., 2007). Secondly, the percentage of cells that are N₂ fixing heterocysts may increase (without an overall increase in vegetative cells which do not fix N₂), although previous studies have failed to demonstrate any effect of varying CO₂ concentrations on heterocyst percentage or cell size (Czerny et al., 2009). Thirdly, the activity of heterocyst cells may increase in response to N limitation, with more N₂ fixed on a per heterocyst cell basis. However, many studies do not quantify heterocyst cell counts or standardize N₂ fixation measurements based on cell counts (Smith, 1984; DeLuca et al., 2002; Zackrisson et al., 2004; Gentili et al., 2005; Zackrisson et al., 2009), making a mechanistic interpretation challenging. Further, the results

from marine settings do not extend well to terrestrial systems, because pH and rising atmospheric CO₂ are confounding factors (Czerny et al., 2009). The mechanistic insights gained from *Trichodesmium*, which is the most common cyanobacterium in marine environments, are poorly applicable because this cyanobacterium is also non-heterocystous (Levitan et al., 2007). In addition, studies on unicellular cyanobacteria such as *Synechococcus* (Fu et al., 2007) are extremely challenging to extend to cyanobacteria-moss associations. In cases where such research is done on cyanobacteria isolates from this system (Gentili et al., 2005), it is unknown how growth stage plays into the results, and the insights we have are mostly from the non-heterocystous species *Trichodesmium* under elevated CO₂ (Levitan et al., 2007). At present, there is no study on a terrestrial cyanobacterium from any system that investigates the effects of N availability, temperature, and CO₂ in a factorial setting.

1.4 Response of N₂ Fixation to Variation in Global Environmental Change Factors

Changes in global environmental change factors associated with anthropogenic activity and a changing climate, including increased N availability, elevated temperature and increased atmospheric CO₂ concentrations, are predicted to influence N₂ fixation by the cyanobacteria-moss association (Lindo et al., 2013). In the case of N availability, it is known that N₂ fixation does not occur in the presence of available N, with associated declines in heterocyst percentage and heterocyst activity. However, the effects of temperature on N₂ fixation by axenic cyanobacteria in terrestrial systems are conflicting between species (Gentili et al., 2005), with a mechanistic understanding currently lacking. At present, there is only one known study of cyanobacteria-moss N₂ fixation under elevated CO₂ (Smith, 1984),

with no study on axenic cyanobacteria from this system. As a result, it is critical to determine the effects of N availability, temperature, and CO₂ on N₂ fixation on a terrestrial cyanobacterium with symbiotic capabilities.

Nitrogen availability in terrestrial ecosystems is predicted to rise over the next 100 years due to the collection of processes that generate available N (IPCC, 2007; Schimel & Bennett, 2004; Gruber & Galloway, 2008; Galloway et al., 2008). In the boreal forest, N mineralization and N deposition bring in NO_x, with NH₃ inputs to the soil N pool predominantly coming from N₂ fixation. With more available N present, cyanobacteria stop fixing N₂ in response to the nitro-stat signal, as previously described. Many N addition and natural N gradient studies corroborate a reduction or elimination of N₂ fixation under increased N availability (Zackrisson et al., 2004; Zackrisson et al., 2009; Ackermann et al., 2012), with one study demonstrating a concomitant reduction in cyanobacteria abundance (Gundale et al. 2011), which may indicate reduced growth rates. Cyanobacteria do not produce heterocysts or fix N₂ when N is readily available, and this is the most likely the mechanism for reduced N₂ fixation under increased N availability. It is established that reductions in N₂ fixation are due to reduced cyanobacterial abundance, and the absence of N₂ fixing heterocysts. However, the effects of increases in temperature and CO₂ in N limited settings remain poorly described.

Temperature increases between 4 and 8 °C are predicted for the boreal forest over the next 100 years (IPCC, 2007). Across all terrestrial systems, nitrogenase activity reaches a maximum near 25 °C (Houlton et al., 2008). Moss-associated cyanobacteria have been observed to fix N₂ at temperatures as low as 0 °C (Smith, 1984) and at temperatures as high as 36 °C, with a response to temperature occurring within minutes (Jean et al., 2012). N₂ fixation decreases sharply beyond 30 °C (Smith, 1984; Jean et al., 2012); however, it is likely

that differences among mosses and the associated cyanobacterial communities will influence the optimum temperature for N₂ fixation in boreal ecosystems. One study on cyanobacteria isolated from the moss *P. schreberi* found that N₂ fixation was greatest at 30 °C for *Calothrix*, with increasing temperature having a positive effect on N₂ fixation, whereas N₂ fixation was greatest at 13 °C for *Nostoc*, with an overall unimodal relationship on N₂ fixation (Gentili et al., 2005). Although the effects of temperature on overall N₂ fixation rates are somewhat described in the literature, studies on the cyanobacteria-moss association often do not quantify heterocyst cells and, as a result, do not determine whether changes in heterocyst percentage or heterocyst activity are driving the observed response.

Atmospheric CO₂ concentrations in the boreal forest are expected to double by the end of the next century (IPCC, 2007). Nevertheless, the effects of CO₂ on terrestrial N₂ fixation by moss-associated cyanobacteria remain enigmatic. At present, there is only one study on the effects of CO₂ on N₂ fixation by moss-associated cyanobacteria, which found a decrease in N₂ fixation in response to increased CO₂ (Smith, 1984); however, cyanobacteria isolates were not microscopically examined, and the mechanism for decreased N₂ fixation in response to elevated CO₂ remains unknown. In marine ecosystems, the effects of CO₂ on N₂ fixation are not consistent among species (Czerny et al., 2009; Levitan et al., 2007; Chinnasamy et al., 2009), and do not extend well to terrestrial ecosystems. For example, increased CO₂ was observed to increase N₂ fixation and growth rates in the marine non-heterocystous cyanobacteria *Trichodesmium* (Levitan et al., 2007), and the freshwater heterocystous cyanobacteria *A. fertilissima* (Chinnasamy et al., 2009). However, elevated CO₂ decreased N₂ fixation and cell division rates in *Nodularia spumigena*, a heterocystous species of cyanobacteria from the Baltic Sea (Czerny et al., 2009). Previous studies by Levitan et al., (2007) suggest that the discrepancy in these results could be attributed to

different growth phases of cyanobacterial cultures, whereby elevated CO₂ concentrations increase N₂ fixation during the exponential phase of growth, and not during the stationary phase. Previous research has failed to detect differences in cell size or heterocyst frequency among *N. spumigena* cultures grown at different CO₂ concentrations (Czerny et al., 2009) and overall, the mechanisms for CO₂ effects on N₂ fixation remain elusive. In general, studies on CO₂ effects often observe net N₂ fixation rates, and do not standardize these rates based upon heterocyst cells (Czerny et al., 2009; Chinnasamy et al., 2009). Nonetheless, studies that examine the effects of CO₂ concentrations concomitantly with other global environmental change factors are scarce, particularly within the terrestrial literature.

Studies have recently begun to examine the effects of global environmental change on N₂ fixation by cyanobacteria; however, there are comparatively few that examine the interactive effects of multiple global environmental change factors. Temperature and light have combined effects on N₂ fixation in cyanobacteria moss-associations, where light tends to have a stimulatory effect on N₂ fixation rates at low temperatures, and an inhibitory effect on N₂ fixation rates at high temperatures (Gundale et al., 2012). Interactive effects of temperature and CO₂ have also been observed in *A. fertilissima*, where elevated CO₂ tends to mitigate the effect of high temperatures on growth and N₂ fixation (Chinnasamy et al., 2009). *Synechococcus* responds positively to both temperature and CO₂, whereas fewer effects are observed in *Prochlorococcus* (Fu et al., 2007). However, this species difference has been presented for unicellular cyanobacteria, and not multicellular cyanobacteria. Since it is expected that increases in these global environmental change factors will occur simultaneously (IPCC, 2007), it is critical to describe the main and combined effects of these changes on N₂ fixation and, moreover, to elucidate the mechanism(s) responsible for the observed response of axenic cyanobacteria from the moss association.

1.5 Objectives

In this thesis I present a two-part study that advances our understanding of the effects of increased N availability, elevated temperature, and increased atmospheric CO₂ concentrations on N₂ fixation by the terrestrial cyanobacterium *N. punctiforme* in axenic culture. In the ‘growth experiment’, my first objective was to examine the pattern of growth and N₂ fixation activity of *N. punctiforme*, with a second objective of comparing how these patterns differ under ambient and elevated CO₂. In satisfying both of these objectives, a timeline of growth and N₂ fixation events is created and used to interpret the findings of further experiments. In the second ‘global environmental change’ experiment, my objective was to explore the interactive effects of N availability, temperature, and atmospheric CO₂ on N₂ fixation by *N. punctiforme*. In both these studies I distinguished between changes in cyanobacterial growth, proportion of heterocysts, and heterocyst activity to mechanistically understand changes in N₂ fixation rates.

1.6 Hypothesis & Predictions

Changes in N₂ fixation and the associated mechanisms are predicted in response to increases in the global environmental change factors (Figure 1.1). Since N availability, temperature, and CO₂, when increased in isolation, are known to influence the N₂ fixation process, I hypothesized that interactive effects would be present among these factors, with growth, heterocyst percentage, and heterocyst activity responsible for the observed response. Given that N₂ fixation is an energetically costly process, I hypothesized that *N. punctiforme* would not fix N₂ in conditions where N is available. Mechanistically, this would arise through the lack of heterocyst cell formation (and activity if heterocysts were present in the original culture), and subsequent slowed growth through the production of fewer vegetative

cells. Given that available N is critical for cyanobacteria, I predicted that *N. punctiforme* would fix N₂ in conditions where N is unavailable. This would correspond with heterocyst production, and demonstrated activity of these heterocysts. Under N limitation, I predicted that N₂ fixation rates would increase primarily due to the stimulatory effect of CO₂ on vegetative cells and the greater demand for N under growth conditions. Since N₂ fixation reaches an optimum at 25 °C, and all my experiments were performed at temperatures below this optimum, I predicted that increased temperature would result in increased N₂ fixation by *N. punctiforme*. This would be due to growth of the cyanobacterial cultures, but also greater heterocyst activity under elevated temperatures. The combined effects of elevated temperature and CO₂ were more challenging to predict, but I predicted that these two environmental change factors should have a synergistic effect on growth, with CO₂ and temperature being stimulatory to cyanobacterial N₂ fixation.

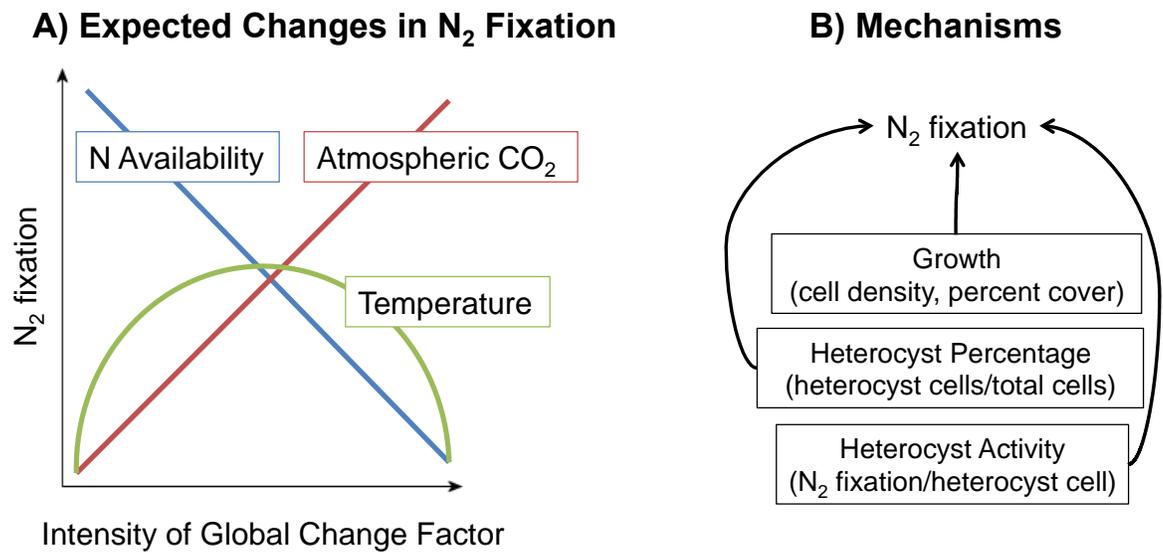


Figure 1.1. A schematic representation of A) expected changes in N₂ fixation under different global environmental change factors and B) the proposed mechanisms for the observed N₂ fixation response. It was expected that N availability will reduce N₂ fixation, whereas atmospheric CO₂ would stimulate N₂ fixation. The relationship between N₂ fixation and temperature was expected to have a unimodal relationship, where N₂ fixation would increase approaching a temperature optimum, but later decline. A combination of growth, heterocyst percentage, and heterocyst activity was expected to be the mechanism driving the observed N₂ fixation response.

2 Methods

2.1 Cyanobacteria Inoculations & Media Recipes

I obtained pure cultures of *N. punctiforme* from the Canadian Phycological Culture Centre (CPCC strain #41 *N. punctiforme* (Kutz.) Hariot). When the cultures arrived at the Biotron facilities at Western University, I stored the cultures in a growth chamber at 18 °C with a consistent light intensity and 12D:12L photoperiod. I transferred them to liquid BG-11 media for maintenance prior to the initiation of my experiments. I followed the media recipes outlined by the University of California at Davis, which are based on Rippka et al. (1979). I ordered BG-11 stock solution (commercially available from Sigma), which contains available N (as sodium nitrate and ferric ammonium citrate), but made the N-free media (BG-11₀) from scratch in the lab; both media formulations are standard recipes outlined for algal culture maintenance (Allen, 1968) and contain equal quantities of other micronutrients. I used bacteriological agar for the BG-11 media, but used noble agar for the BG-11₀ to ensure that nitrogen-containing impurities were not introduced to the N-free vials.

I autoclaved the media in a 2 L flask at 121 °C on a liquid cycle for 20 minutes. The autoclaving process was proven successful by visually inspecting autoclave tape on the flasks. I allowed the media preparation to cool to approximately 50 °C, at which time I transferred the media to 50 mL vials. I filled each vial to the 25 mL mark and allowed it to cool to room temperature over 24 h. I subsequently inoculated solidified vials with 30 µL of *N. punctiforme* cell suspension in BG-11 liquid media. Prior to inoculating the vials, I applied a series of 10 µL aliquots of the cell suspension to each of two grids on a haemocytometer to quantify cell densities and ensured that they were consistent across experimental vials. I used the stock culture in BG-11 media (described above) to create an

inoculum culture with a cell count of 4.92×10^5 cells mL⁻¹ that was then used in both experiments. I performed microscopic analysis at the start of both experiments and demonstrated that heterocysts were infrequent, and made up less than 0.1% of all cells in the inoculum culture. I allowed the axenic cell cultures to grow on solid media for 30 or 90 d depending upon the experiment (described below).

2.2 Determination of Cell Culture Growth & Percentage

Heterocysts

To determine cell culture growth, I developed and employed two different methods. For the first (growth) experiment, I created a protocol for image analysis to determine the amount of growth of cyanobacteria on the agar surface. Here, I captured digital images of the vial surface using an Olympus PEN mini digital camera under auto-focus mode and tripod set-up. I captured the images from approximately 7 cm above the vials, but this distance varied slightly on occasion to ensure the best image capture was possible. I calibrated all images using the known diameter of the vials (27 mm) to ensure that pixel quantity across images did not alter calculations of cyanobacterial cover. To easily distinguish between agar surface and cyanobacteria cells, I converted the images to an 8-bit format, which allowed the software to use black and white images for all quantifications of surface area. I removed any remaining background noise outside of the agar surface using either the eraser tool or the paintbrush tool with the colour set to white, where applicable. I measured all of the images using the particle analysis function with the minimum particle size set to 5 pixels, and subsequently recorded cyanobacterial cover in mm². At the beginning of the experiment, the initial surface area of the vial was 572.56 mm²; I made adjustments for any semi-destructive sampling that removed some of the agar surface at established timepoints by subtracting the

surface area of the agar core removed from the final image analysis calculations. I presented cyanobacterial cover as the percentage of agar surface covered by cyanobacteria cells (% cover).

In both experiments, I assessed changes in cell densities among treatments through direct cell counts of both vegetative and heterocyst cells grown on agar. I obtained micro-core samples (e.g. cylinder shaped cores) of cyanobacteria cells from each 50 mL vial using a 2 mm diameter plastic dropper inserted at a depth of 3 mm into the agar surface. Next, I transferred this core to a 1.5 mL Eppendorf tube containing distilled water, and homogenized it to form a cell suspension via vortex mixing. In the growth experiment, cell densities increased rapidly. As a result, I transferred the cell cores (as described previously) to 0.1 mL of distilled water until day 20, at which time the volume of distilled water was changed to 0.2 mL. In the global environmental change experiment, cell densities were much greater since the experiment was 90 d long. Thus, I used a volume of 0.5 mL distilled water to create these cell suspensions. In cases where I could not easily dissociate the cyanobacteria cells using a vortex, I used the blunt end of a metal microscope tool to simulate a mortar and pestle in the Eppendorf tube, thereby breaking the cells apart into filaments. To prevent cross-contamination, I used a different Eppendorf tube for each vial, and thoroughly rinsed the dropper with distilled water between samples. All measurements of cell counts were taken immediately after vortex mixing to ensure that cyanobacteria cells remained viable and therefore could be seen under the epifluorescence microscope. I measured the cyanobacteria cells using haemocytometry, with cell counts performed under epifluorescence microscopy with Texas Red filter to visualize the vegetative cells. Since the heterocyst cells do not fluoresce, I was able to morphologically distinguish them from the vegetative cells. I calculated the cell densities in each vial using the average of two 10 μ L aliquots for each

sample. I presented cyanobacterial abundance as the total number of each cell type (vegetative, heterocyst and total cells) per vial. The number of cells in each vial was mathematically determined by using the concentration of cyanobacterial cells in each Eppendorf suspension with a dilution-factor correction, and mathematically extrapolated to 572.56 mm^2 agar surface, and the corresponding 3 mm depth of cyanobacteria growth.

2.3 Determination of N_2 Fixation by the Acetylene Reduction

Assay

The acetylene reduction assay (ARA) is a well-established technique for the quantification of N_2 fixation rates, and commonly used in both axenic cyanobacteria (Levitan et al., 2007; Czerny et al., 2009; Chinnasamy et al., 2009) and moss-associated cyanobacteria studies (Hardy, 1968; Bergersen, 1971; Zackrisson et al., 2004; Lindo & Whiteley, 2011). This assay quantifies the reduction of acetylene gas (C_2H_2) to ethylene gas (C_2H_4), as a proxy for the conversion of N_2 to NH_3 at a rate of three moles C_2H_4 produced per mole of N_2 fixed (DeLuca et al., 2002). As described by Bergersen (1970), the nitrogenase enzyme converts acetylene to ethylene using the same quantity of energy (ATP) required to fix N_2 . After comparing these processes stoichiometrically, the moles of N_2 fixed are converted to moles of ammonia produced using a 1:2 conversion ratio, and the moles of NH_3 produced are converted to the mass of ammonia produced by multiplying by the molar mass of NH_3 ($17.031 \text{ g mol}^{-1}$). This stoichiometric approach is similar to the one used in some previous studies (Gavazov et al., 2010). Within my study, I followed standard protocol for ARA and stoichiometric quantification of N_2 fixation (as described above).

I added rubber stoppers to all experimental vials, removed 10% of the headspace using a syringe (2.5 mL), and replaced it with acetylene gas. I allowed the vials to incubate at

experimental conditions for 24 h. Next, I removed 1 mL of headspace from each vial and injected it into a gas chromatograph (GC) for quantification of ethylene gas (C₂H₄). The GC was a Shimadzu 2014 with a flame ionization detector (FID) (250 °C), a Poropak T80/100 packed column (200 °C), and injector (200 °C) with helium (He) used as a carrier gas. I calibrated the GC using a three-point calibration curve of known quantities of ethylene gas (C₂H₄), which allowed me to directly obtain the output of C₂H₄ production in μmol. I presented N₂ fixation measurements as C₂H₄ produced per vial (μmol C₂H₄ vial⁻¹). I standardized N₂ fixation on a per heterocyst basis as heterocyst activity (μmol C₂H₄ heterocyst⁻¹), and on a surface area basis as cyanobacterial activity (μmol C₂H₄ percent cover⁻¹). In each case, I divided the amount of C₂H₄ produced per vial by the number of heterocysts per vial (heterocyst activity), and the percentage cover per vial (cyanobacterial activity).

2.4 Experimental Conditions in the Biotron Biomes

I performed both the growth experiment and global environmental change experiment using the Biomes in the Biotron Institute for Experimental Climate Change Research at Western University (described in Dieleman et al., 2014). The Biomes are environmentally controlled greenhouses (N = 6) that maintain light, humidity, and air circulation as constant parameters, while allowing for manipulation of temperature and CO₂. The Biomes are located on the top of a five-story building and face south without obstruction from other buildings or trees. To ensure that edge effects do not interfere with experiments, there are two staging greenhouses flanking the six experimental Biomes. Experimental conditions in the Biomes are pre-set for maximum experimental use with two replicate Biomes at each of three temperature set points (described below). For my study, there were two Biomes at each experimental temperature, with one containing ambient (430 ppm) atmospheric CO₂ and the

other Biome containing elevated (750 ppm) atmospheric CO₂ to match predicted future climate change scenarios (IPCC, 2007).

2.5 Experiment 1: Growth Experiment

In the first experiment, I examined the effects of ambient and elevated CO₂ on growth and N₂ fixation by *N. punctiforme*. I inoculated six vials containing BG-11₀ media with 30 µL of *N. punctiforme*, and placed these vials in each of two experimental Biomes (one at ambient and one at elevated CO₂). Each Biome had a temperature set point of fluctuating ambient conditions based on the average daily maximum and minimum temperature over the past five years for London Ontario (average daily temperature for July and August was 22 °C): the two Biomes differed in atmospheric CO₂ as described above. I allowed the experiment to run for 30 d, and assessed cyanobacterial cover every two days starting at 0 d for each vial using the image analysis protocol, and measured N₂ fixation (ARA-GC) and cell counts (haemocytometry) on 0, 3, 5 d and subsequently every 5 d until 30 d. I used two blank vials containing BG-11₀ agar without acetylene, and two control vials containing both BG-11₀ agar and acetylene (but no cyanobacteria) to correct N₂ fixation measurements obtained in the ARA-GC protocol. I measured five variables during this experiment: cyanobacterial percent cover, cyanobacterial abundance, N₂ fixation, heterocyst activity, and cyanobacterial activity. I observed the effects of CO₂ on these variables, and constructed a timeline of growth and fixation events for *N. punctiforme*. I related N₂ fixation to growth and used this as a basis for Experiment 2.

2.6 Experiment 2: Global Environmental Change Experiment

In the second experiment, I examined the effects of increases in N availability, temperature, and atmospheric CO₂ on N₂ fixation by *N. punctiforme*. I filled vials with BG-

11 (N = 36) and BG-11₀ (N = 36) media and equally divided them among the six Biomes, prior to inoculating them with cyanobacteria as described above. Two Biomes (ambient and elevated CO₂) were used at each of three temperature set points, which were non-fluctuating during the experiment: 11.5, 15.5, and 19.5 °C. This experiment therefore had a factorial design of the three global environmental change factors, with CO₂ and temperature manipulated at the Biome-level, and N availability manipulated using the media (2 CO₂ × 3 temperature × 2 N availability × 6 replicates = 72 experimental units). Because the temperature set-points were much lower than in the growth experiment, I chose not to obtain measurements of N₂ fixation using the ARA-GC protocol until 30 d, and continued every ten days until the culmination of the experiment at 90 d. As in the growth experiment, I corrected all measurements of N₂ fixation using blanks and controls. At the end of the growth experiment, I quantified vegetative and heterocyst cells using the haemocytometry protocol, and mathematically extrapolated these counts to the number of each cell type in a given vial as previously described.

2.7 Statistical Analyses

I performed all statistical analyses using the R (2.15.1) language and computing environment (R Core Team, 2012). Since all experimental designs were balanced, with equal sample size in each treatment groups, I was able to write syntax using type I sums of squares. For both experiments, I selected a repeated measures analysis of variance (RM-ANOVA) with N availability, temperature, CO₂ and time as fixed effects, and incorporated ‘vial’ as a random effect using an error term. I used Tukey HSD post-hoc tests for each analysis, and also constructed interaction plots to assist in the interpretation of results. Figures were plotted using the ‘sciplot’ packages, with additions from the ‘car’ and ‘lme4’ packages where required. I have attached all the R code used to generate all analyses and figures within this

thesis (Appendix A). I have also archived all data, calculations, and summary statistics in FigShare (DOI: <http://dx.doi.org/10.6084/m9.figshare.1137139> through [.1137142](http://dx.doi.org/10.6084/m9.figshare.1137142)).

3 Results

3.1 Growth Experiment

3.1.1 Cyanobacterial Cover

Nostoc punctiforme covered the entire agar surface under both ambient CO₂ and elevated CO₂ at the end of the experiment with no detectable difference in average cyanobacterial cover between ambient CO₂ (46%) and elevated CO₂ (47%) (RM-ANOVA: $F_{1,10} = 0.37$, $P = 0.56$; Figure 3.1). Cyanobacterial cover (measured as the percentage of the agar surface covered by cyanobacteria) differed among the sampling times (RM-ANOVA: $F_{15,150} = 478.13$, $P < 0.001$), such that the growth of the cyanobacteria on the agar surface followed a typical bacterial growth curve, with lag, exponential, and stationary phases present (Figure 3.1). The rate of growth was the greatest between 15 and 20 days, where the curve is the steepest for both ambient CO₂ and elevated CO₂ treatments (Figure 3.1). No significant effect of CO₂ × time was detected (RM-ANOVA: $F_{15,150} = 0.41$, $P = 0.98$), indicating that the CO₂ effect on cyanobacterial cover was consistent across all time points during the growth experiment. The exponential phase of growth occurred between 6 and 22 d (Figure 3.1). Outside of this time period, growth was stagnant during both the lag phase (0 – 5 d) and stationary phase (22 – 30 d). No decline phase was present during the experiment for either ambient CO₂ or elevated CO₂.

3.1.2 Cyanobacterial Abundance

Cyanobacterial abundance (measured as the total number of cyanobacteria cells per vial) differed between ambient CO₂ and elevated CO₂ (RM-ANOVA: $F_{1,10} = 5.44$, $P = 0.04$), such that elevated CO₂ had an overall positive effect on cyanobacterial abundance (Figure 3.2).

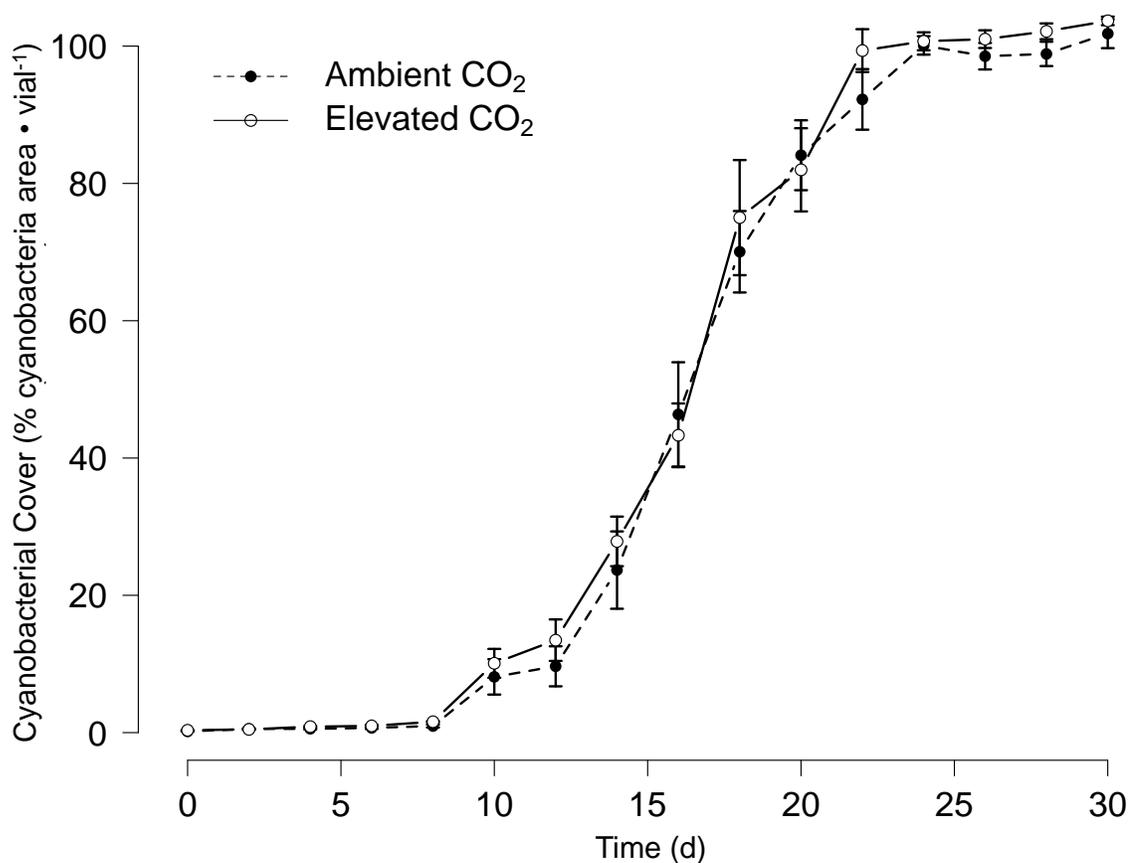


Figure 3.1: Cyanobacterial cover during the growth experiment. The growth of axenic *N. punctiforme* was measured by percent cover of culture cells on the surface of 27 mm diameter agar vials over 30 days under ambient (430 ppm) and elevated (750 ppm) atmospheric CO₂ conditions. Ambient CO₂ is shown using a dotted line with a solid circle, whereas elevated CO₂ is shown using a solid line with an open circle. All data are plotted as mean \pm SD, and based upon six replicate samples per each biome at ambient and elevated CO₂ (overall N = 192).

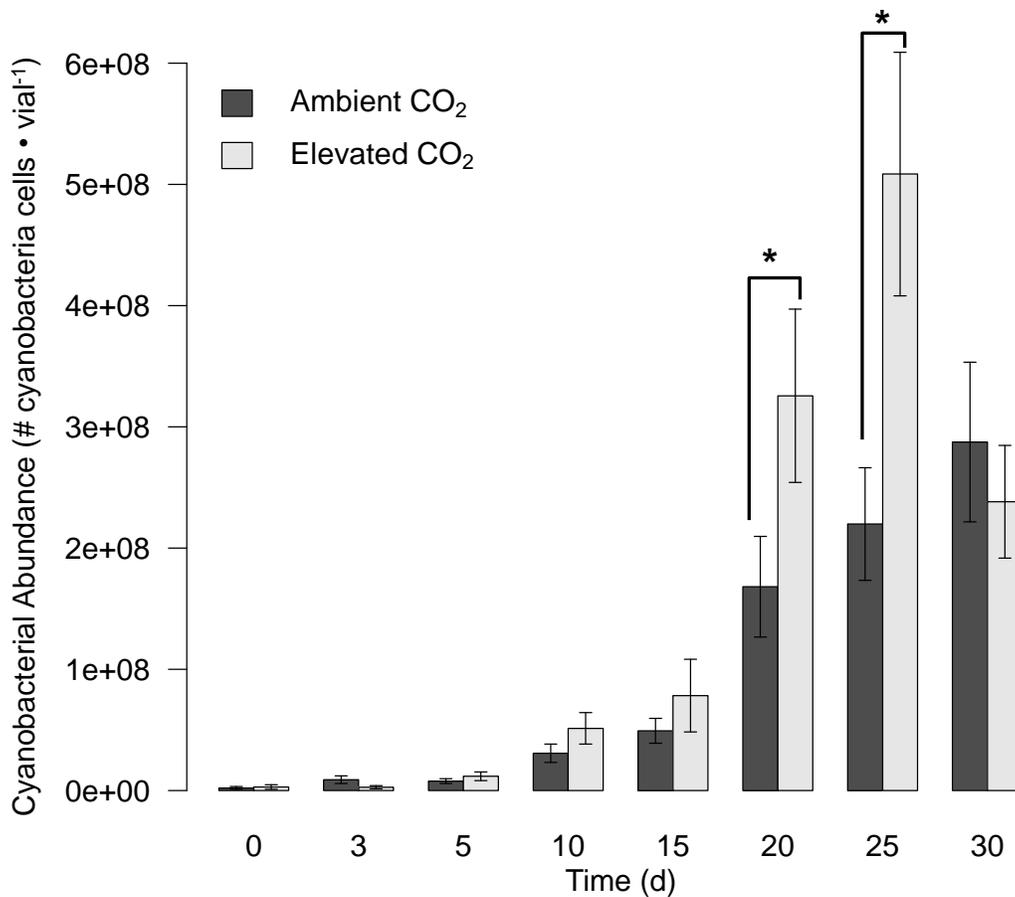


Figure 3.2: Cyanobacterial abundance, as measured by the total number of cyanobacterial cells, during the growth experiment. Growth of axenic *N. punctiforme* was observed on the surface of 27 mm diameter agar vials over 30 days under ambient (430 ppm) and elevated (750 ppm) atmospheric CO₂ conditions. Ambient CO₂ is shown using a dark grey bar, whereas elevated CO₂ is shown using a light grey bar. All data are plotted as mean \pm SD, and based upon six replicate samples per CO₂ treatment (overall N = 96). Pairwise differences are shown using an asterisk symbol (*) with a bar.

Time influenced cyanobacterial abundance (RM-ANOVA: $F_{7,70} = 25.89$, $P < 0.001$), with maximum abundance observed under ambient CO_2 at 30 days, and elevated CO_2 at 25 days, indicating that the elevated CO_2 culture was entering the decline phase at the end of the experiment, whereas the ambient CO_2 culture was not. A significant effect of $\text{CO}_2 \times \text{time}$ (RM-ANOVA: $F_{7,70} = 3.91$, $P < 0.001$) was observed on cyanobacterial abundance, likely driven by the pairwise differences between ambient and elevated CO_2 at days 20 and 25 (Tukey's HSD: $P < 0.05$). Vegetative cells followed the same trend as total cells, and ranged between 82 and 100% of the cells in each vial under ambient CO_2 , and 53 and 100% of the cells in each vial under elevated CO_2 .

3.1.3 Heterocyst Counts & Percentage

The number of heterocyst cells (measured as the total number of heterocyst cells per vial) were an average of 3.63×10^6 cells vial⁻¹ under ambient CO_2 and 4.36×10^6 cells vial⁻¹ under elevated CO_2 . There was no statistically significant effect of CO_2 on heterocyst percentage (RM-ANOVA: $F_{1,10} = 0.06$, $P = 0.81$; Figure 3.3). However, heterocyst percentage (measured as the proportion of heterocyst cells multiplied by 100) varied among time periods (RM-ANOVA: $F_{7,70} = 13.17$, $P < 0.001$), with the maximum percentage of heterocyst cells at day 5 for both ambient and elevated CO_2 treatments occurring at 12% and 20%, respectively (Figure 3.3). No statistically significant effect of $\text{CO}_2 \times \text{time}$ was observed (RM-ANOVA: $F_{7,70} = 1.62$, $P = 0.15$), indicating that the CO_2 effect was consistent throughout the experiment. After a steady increase between days 0 and 5, heterocyst percentage sharply decreases and plateaued at 3% under both ambient and elevated CO_2 .

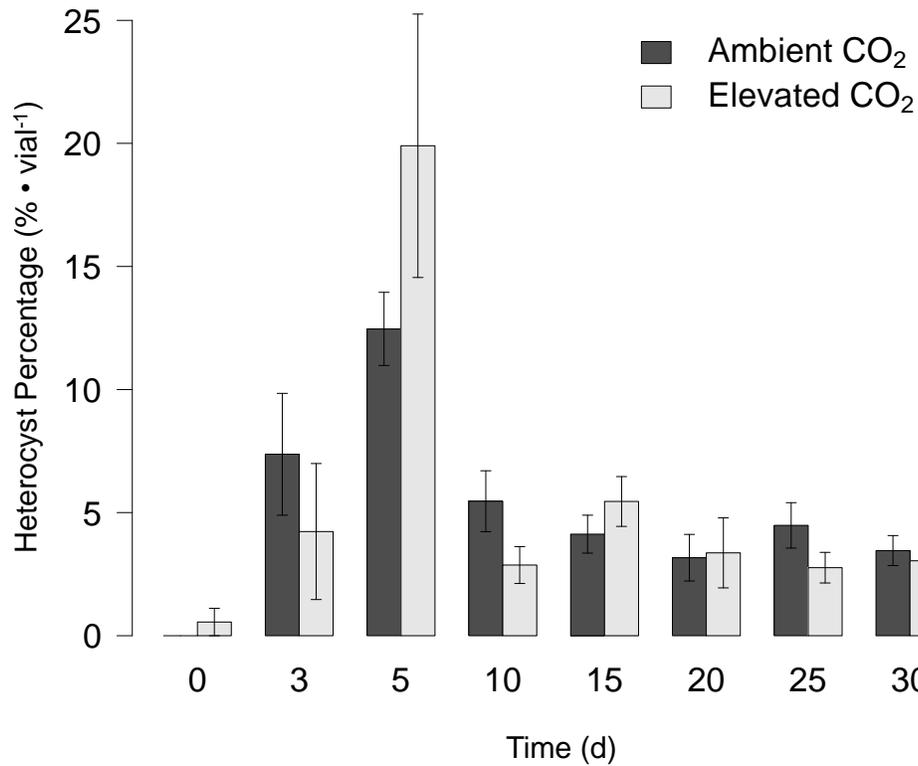


Figure 3.3: Heterocyst percentage of axenic *N. punctiforme* during the growth experiment. Cells were grown on the surface of 27 mm diameter agar vials over 30 days under ambient (430 ppm) and elevated (750 ppm) atmospheric CO₂ conditions. Ambient CO₂ is shown using a dark grey bar, whereas elevated CO₂ is shown using a light grey bar. All data are plotted as mean \pm SD, and based upon six replicate samples per CO₂ treatment (overall N = 96).

3.1.4 N₂ Fixation

N₂ fixation, as measured by ethylene production per vial, did not differ between ambient CO₂ and elevated CO₂ treatments (RM-ANOVA: $F_{1,10} = 2.04$, $P < 0.18$; Figure 3.4), although N₂ fixation increased throughout time (RM-ANOVA: $F_{7,70} = 64.74$, $P < 0.001$). At ambient CO₂, average N₂ fixation was 2.14 $\mu\text{mol vial}^{-1}$, versus 2.45 $\mu\text{mol vial}^{-1}$ under elevated CO₂ conditions. No significant interaction effect of CO₂ and time was detected (RM-ANOVA: $F_{7,70} = 0.93$, $P = 0.48$). At all sampling times between 0 and 10 d, average N₂ fixation rates were consistently below 0.64 $\mu\text{mol vial}^{-1}$, with an exponential increase until day 20, and plateau between days 20 and 30 (Figure 3.4). N₂ fixation and cyanobacterial cover were strongly and positively correlated at all time points, with R values ranging between 0.58 and 0.85 (Pearson's Correlation: $R \leq 0.05$ for all data).

3.1.5 Cyanobacterial Activity

Cyanobacterial activity (calculated as N₂ fixation divided by percent cover) was influenced by CO₂, such that elevated CO₂ increased cyanobacterial activity relative to ambient CO₂ (RM-ANOVA: $F_{1,10} = 6.09$, $P = 0.03$; Figure 3.5). Time also increased cyanobacterial activity (RM-ANOVA: $F_{7,70} = 70.42$, $P < 0.001$), where maximum cyanobacterial occurred on day 5 for both ambient and elevated CO₂ treatments (RM-ANOVA: $F_{7,70} = 4.38$, $P < 0.001$), where respective values were 0.32 $\mu\text{mol percentage cover}^{-1}$ and 0.52 $\mu\text{mol percentage cover}^{-1}$. Cyanobacteria were not yet active at day 0, and cyanobacterial activity was observed to sharply decline beyond day 5 (Figure 3.5). Cyanobacterial activity reached a maximum on day 5 (Figure 3.5), which precedes the exponential phase of growth beginning near day 10 (Figure 3.1). Between days 10 and 30, cyanobacterial activity was fairly constant.

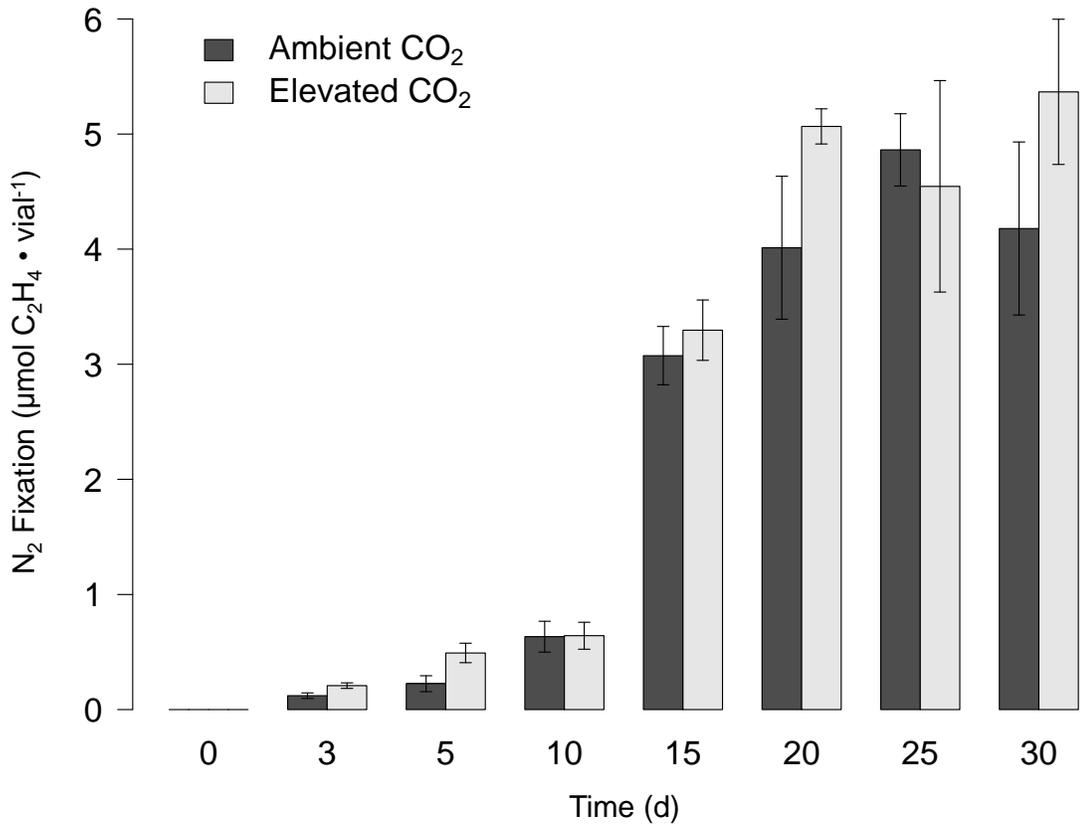


Figure 3.4: N₂ fixation of axenic *N. punctiforme* as measured by the ethylene (C₂H₄) produced by *N. punctiforme* cultures during the growth experiment. Samples were grown on 27 mm diameter agar vials over 30 days under ambient (430 ppm) and elevated (750 ppm) atmospheric CO₂ conditions. Ambient CO₂ is shown using a dark grey bar, whereas elevated CO₂ is shown using a light grey bar. All data are plotted as mean ± SD, and based upon six replicate samples per CO₂ treatment (overall N = 96).

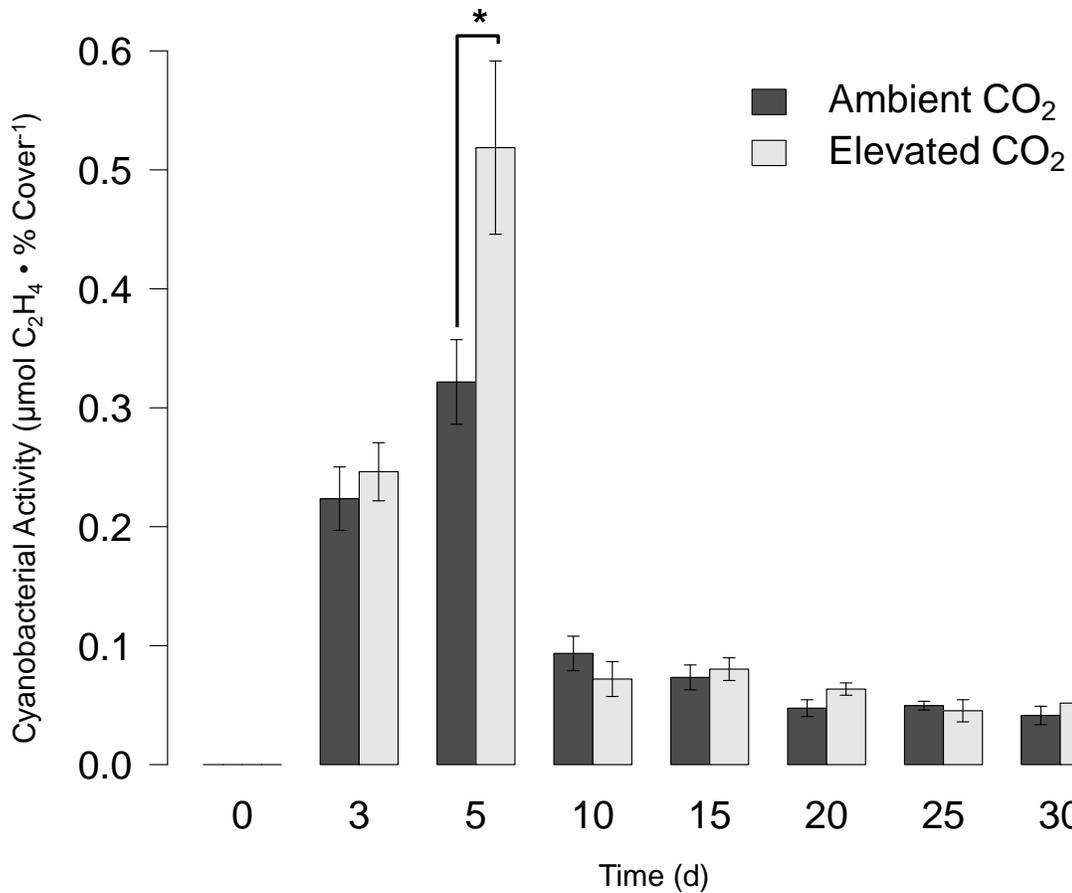


Figure 3.5: Cyanobacterial activity of axenic *N. punctiforme* as measured by the ethylene (C₂H₄) produced per percentage cover of cultures during the growth experiment. Samples were grown on 27 mm diameter agar vials over 30 days under ambient (430 ppm) and elevated (750 ppm) atmospheric CO₂ conditions. Ambient CO₂ is shown using a dark grey bar, whereas elevated CO₂ is shown using a light grey bar. All data are plotted as mean ± SD, and based upon six replicate samples per CO₂ treatment (overall N = 96). Pairwise differences are shown using a symbol (*) with a bar.

3.1.6 Heterocyst Activity

Heterocyst activity (calculated as N₂ fixation per heterocyst cell) between ambient CO₂ and elevated CO₂ was not statistically different (RM-ANOVA: $F_{1,10} = 0.05$, $P = 0.82$; Figure 3.6), with average values of 7.08×10^{-7} $\mu\text{mol C}_2\text{H}_4$ heterocyst⁻¹ and 6.78×10^{-7} $\mu\text{mol C}_2\text{H}_4$ heterocyst⁻¹ for each CO₂ treatment. But, there was a significant difference in heterocyst activity on different days of the experiment (RM-ANOVA: $F_{7,70} = 11.19$, $P < 0.001$), with heterocyst activity reaching a maximum at day 15 for both ambient CO₂ and elevated CO₂ treatments at values of 1.92×10^{-6} and 1.45×10^{-6} $\mu\text{mol C}_2\text{H}_4$ heterocyst⁻¹ (Figure 3.6). Although heterocyst activity declines after reaching a maximum at day 15, a secondary increase is observed at day 30. No effect of CO₂ \times time was observed (RM-ANOVA: $F_{7,70} = 0.91$, $P = 0.50$).

3.1.7 Timeline of Cyanobacterial Growth & N₂ Fixation

Events

Based on the analyses above, a timeline of cyanobacterial growth and fixation activity events was constructed using the main findings for each of the response variables in the growth experiment, highlighting any statistically significant effects observed, maximum levels, and initiation of heterocyst production (Figure 3.7). The tight coupling of N₂ fixation and growth, in addition to the timing of other major events, was used as a framework to interpret the results obtained in the global environmental change experiment.

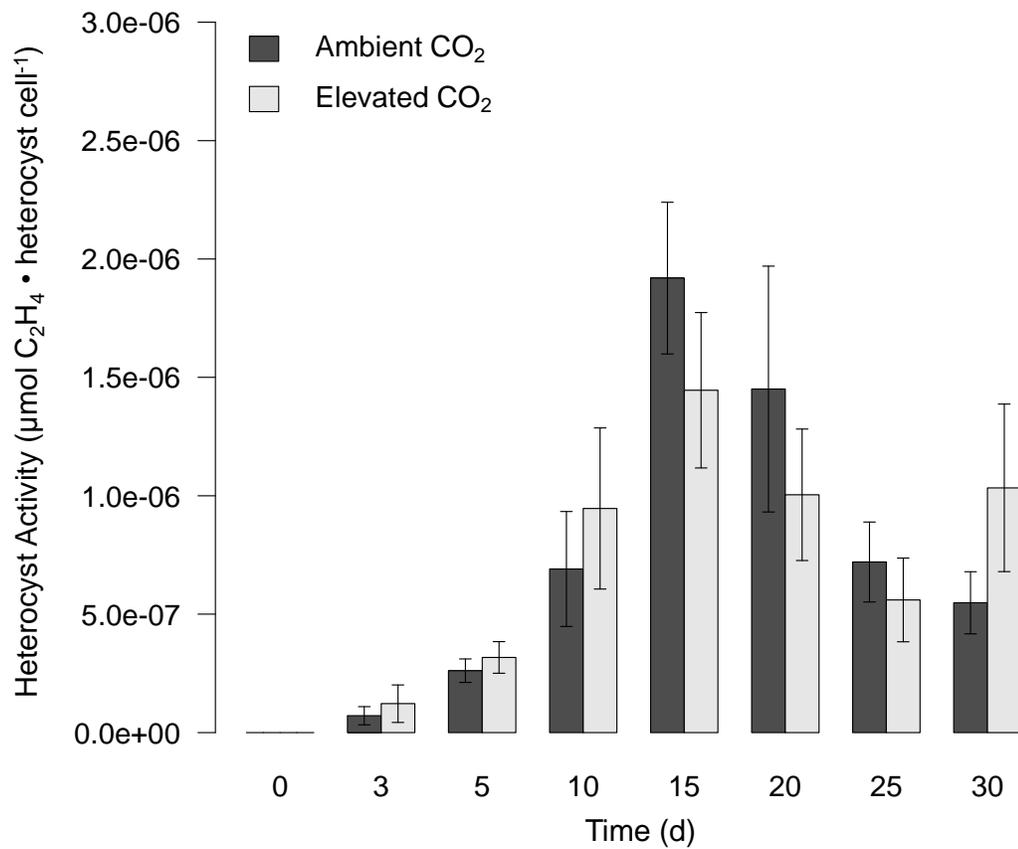


Figure 3.6: Heterocyst activity of axenic *N. punctiforme* during the growth experiment. Heterocyst activity was calculated by the ethylene (C₂H₄) produced per heterocyst cell of *N. punctiforme* cultures on 27 mm diameter agar vials over 30 days under ambient (430 ppm) and elevated (750 ppm) atmospheric CO₂ conditions. Ambient CO₂ is shown using a dark grey bar, whereas elevated CO₂ is shown using a light grey bar. All data are plotted as mean \pm SD, and based upon six replicate samples per CO₂ treatment (overall N = 96).

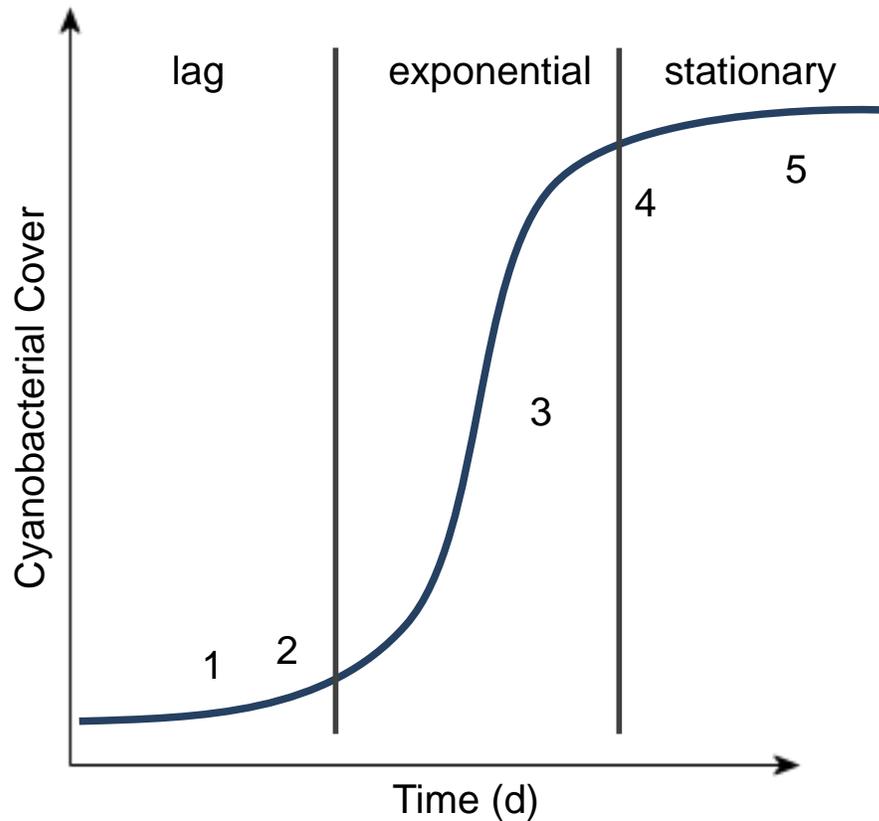


Figure 3.7: Timeline of growth and N_2 fixation events for the cyanobacterium *N. punctiforme*. Samples were grown on 27 mm agar vials during the 30 day growth experiment. In this experiment, CO_2 had a statistically significant effect on cyanobacterial activity, and N_2 fixation and growth followed the same pattern. The numbers shown on the curve represent the timing of critical growth and N_2 fixation events: 1) first heterocysts observed, 2) maximum cyanobacterial activity and maximum heterocyst percentage, 3) maximum heterocyst activity and growth rate, 4) maximum N_2 fixation, and 5) maximum cyanobacterial cover.

3.2 Global Environmental Change Experiment

The global environmental change experiment was designed using a factorial combination of two N availability treatments, three temperature, and two CO₂ treatments, as described above with measurements obtained during a 90 day period. However, due to the absence of N₂ fixation in conditions with available N, and the concomitant lack of heterocyst production and heterocyst activity, the conditions with available N and without available N were analyzed separately, with a focus on N unavailable conditions to interpret the effects of CO₂ and temperature on N₂ fixation (Figure 3.8).

3.2.1 N₂ Fixation and Cyanobacterial Abundance Under N Available Conditions

N₂ fixation was virtually absent in conditions with N available, with observed values consistently less than 0.04 μmol vial⁻¹ (Figure 3.9). The factors CO₂ (RM-ANOVA: $F_{1,30} = 0.50$, $P = 0.48$), temperature (RM-ANOVA: $F_{2,30} = 2.28$, $P = 0.12$), and time (RM-ANOVA: $F_{6,180} = 1.92$, $P = 0.07$) did not have any influence on N₂ fixation under N available conditions. Moreover, no significant effects of CO₂ × time (RM-ANOVA: $F_{6,180} = 1.70$, $P = 0.12$), temperature × time (RM-ANOVA: $F_{12,180} = 0.73$, $P = 0.72$), or CO₂ × temperature × time ($F_{12,180} = 1.61$, $P = 0.09$) were observed, indicating that any observed effects of the global environmental change factors would be consistent throughout the global environmental change experiment for N available conditions.

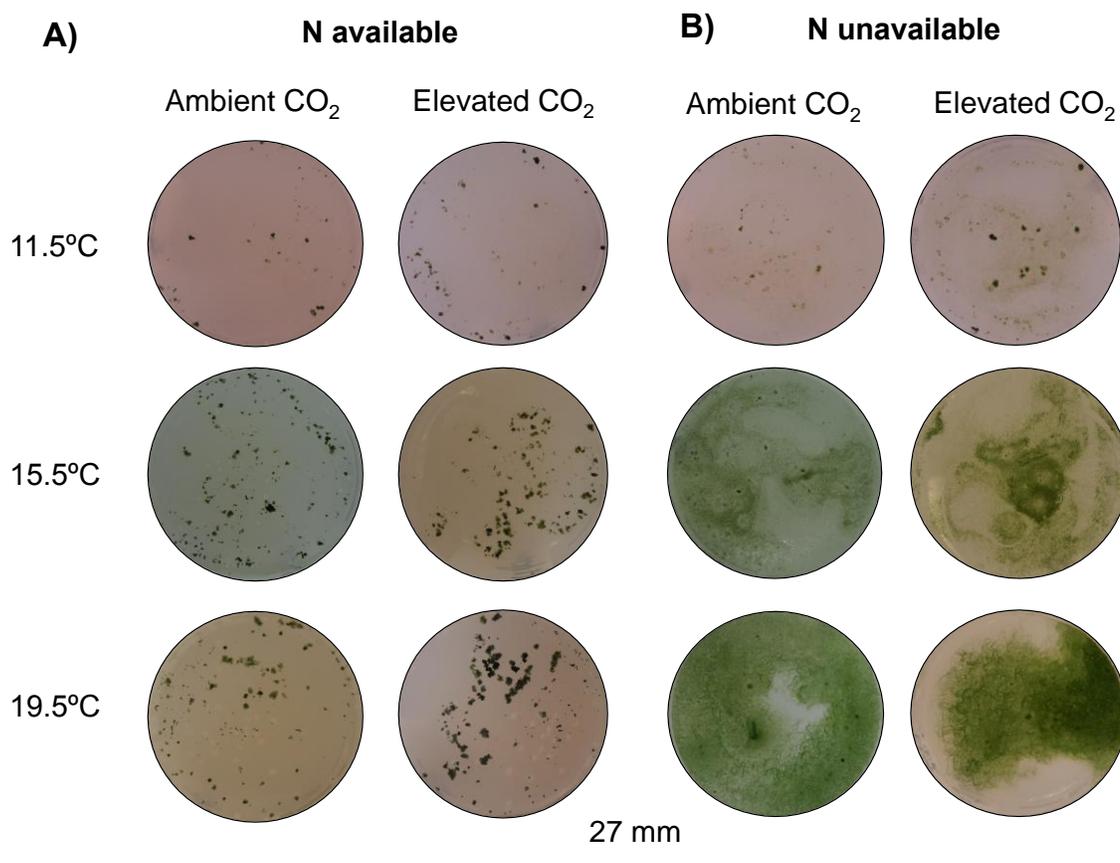


Figure 3.8: *Nostoc punctiforme* on 27 mm diameter agar vials in conditions with A) N available and B) N unavailable after 30 days of growth. In conditions where N was available, less cyanobacteria cover was present, and cyanobacteria grow in isolated colonies, rather than in a mat-like shape as observed under conditions where N was unavailable. Images were captured on an Olympus PEN-mini digital camera and show the surface of the agar (grey) with cyanobacterial growth (green).

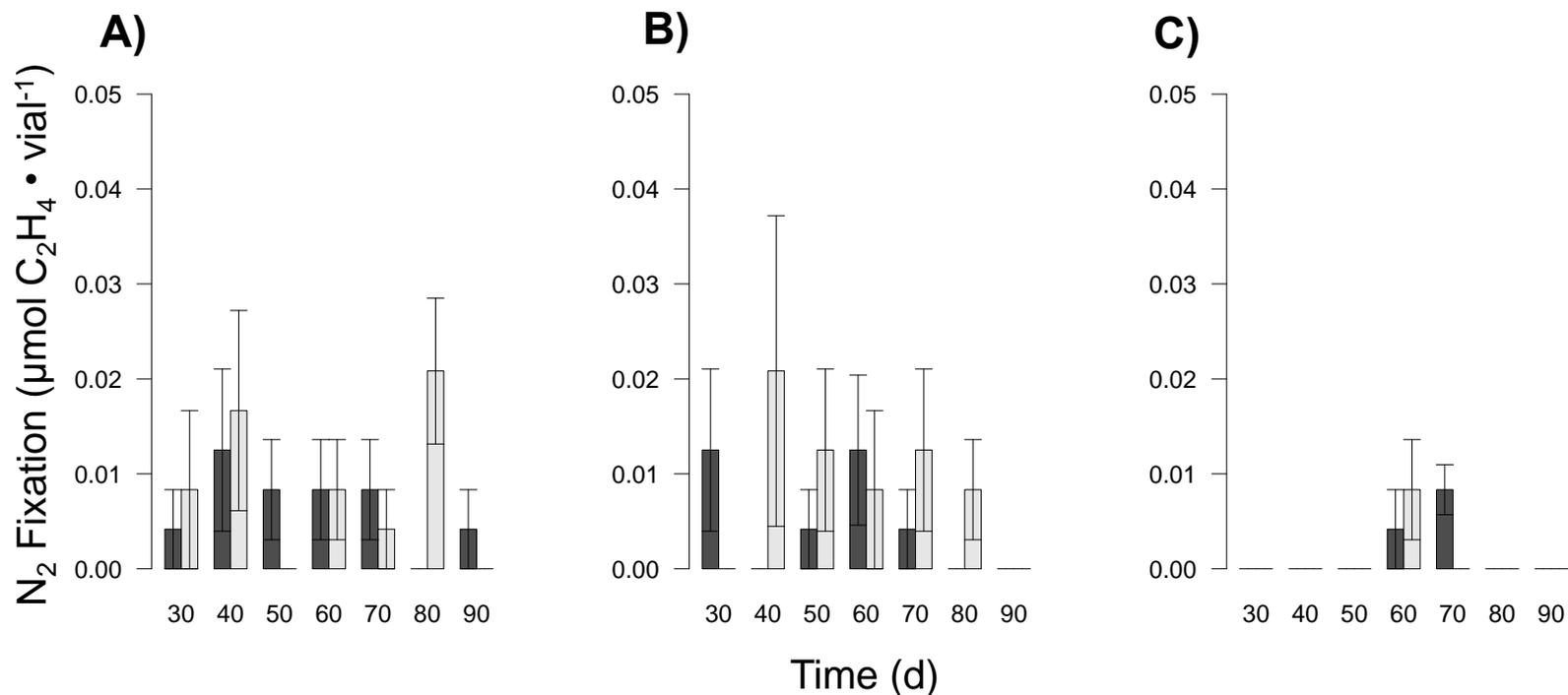


Figure 3.9: N_2 fixation of axenic *N. punctiforme* as measured by the ethylene (C_2H_4) produced per 27 mm diameter agar vials over 90 days. Cultures were grown at A) 11.5 °C, B) 15.5 °C, and C) 19.5 °C on BG-11 media. The conversion from ethylene production to N_2 fixation relies upon a 3:1 molar ratio. Ambient (430 ppm) and elevated (750 ppm) atmospheric CO_2 conditions are marked using dark grey bars and light grey bars, respectively. Values obtained for N_2 fixation in conditions with available N were not statistically different than zero. All data are plotted as mean \pm SD with measurements taken on 36 vials (overall N = 252).

Likewise, there were no significant differences in cyanobacterial abundance present between CO₂ treatments (RM-ANOVA: $F_{1,30} = 0.84$, $P = 0.37$), or temperature treatments (RM-ANOVA: $F_{2,30} = 1.14$, $P = 0.34$) under N available conditions (Figure 3.1). Further, there was no significant effect of CO₂ × temperature (RM-ANOVA: $F_{2,30} = 0.29$, $P = 0.75$). Overall, cyanobacterial cover was minimal in available N treatments compared to N limitation treatments (Figure 3.8).

3.2.2 N₂ Fixation and Cyanobacteria Abundance Under N Limitation Conditions

Under conditions where N was unavailable, N₂ fixation was influenced by CO₂, such that overall N₂ fixation rates were higher for ambient CO₂ (1.35 μmol vial⁻¹) than elevated CO₂ (1.69 μmol vial⁻¹) (RM-ANOVA: $F_{1,30} = 21.77$, $P < 0.001$; Figure 3.1). Temperature also influenced N₂ fixation (RM-ANOVA: $F_{2,30} = 9.77$, $P < 0.001$), with overall higher N₂ fixation rates observed for 11.5 and 19.5 °C than 15.5 °C (Tukey's HSD: $P < 0.05$). However, a statistically significant effect of CO₂ × temperature was revealed (RM-ANOVA: $F_{2,30} = 5.08$, $P = 0.01$), with further interpretation using interactions plots and Tukey's HSD tests demonstrating a strong positive CO₂ effect at 11.5 °C (2.52 μmol vial⁻¹ versus 3.71 μmol vial⁻¹), but no CO₂ effect at 15.5 °C, and a transient positive CO₂ effect at 19.5 °C between days 70 and 90 (Figure 3.1). No significant effect of CO₂ × time was present (RM-ANOVA: $F_{6,180} = 1.41$, $P = 0.22$). Time also had a statistically significant effect on N₂ fixation rates (RM-ANOVA: $F_{6,180} = 53.83$, $P < 0.001$), and a statistically significant

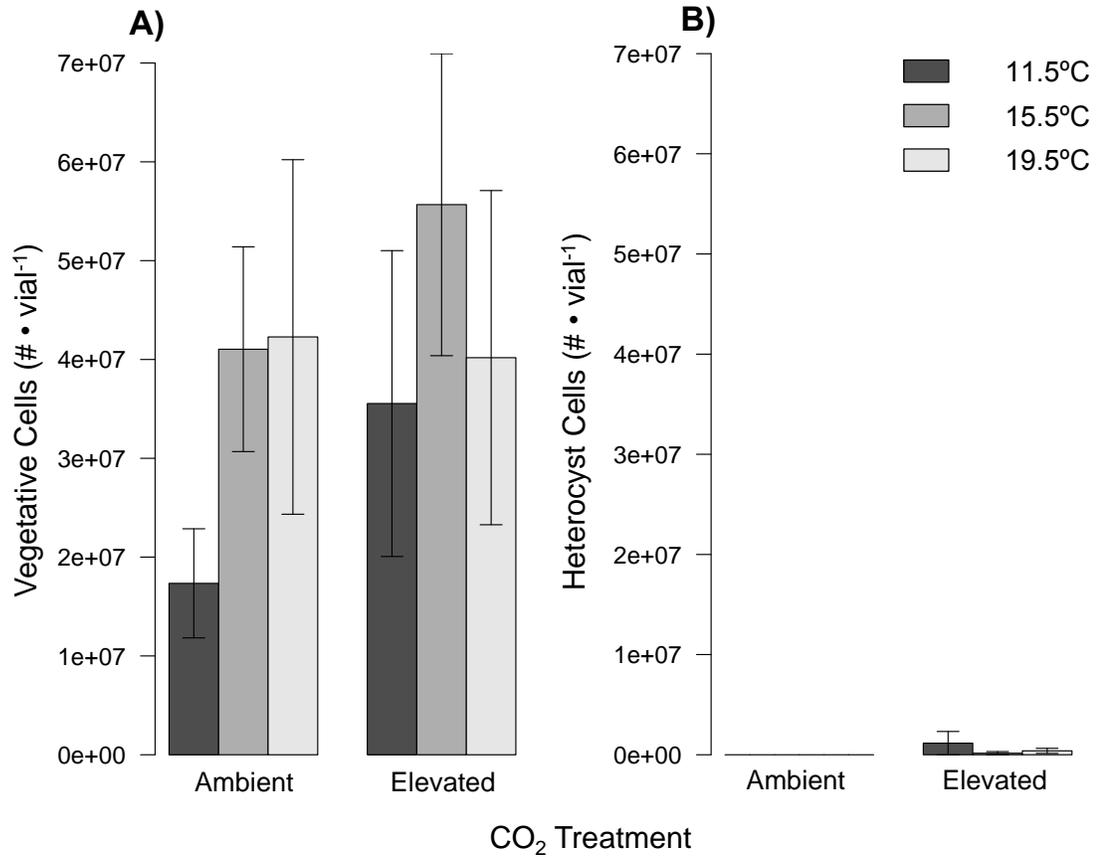


Figure 3.10: A) Vegetative and B) heterocyst cell densities for *N. punctiforme* cultures on 27 mm diameter agar vials at day 90 under ambient and elevated CO₂ treatments on available N media. Temperatures are indicated using color, with 11.5 °C in dark grey, 15.5 °C in medium grey, and 19.5 °C in dark grey. Heterocysts were not produced to a significant extent in conditions with available N. All data are presented as mean ± SD with an overall N = 36.

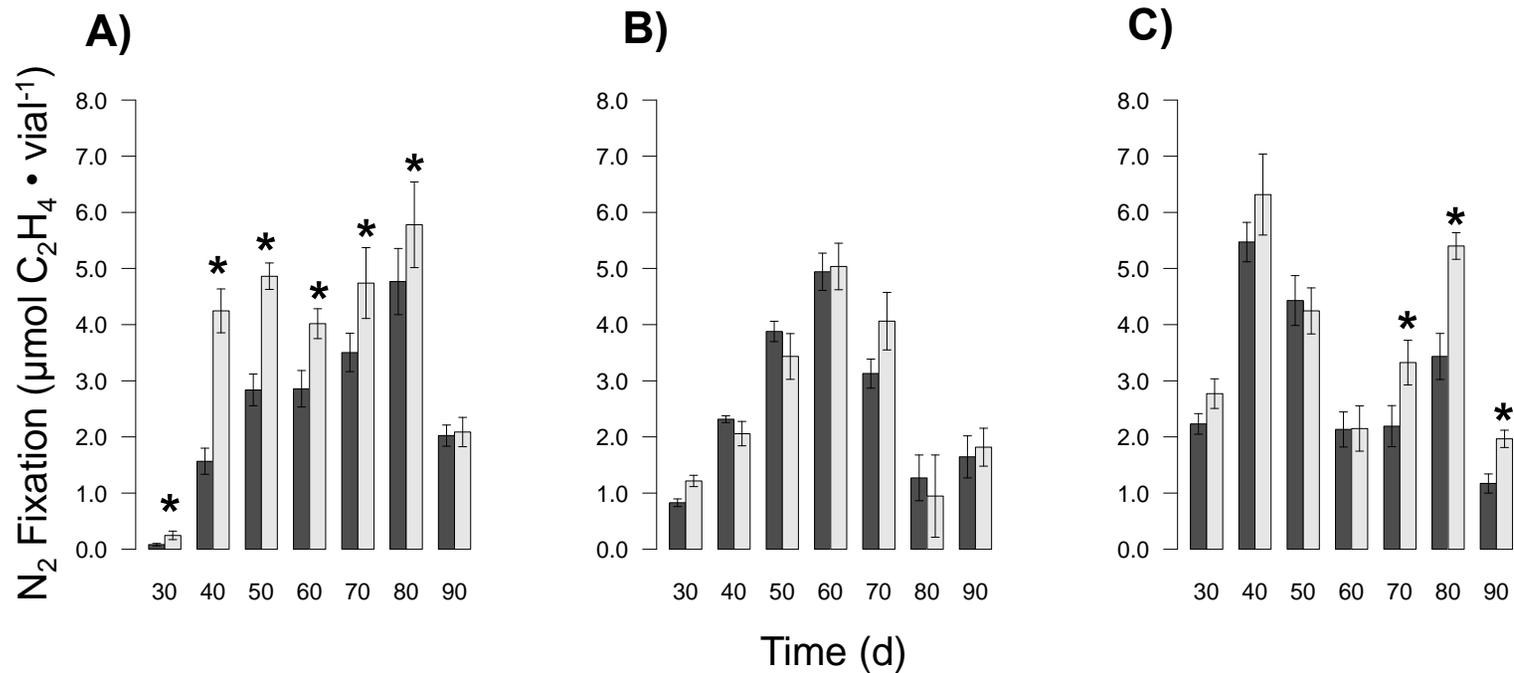


Figure 3.11: N_2 fixation of axenic *N. punctiforme* as measured by the ethylene (C_2H_4) produced per 27 mm diameter agar vial over 90 days during the global environmental change experiment. Vials were grown at A) 11.5 °C, B) 15.5°C, and C) 19.5°C. The conversion from ethylene production to N_2 fixation relies upon a 3:1 molar ratio. Ambient (430 ppm) and elevated (750 ppm) atmospheric CO_2 conditions are marked using dark grey bars and light grey bars, respectively. Vials at 11.5 °C reached maximum N_2 fixation at day 80, whereas this maximum occurred at day 60 at 15.5 °C. In contrast, two maxima were present at 19.5 °C at days 40 and 80. All data are plotted as mean \pm SD with measurements taken on 36 vials (overall N = 252). Pairwise differences are shown using an asterisk (*).

effect of temperature \times time (RM-ANOVA: $F_{12,180} = 30.95$, $P < 0.001$), indicating that different temperatures peak at different times during the global environmental change experiment, where N_2 fixation peaked at day 80 for 11.5°C, day 60 for 15.5°C, and at days 40 and 80 for 19.5°C (Figure 3.11). A statistically significant effect three-way interaction of $CO_2 \times$ temperature \times time was also detected (RM-ANOVA: $F_{12,180} = 2.51$, $P < 0.01$).

There was no significant effect of any of the treatment variables on cyanobacterial abundance at the end of 90 days in vials under N free conditions using RM-ANOVA (CO_2 : $F_{1,30} = 0.08$, $P = 0.79$; temperature: $F_{2,30} = 0.88$, $P = 0.43$; $CO_2 \times$ temperature: $F_{2,30} = 1.08$, $P = 0.35$). There was no statistically significant effect of CO_2 on heterocyst percentage (RM-ANOVA: $F_{1,30} = 0.08$, $P = 0.77$; Figure 3.12). However, a statistically significant effect of temperature on heterocyst percentage was present (RM-ANOVA: $F_{2,30} = 3.57$, $P = 0.04$), such that vials incubated at 11.5 °C (2%) had fewer heterocysts than either 15.5 °C (5%) or 19.5 °C (4%) (Tukey's HSD: $P < 0.05$). The $CO_2 \times$ temperature interaction was not statistically significant (RM-ANOVA: $F_{2,30} = 1.43$, $P = 0.25$).

3.2.3 Cyanobacterial N_2 Fixing Activity Under N Limitation Conditions

Observations of the amount of N_2 fixation activity per heterocyst cell (heterocyst activity) demonstrated that there was no statistically significant effect of CO_2 on heterocyst activity (RM-ANOVA: $F_{1,30} = 0.59$, $P = 0.45$), which was remarkably consistent across the ambient CO_2 and elevated CO_2 treatments (Figure 3.13). Temperature also did not influence heterocyst activity (RM-ANOVA: $F_{2,30} = 1.09$, $P = 0.35$), although heterocyst activity was qualitatively higher at 11.5 °C ($3.74 \times 10^{-7} \mu \text{ mol } C_2H_4 \text{ heterocyst}^{-1}$) and 19.5 °C ($3.14 \times 10^{-7} \mu \text{ mol } C_2H_4 \text{ heterocyst}^{-1}$) than 15.5 °C ($1.75 \times 10^{-7} \mu \text{ mol } C_2H_4 \text{ heterocyst}^{-1}$).

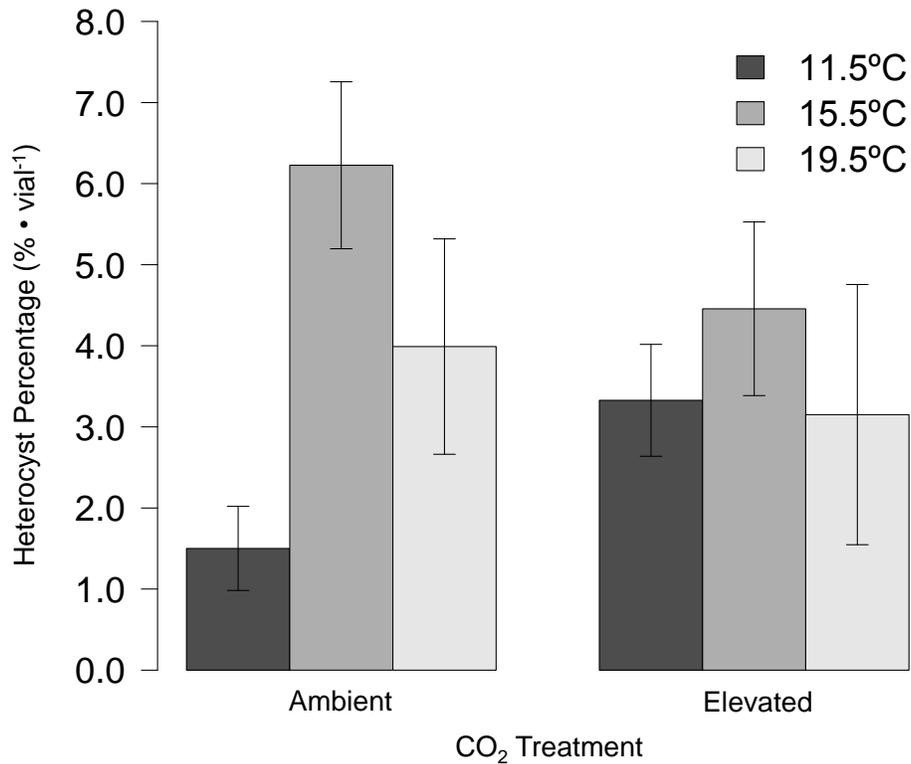


Figure 3.12: Heterocyst percentage for *N. punctiforme* cultures on 27 mm diameter agar vials at day 90 during the global environmental change experiment for under ambient and elevated CO₂ treatments. Data shown are for vials that were grown on media without available N. Temperatures are indicated using color, with 11.5 °C in dark grey, 15.5 °C in medium grey, and 19.5 °C in dark grey. Heterocyst percentage was significantly greater at 15.5 °C and 19.5 °C than 11.5 °C. All data are presented as mean ± SD with an overall N = 36.

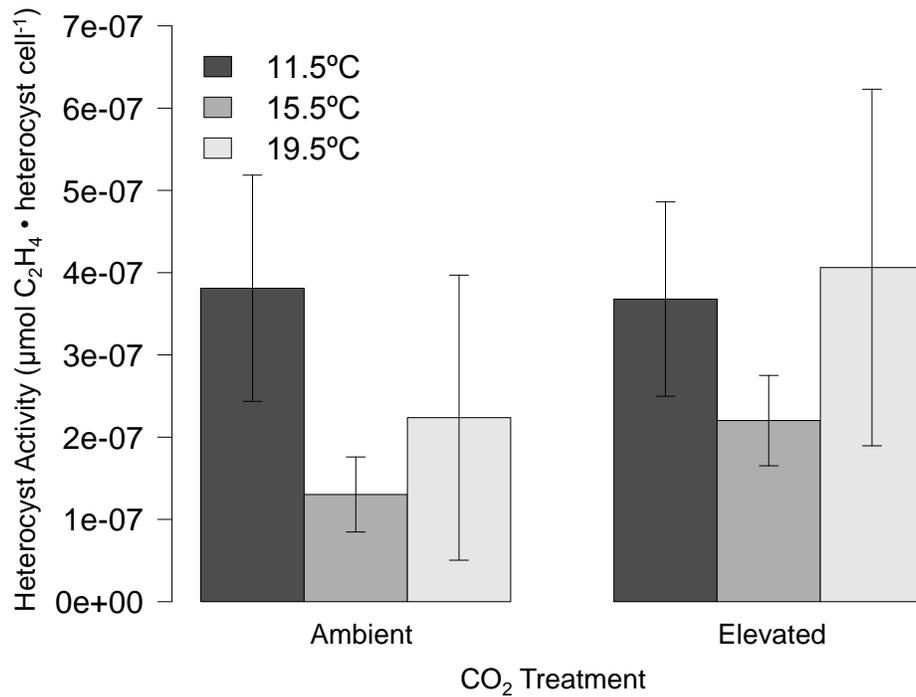


Figure 3.13: Heterocyst activity for *N. punctiforme* cultures on day 90 of the global environmental change experiment. Samples were grown using 27 mm diameter agar vials at day 90 under ambient and elevated CO₂ treatments on media without available N. Temperatures are indicated using color, with 11.5 °C in dark grey, 15.5 °C in medium grey, and 19.5 °C in dark grey. Heterocyst activity was greater at 11.5 and 19.5 °C, compared with 15.5 °C under both ambient and elevated CO₂ conditions. All data are presented as mean ± SD with an overall N = 36.

4 Discussion

4.1 Growth of Axenic Cultures of *Nostoc punctiforme*

My study used the multicellular cyanobacterium *Nostoc punctiforme* as a model species to explore the effects of global environmental change on N₂ fixation. This cyanobacterium was selected because it is terrestrial, filamentous, and known to associate with mosses (Dodds et al., 1995; Meeks & Elhai, 2002; Gentili et al., 2005). In the growth experiment, I examined the effects of ambient and elevated CO₂ on growth and N₂ fixation activity in axenic cultures of *N. punctiforme*. This experiment was performed to gain mechanistic insights into N₂ fixation patterns with respect to growth, and the timing of developmental events for cyanobacteria in N-limited settings. This experiment revealed that N₂ fixation and growth are strongly correlated, and that N₂ fixation rates are highly dependent upon the growth stage of the culture. I observed a stimulatory effect of CO₂ on cyanobacterial activity at day 5, with a similar trend for heterocyst percentage. At the culture scale, I determined that there is a temporal separation between heterocyst formation (3 d), maximum heterocyst percentage (5 d), and maximum heterocyst activity (15 d), demonstrating that N₂ fixation is a carefully orchestrated process driven by differential gene expression; specifically, with up-regulation of genes associated with heterocyst formation (Campbell et al., 2007). Collectively, the findings in this experiment served as a critical framework for interpreting the results of the second experiment (global environmental change experiment).

Since *N. punctiforme* is a terrestrial cyanobacterium, I used solid media to carry out the experiments in this thesis. Growth of axenic cultures was quantified using both cyanobacterial cover and cyanobacterial abundance. I believe that cyanobacterial cover was a

more realistic measure for moss systems where cyanobacteria grow is by spreading across the moss shoots (Gavazov et al., 2010). However, the use of solid media in my thesis makes the results hard to compare with the bulk of other studies, mostly on aquatic cyanobacteria that use liquid media (Levitan et al., 2007; Chinnasamy et al., 2009; Czerny et al., 2009) and cells are freely distributed in three-dimensional space (media volume). In my study, some cyanobacteria cells were able to penetrate the agar surface and grow into the media, but these cells likely would not have been able to scavenge the acetylene gas used in the N₂ fixation assay, and as a result would not demonstrate N₂ fixation activity.

Growth by cyanobacterial cover and N₂ fixation were strongly and positively correlated, while greater cyanobacterial abundance (vegetative cells) as seen on days 20 and 25 of the growth experiment under elevated CO₂ conditions, did not translate to greater N₂ fixation rates. This is likely because vegetative cells are photosynthetic but not N₂ fixing (Meeks & Elhai, 2002). Although the production of vegetative cells (Czerny et al., 2009) or chlorophyll biomass (Chinnasamy et al., 2009) are often used to measure growth in experiments on aquatic cyanobacteria, these measures are not entirely adequate for cyanobacteria in moss systems. Hence, I propose that cyanobacterial cover is a good representative measure of growth in moss systems, as well as being a biologically relevant correlate for N₂ fixation; as such, I use this to form the relationship between growth and N₂ fixation rates prior to initiating the global environmental change experiment. Moreover, measures of cyanobacteria cell densities do still inform us of key events in the culture growth cycle.

To my knowledge, my study is the first to measure growth rates for a terrestrial cyanobacterium from the cyanobacteria-moss association. I observed lag, log, and stationary phases of the cyanobacteria cultures with all vials ultimately achieving full coverage on the

agar surface. The observed growth stages are consistent with other other bacteria (including cyanobacteria), which experience a period of slow growth when initially introduced to a nutrient filled environment (lag), followed by exponential growth upon nutrient utilization (log), and stationary growth upon nutrient exhaustion. Should nutrients not be replenished during stationary phase growth, the culture will ultimately enter a decline phase due to resource limitation. Here, I observed that the middle of the exponential phase occurred around day 16 for both ambient and elevated CO₂ cultures in the growth experiment. At day 16, growth was the most rapid, and I would expect that resources were likely being consumed at the highest rate at this time (Davis et al., 2009). However, I observed that the overall growth (cyanobacterial abundance) cycle was slightly different for ambient and elevated CO₂, such that elevated CO₂ cultures appeared to be entering a decline phase by the end of the experiment, whereas ambient CO₂ cultures did not; this highlights growth as a confounding factor in studies on cyanobacterial N₂ fixation. To address the relationship between growth and the rates of other microbial processes, many studies use mid-log phase cells to eliminate growth as a confounding factor in experiments on axenic cyanobacteria (Kerson et al., 1984; Buikema et al., 1991; Shirai et al., 1991). However, it is unlikely that cyanobacteria in the boreal forest would all be present at the same growth stage, so this was not performed for my study. At the ecosystem-scale, little is known about the relative species composition of cyanobacterial communities, and how the demands for available N change when the cyanobacterium is in association with the moss. However, if future studies require analyses at the genetic or protein level, I would suggest the use of mid-log phase cultures to adequately remove growth as a confounding factor. Given that controlling for growth is challenging in studies on the cyanobacteria-moss association, I suggest that studies seeking a

mechanistic understanding of N₂ fixation focus on axenic cultures of cyanobacteria, rather than moss systems.

Elevated CO₂ has been shown to increase growth in non-heterocystous *Trichodesmium* (Levitan et al., 2007), and reduce growth through reduced cell division rates in the heterocystous species *N. spumigena* (Czerny et al., 2009). In another study, CO₂ concentration had a unimodal effect on growth in the cyanobacterium *A. fertilissima*, with a decline in growth when culture were incubated at CO₂ concentrations above 6% in the headspace (Chinnasamy et al., 2009). I suggest that the CO₂ effect on growth depends upon the growth stage of the culture, similar to Levitan et al. (2007), who demonstrated that the CO₂ effect on N₂ fixation was growth stage dependent. I posit that the discrepancy in results obtained for heterocystous cyanobacteria for the effects of CO₂ within the literature are likely due to growth as a confounding factor on N₂ fixation rates. My study suggests that CO₂ stimulation of growth in *N. punctiforme* is transient, and further that this transient effect will not result in increased N₂ fixation. Given that cyanobacteria growth in moss-systems is slightly different than in agar systems, it would be interesting to observe whether the results obtained in the present study are applicable when the cyanobacterium is associated with the moss.

Although the mechanism for cyanobacterial colonization of mosses is poorly described within the literature, it is demonstrated that hormogonia, small motile filaments of cyanobacteria cells (Rippka et al., 1979; Meeks & Elhai 2002), are the dispersal mechanism by which cyanobacteria colonize mosses in terrestrial ecosystems (Dodds et al., 1995; Meeks & Elhai, 2002). I postulate that hormogonia production likely played a role in the rate at which cyanobacteria spread across the agar and therefore that the mechanistic understanding of cyanobacterial cover can be extended to the intact cyanobacteria-moss association. Under

the microscope, cyanobacteria were present as lengthy filaments, tightly coiled cells, and hormogonia, which are short linear filaments used for dispersal (Meeks & Elhai, 2002; Meeks et al., 2002). Although it was not formally quantified in my study, hormogonia only appeared to be present in conditions where N was unavailable. Given that hormogonia are often produced in response to environmental stress such as nutrient limitation (Meeks et al., 2002), they may not be produced in conditions with available N and therefore the cyanobacterium may be unable to colonize the moss. Previous studies suggest that the N₂ fixing capability of the cyanobacterium is the benefit to the moss partner in the symbiotic association (Berg et al., 2013). In order to colonize mosses, it is likely that cyanobacteria must be in an exponential stage of growth (thereby fixing more N₂) and under nutrient stress (with hormogonia formation) to facilitate colonization of the moss shoots. In the presence of available N, it is likely that cyanobacteria will not form heterocysts or demonstrate heterocyst activity, and moreover will fail to produce hormogonia and participate in symbiosis with mosses.

Given that heterocyst cells are known to be the site of N₂ fixation (Dixon & Kahn, 2004), I focus my mechanistic interpretation of N₂ fixation on heterocyst percentage and activity, rather than metrics using the vegetative cells. Heterocysts first appeared as a significant cell type on day 3 in the growth experiment, with the maximum heterocyst percentage occurring at day 5. Previous studies have not found heterocyst percentage or cell size to be the mechanism for increased N₂ fixation under elevated CO₂ (Czerny et al., 2009). Given that the increase in heterocyst percentage was transient, and not statistically significant, I posit that increased heterocyst percentage is only part of the mechanism for increased N₂ fixation in terrestrial cyanobacteria. There is a genetic basis for both the production of heterocysts and the spacing of heterocysts between vegetative cells in

cyanobacteria filaments (Meeks & Elhai, 2002). It is possible that the genetic regulation of this process is sufficiently tight that it is not plastic in response to environmental conditions. During exponential growth, cyanobacteria consume more N than during stationary growth (Levitan et al., 2007), suggesting that an increase in heterocyst percentage, and thereby available N, must precede the exponential growth stage. My findings are consistent with previous findings (Levitan et al., 2007), in that heterocyst percentage reached a maximum prior to the exponential growth stage. I demonstrated that heterocyst activity did not reach a maximum until day 15, suggesting that there is a temporal separation between heterocyst production, maximum heterocyst percentage, and maximum heterocyst activity. Collectively, my findings suggest that N₂ fixation is dependent upon the timing of heterocyst production, maximum heterocyst percentage, and maximum heterocyst activity; heterocyst formation must precede exponential growth and the associated spike in N₂ fixation that occurs during the exponential phase.

4.2 Growth of Axenic Cultures of *Nostoc punctiforme* In Response to Variation in Global Environmental Change Factors

The overarching goal of the global environmental change experiment was to describe the main and combined effects of N availability, temperature, and CO₂ on N₂ fixation in *Nostoc punctiforme*. I correctly predicted that N₂ fixation would be minimal in conditions with available N present, with heterocysts and their N₂ fixation activity essentially absent. My corresponding prediction of reduced vegetative cell growth was also correct. Likewise, I predicted and observed that biologically relevant N₂ fixation occurs in conditions where fixed N₂ is unavailable, with more heterocysts and their N₂ fixation activity in this N availability

scenario. I found an overall stimulatory effect of CO₂ on N₂ fixation that was temperature dependent, whereby increased N₂ fixation was observed at 11.5 °C under elevated CO₂, although we failed to observe this effect at 15.5 °C, and observed a transient stimulation at 19.5 °C. Although I predicted that CO₂ and temperature would have a synergistic effect on N₂ fixation rates, this effect was antagonistic instead. Together, my findings suggest that the mechanism for increased N₂ fixation under elevated CO₂ is due to a temperature-dependent growth cycle, and a result of increased cyanobacterial activity and heterocyst percentage preceding the exponential growth phase of the cyanobacteria culture.

4.2.1 Effects of elevated atmospheric CO₂ on cyanobacterial growth

In previous studies, CO₂ has been observed to have a stimulatory effect on growth of non-heterocystous species such as *Trichodesmium* (Levitan et al., 2007); however, there are discrepancies in the results for heterocystous species. For example, *N. spumigena* displayed reduced cell division rates in response to CO₂ concentrations across a range of 0 to 800 ppm (Czerny et al., 2009), whereas *A. fertilissima* experienced an increase in chlorophyll biomass with increases of 6% CO₂ concentration (Chinnasamy et al., 2009). The overall stimulatory effect of CO₂ observed in the global environmental change experiment did not translate to a great discrepancy in N₂ fixation rates or cyanobacterial abundance, because the strength of the CO₂ effect was dependent upon the temperature and accordingly, the growth stage of the culture (as described above). Previous studies support that the strength of the CO₂ effect on N₂ fixation in non-heterocystous cyanobacteria is growth stage dependent (Levitan et al., 2007), but temperature was maintained constant at 25 °C. In heterocystous species, it is more challenging to interpret the CO₂ effect, since biomass is sometimes measured using cell

division rates (Czerny et al., 2009), but also by chlorophyll biomass, which may not be standardized on a per cell basis (Chinnasamy et al., 2011). The previous study that examined CO₂ effects on N₂ fixation in mosses did not quantify cyanobacterial abundance (Smith, 1984), leaving the effects of CO₂ on the cyanobacterial communities enigmatic. Since my study demonstrates that the CO₂ effect is dependent upon the growth rate (due to temperature), and the growth stage, it is possible that cyanobacterial growth rates are also of importance at the ecosystem-level (e.g. moss associated systems). Evidently, a CO₂ experiment in an *in situ* ecosystem setting would be an ideal next step in the mechanistic interpretation of CO₂ effects on N₂ fixation.

4.2.2 Effects of elevated temperature on cyanobacterial growth

Average growth rates are a function of temperature in heterocystous cyanobacteria (Spencer et al., 2011; Belnap, 2003), whereby cultures at higher temperatures grow faster than cultures at lower temperatures. This relationship is likely unimodal and elevated temperatures beyond some species-specific optimum likely negatively impact growth rates. Qualitatively, it was evident that the spread of the culture across the agar was more strongly related to temperature than to CO₂ in the global environmental change experiment because vials at 19.5 °C displayed more cover than those at 15.5 or 19.5 °C upon 30 days of growth (Figure 3.9). I propose that increased temperature will allow greater cyanobacterial infection of mosses, given that cyanobacterial cover (spread of the agar surface) is likely closely related to the rate at which cyanobacteria can colonize the moss.

N₂ fixation rates are also strongly influenced by temperature (Belnap, 2003), particularly in axenic cyanobacteria from the moss-association (Gentili et al., 2005). To add

to this body of knowledge, I demonstrate that N_2 fixation is a cyclical process that closely follows growth. At 11.5 °C, the cultures were observed in exponential phase, followed by a stationary state at day 90, as suggested by N_2 fixation rates correlating to growth. In contrast, the cultures at 15.5 °C experienced an exponential, stationary, and decline phase, with what appeared to be a secondary exponential phase commencing at day 90 of the global environmental change experiment, while two successive growth cycles were observed at 19.5 °C. At each temperature, the CO_2 treatment did not appear to influence the growth stage of the culture, suggesting that the effect of temperature on N_2 fixation cycles is the strongest. At the conclusion of the second experiment, no differences in cyanobacterial abundance were present between the temperature treatments. Together, these findings suggest that it is the growth stage of the cyanobacteria cells, not the abundance that dictates N_2 fixation rates. In ecosystem studies, the growth stage of the cyanobacteria cells often remains unknown, which can potentially lead to a discrepancy in the effects of temperature on N_2 fixation rates.

4.2.3 Effects of N availability and N limitation on cyanobacterial growth

In the global environmental change experiment, available N lead to a sharp decrease in cyanobacterial abundance, whereby the abundance of cyanobacteria was an order of magnitude lower for vials that contained available N. From an N availability perspective, the cyanobacteria growth was also dramatically lower on BG-11 agar with available N, compared to BG-11₀ agar without available N. It is possible that available N would reduce the ability of cyanobacteria to colonize the moss, since it reduces the ability of cyanobacteria to spread on the agar, and this is closely related to how cyanobacteria will spread in a moss system. N_2 fixation was also absent in conditions with available N; since cyanobacteria are

inactive in conditions where N is available, the mechanism for this is a combination of reduced cyanobacterial abundance and thereby less N₂ fixation.

Given that the presence or absence of N was used to construct treatments within this study, I could not determine whether the effects of N availability on cyanobacterial growth were indicative of a threshold at which cyanobacterial abundance decreases in response to available N, or whether small influxes of N are non-problematic for cyanobacterial abundance. Given that N is a required nutrient for cyanobacterial growth and development (Campbell et al., 2007), it is likely that smaller influxes of N will not have significant effects on cyanobacterial abundance. Further, it is known that cyanobacteria preferentially use ammonium (NH₄⁺) over nitrate (NO₃⁻), suggesting that the type of available N may also have differing effects on cyanobacterial abundance (Liu et al., 2013).

4.2.4 N₂ fixation of axenic cultures of *Nostoc punctiforme*

Previous studies have measured the abundance of cyanobacteria cells on moss shoots to understand N₂ fixation rates in terrestrial ecosystems (Gundale et al., 2011; Lindo and Whiteley, 2011). Although my study was carried out on axenic cultures of cyanobacteria to acquire a mechanistic understanding of N₂ fixation, my interpretation of the results can be extended to the cyanobacteria in association with mosses. Given that cyanobacterial cover and N₂ fixation were closely related, it suggests that an increase in growth due to vegetative cell production was not the mechanism for increased N₂ fixation under elevated CO₂ observed in the global environmental change experiment. Although it is expected that vegetative cells are photosynthetic and supply heterocyst cells with energy and resources for N₂ fixation (Levitan et al., 2007; Meeks and Elhai, 2002; Dixon and Kahn, 2004), it is evident that cyanobacterial abundance does not always correspond to an increase in observed N₂ fixation rates.

In the growth experiment, N₂ fixation accelerated rapidly at day 15, which corresponded to the middle of the exponential growth cycle. Hence, it appears that cyanobacteria produce fixed N₂ to sustain rapid growth of vegetative cells. Given that vegetative cells are photosynthetic and nutrient depleting (Dixon and Kahn, 2004; Campbell et al., 2007; Meeks et al., 2001), it is likely that available N production is a precursor to rapid growth. As a result, it is unlikely that increased cyanobacterial abundance can be the mechanism for increased N₂ fixation, since available N production must occur before this increase in growth. Indeed, it has previously been showed that N₂ fixation rates are much greater during exponential growth than stationary growth (Levitan et al., 2007). Studies on the cyanobacteria-moss association often do not determine the abundance of cyanobacteria cells (Smith, 1984; Zackrisson et al., 2004; Zackrisson et al., 2009), and moreover, estimating the growth stage of these cells is difficult in studies that do consider cyanobacterial abundance (Gundale et al., 2011). Here, I report empirical evidence that N₂ fixation precedes rapid vegetative cell production.

4.3 N₂ Fixation of Axenic Cultures of *Nostoc punctiforme* In Response to Variation In Global Environmental Change Factors

4.3.1 Effects of elevated atmospheric CO₂ conditions on N₂ fixation

No effect of CO₂ on N₂ fixation was observed during the growth experiment. However, this experiment was run at 22 °C, and it is known that temperature has a profound effect on cyanobacterial N₂ fixation rates (Belnap, 2003), with secondary effects on growth

rates as shown in my study, whereby the rate of successive growth cycles dictated the rate at which N₂ was fixed. However, stimulatory effects of CO₂ on cyanobacterial abundance did not translate to increased N₂ fixation at days 20 and 25, therefore I postulate that the CO₂ effect was either absent or temperature dependent on N₂ fixation rates. My observation of a transient and stage dependent effect on cyanobacterial abundance suggests that the decreased N₂ fixation previously observed in moss systems under elevated CO₂ cannot be properly described without examining the stage of cells (Smith, 1984). Given that the number of cyanobacterial cells present and their growth stage is often not quantified concomitantly in experiments on cyanobacteria-moss associations (DeLuca et al., 2002; Gundale et al., 2012; Zackrisson et al., 2004; Zackrisson et al., 2009), I suggest that future experiments on CO₂ effects in moss-systems will likely be negatively affected by disregarding the important implications of cyanobacteria growth rates and stages.

Since my study was the first to explore CO₂ effects on N₂ fixation in cyanobacteria from the moss association, it is unknown how species differences between cyanobacterial communities will contribute to the observed effects of CO₂ on N₂ fixation. Cyanobacteria from different genera associate with mosses (Gentili et al., 2005; Lindo and Whiteley, 2011; Jean et al., 2012); however, I do not yet have evidence of how the CO₂ effect on N₂ fixation varies between species. Given that *Calothrix* and *Nostoc* possess different temperature maxima for N₂ fixation rates (Gentili et al., 2005), it is possible that the response of cyanobacteria to other global environmental change factors will also be species dependent. I suggest that future studies on pure cultures use multiple strains of cyanobacteria, to ensure that an accurate scope of CO₂ effects is obtained.

At the conclusion of the global environmental change experiment, heterocyst percentage appeared to be similar across all CO₂ and temperature treatments with N

unavailable. Based upon the findings of my growth experiment, I believe that heterocyst percentage does not vary significantly in response to variation in global environmental change factors other than available N.

4.3.2 Effects of elevated temperatures on N₂ fixation

The growth experiment demonstrated that N₂ fixation and growth are concerted processes, dictated by the rate at which cyanobacteria spread on the agar and presumably require N to produce vegetative cells. With this as the basis for the global environmental change study, I was able to relate the obtained measurements of N₂ fixation to growth rate and stage in the cyanobacterial culture. I found that CO₂ had a stimulatory effect on N₂ fixation, but that the extent of this was temperature dependent. My study is the first to demonstrate an interactive effect of CO₂ and temperature on a terrestrial cyanobacterium from the moss association. Previous studies demonstrated that CO₂ had a beneficial effect on N₂ fixation at elevated temperature for marine species (Chinnasamy et al., 2009), but I observed that CO₂ stimulates N₂ fixation at low temperature in a terrestrial species. At the conclusion of the global environmental change study, I found no differences in vegetative cell production between the CO₂ treatments at each temperature, suggesting that vegetative cell production was not the mechanism for increased N₂ fixation. However, N₂ fixation rates at day 90 of the global environmental change experiment were similar, so this could be the reason for a lack of a difference. Critically, the stimulatory effect of CO₂ was observed at current boreal ambient conditions (11.5 °C), but absent at 15.5 and 19.5 °C, which represent temperature increases.

Since temperature and atmospheric CO₂ concentrations are both expected to increase in boreal ecosystems (IPCC, 2007), I suggest that rising temperatures will likely negate the beneficial effect of CO₂ on N₂ fixation rates. However, it cannot be excluded that differences

in cyanobacterial community composition will contribute to the response to increases in temperature and atmospheric CO₂ concentrations. For example, *Calothrix* experiences increased N₂ fixation at 30 °C (Gentili et al., 2005), a temperature that above the thermal optimum for *Nostoc* (Gentili et al., 2005) and terrestrial nitrogenase activity as a whole (Houlton et al., 2008).

4.3.3 Effects of N availability and N limitation on N₂ fixation

N₂ fixation did not occur to any biologically relevant extent in conditions where available N was present, with no associated effects of temperature or CO₂. Previous literature supports that N₂ fixation in mosses sharply declines in response to available N, and that the mechanism for this may be genetic (Vintila & El-Shehawy, 2007) or environmental (Zackrisson et al., 2004; Zackrisson et al., 2009; Gundale et al., 2011). However, the threshold at which this occurs is enigmatic. Global environmental change studies have shown that N₂ fixation responds to temperature changes in minutes (Jean et al., 2012), suggesting the effects of N₂ fixation to other global environmental change factors could also be instantaneous. In previous studies on the cyanobacteria-moss association, N₂ fixation rates were often not correlated with cyanobacterial abundance (Smith, 1984), leaving the mechanism responsible for reductions in N₂ fixation in response to available N unknown. Here, I demonstrated that N₂ fixation decreases in response to available N, and reveal that reduced heterocyst abundance and activity can be attributed to this decrease.

N₂ fixation occurred to a significant extent in conditions where N was absent. Given that vials were consistently inoculated with cyanobacteria that were grown in BG-11 media with a low heterocyst count, I support the suggestion that an environmental signal called a

‘nitro-stat’ (Menge & Hedin, 2009) allows cyanobacteria to sense an environment lacking available N and commence producing heterocyst cells to fix unavailable atmospheric N₂. I previously confirmed that cell densities of cyanobacteria in these conditions are an order of magnitude greater than conditions with available N present, and propose that the ‘nitro-stat’ signal is concomitant with cyanobacteria growth.

My results are consistent with previous studies that demonstrate available N reduces N₂ fixation rates in moss systems (Zackrisson et al., 2004; Zackrisson et al., 2009). Here, I propose that the abundance of cyanobacteria cells may decrease in response to available N and result in moss systems that lack cyanobacteria and their N fixing ability. Indeed, previous studies have shown that cyanobacterial abundance is lower in scenarios with available N (Gundale et al., 2011), which adds support to my findings. Together, these findings suggest that available N may eliminate N₂ fixation in mosses due to a reduction in cyanobacterial abundance led by a decrease in vegetative cells. In both N availability scenarios, there was no demonstrated effect of temperature or CO₂ on cyanobacterial abundance, suggesting that available N will be the predominant global environmental change factor influencing the ability of cyanobacteria to colonize mosses. Since I also showed that cyanobacterial cover decreased in response to available N, I have multiple points of evidence suggesting that increases in N availability at the ecosystem scale will likely result in reduced N₂ fixation rates. Further, I demonstrated that a decrease in cyanobacterial abundance is likely the mechanism for the observed decrease in N₂ fixation rates in response to available N in cyanobacteria-moss studies (Zackrisson et al., 2004; Zackrisson et al., 2009), although prior studies have often failed to identify a mechanism since cyanobacterial isolates are not typically microscopically examined.

My study consisted of a binary classification for N availability, whereby available N was either present or absent for each N availability treatment. However, this scenario is likely non-representative at the ecosystem scale. Although influxes of available N from anthropogenic and microbial processes occur (Gruber & Galloway, 2008), it is likely that N availability will rise gradually over time rather than instantaneously (IPCC, 2007). Given that N deposition rates are expected to increase over the course of the next century (IPCC, 2007), it is possible that an influx of NO_x will result in decrease of cyanobacterial abundance and their N_2 fixation, and that this will have ecosystem-level effects on the composition of the soil N pool. Since the level at which available N reduces cyanobacterial abundance is unknown, I propose that it is critical to quantify this threshold.

In conditions where N is available, cyanobacteria do not form heterocysts. As a result, cyanobacteria do not fix N_2 , or display cyanobacterial activity to any significant extent. These findings are consistent with genetic studies on N_2 fixation (Vintila & El-Shehawy, 2007), as well as ecological studies (Zackrisson et al., 2004; Zackrisson et al., 2009), which consistently show that available N attenuates N_2 fixation rates. My research adds to the field, as I am able to demonstrate that the decrease in N_2 fixation rates in conditions with available N correspond with a decrease in cyanobacterial abundance, cyanobacterial activity, and heterocyst percentage. It is likely that cyanobacteria in association with mosses will show the same response as the *N. punctiforme* used within my study. The amount of ammonia (NH_3) produced on average under each combination of temperature and CO_2 suggests that increases in N_2 fixation due to elevated CO_2 are greatest under ambient temperature. Given that the CO_2 stimulation was absent at 15.5 °C, and minimal at 19.5 °C, this suggests that increases in temperature and CO_2 will result in reduced N_2 fixation in boreal forest ecosystems.

4.4 Conclusions

The results obtained in the present study contribute to the growing body of literature in global environmental change research for boreal forest ecosystems. To my knowledge, my study is the first to document ambient CO₂ and elevated CO₂ growth curves for a cyanobacterium that is terrestrial, axenic, and symbiotically competent. This is the first study to demonstrate that elevated CO₂ is stimulatory on N₂ fixation in a terrestrial cyanobacterium. In doing so, I demonstrated that N₂ fixation and growth closely follow each other. I also show that there is a statistically significant effect of CO₂ on cyanobacterial activity. Moreover, heterocyst production occurs within the first 72 hours of culture establishment, hence heterocysts do not reach their maximum activity until day 15, where the culture displays its maximum growth rate. This is also temporally separated from the occurrence of maximum N₂ fixation, which did not occur until 20 days after vial inoculation. I suggest that this is due to an increased growth demand under elevated CO₂ that stimulates heterocyst percentage and activity. As we saw in the second experiment, this result is mediated by temperature, and therefore growth stage. This result can explain the discrepancy in CO₂ effects previously documented in the literature. Collectively, these findings demonstrated that N₂ fixation in cyanobacteria is dependent upon both growth rate and growth stage. The results gained in this thesis present a mechanistic understanding of N₂ fixation by *N. punctiforme* when it is not in association with mosses. However, it is possible that these results will not hold up in conditions where the cyanobacterium is still in association with the moss. As such, it is possible that the underlying energetics and physiology of the symbiosis are rather different than the effects that are observed when the cyanobacterium is present in isolation.

Using these findings as a basis for my second experiment, I examined the effects of increased N availability, temperature, and CO₂ on *N. punctiforme*. In doing so, I demonstrated that N₂ fixation does not occur in conditions where combined N is available, and that heterocyst production and heterocyst activity are absent under such conditions. Here, it is likely that N available conditions allow the cyanobacterium to remain in the vegetative cell cycle due to an absence of nutrient limitation, which triggers differentiation of vegetative cells into more specialized structures (Meeks et al., 2001; Meeks et al., 2002). I found that N₂ fixation occurs in conditions where available N is absent, and demonstrated that CO₂ stimulation of N₂ fixation occurs at boreal ambient temperature (11.5°C). It is possible that overall reductions in N₂ fixation might be observed, both through influxes of available N and the combined effect of temperature moderating cyanobacterial growth under elevated CO₂. Overall, this collection of findings suggests that any increases in N₂ fixation under elevated CO₂ will be negated by concomitant increases in temperature. Future studies should test the response of cyanobacteria in association with mosses, to further extend the implications of this research to an ecosystem scale.

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Appendices

Appendix A. The R code attached to this thesis was used to generate all plots and analyses in this document.

```
# Permission to Write Presentation.
# Danielle Griffith.
# May 2, 2014.

#####[30 DAY EXPERIMENT]#####

# Set working directory
setwd("/Users/daniellegriffith/Google Drive/R Analysis")
getwd()

# Read in data for percent cover. Data points in this set were
obtained by taking measurements of percent cover every two days for
a period of 30 days.
biomass <- read.csv("full.cover.set.csv")
biomass

# Load required packages.
require(sciplot)

# Subset into required frames based upon carbon.
ambient <- subset(biomass, biomass$vial <= 6)
head(ambient)

elevated <- subset(biomass, biomass$vial >= 7)
head(elevated)

# (1) Percent cover graph.
par(ps=16, cex=1.3, las=1)
lineplot.CI(x.factor=time, x.cont=TRUE, response=cover, group=carbon,
data=biomass, ylab="Growth (% Cover)", xlab="Time (Day)",
legend=TRUE, fixed=TRUE, leg.lab=c(expression(paste("Ambient
CO"[2])),expression(paste("Elevated CO"[2]))), x.leg=0.5, y.leg=102,
pch=c(19,19), err.width=0.05, lty=1,lwd=2, las=1,
col=c("peru","lightskyblue"), frame.plot=FALSE, axes=FALSE)
axis(2)
axis(1)

# (1) Percent cover graph in black and white.
par(ps=16, cex=1.3, las=1)
lineplot.CI(x.factor=time, x.cont=TRUE, response=cover, group=carbon,
data=biomass, ylab="Growth (% Cover)", xlab="Time (Day)",
legend=TRUE, fixed=TRUE, leg.lab=c(expression(paste("Ambient
CO"[2])),expression(paste("Elevated CO"[2]))), x.leg=0.5, y.leg=102,
err.width=0.05, lwd=2, las=1, frame.plot=FALSE, axes=FALSE)
axis(2)
axis(1)
```

```

# (1) ANOVA using the repeated measures model.
anova.cover <-
aov(cover~factor(carbon)*factor(time)+Error(factor(vial)),
data=biomass)
summary(anova.cover) #no significant differences in % cover due to
carbon
quartz()
par(mfrow=c(2,2))
plot(aov(cover~factor(carbon)*factor(time), data=biomass))
TukeyHSD(aov(cover~factor(carbon)*factor(time), data=biomass))

# (1) Interaction plots.
par(mfrow=c(1,2))
lineplot.CI(x.factor=time, x.cont=TRUE, response=cover, data=biomass,
ylab="Growth (% Cover)", xlab="Time (Day)", xlim=c(0,30),
err.width=0.05, las=1, frame.plot=TRUE) #main effect of time
lineplot.CI(x.factor=as.numeric(carbon), x.cont=TRUE, response=cover,
data=biomass, ylab="Growth (% Cover)", xlab="Carbon", las=1,
frame.plot=TRUE)

# Adding in percent cover for standardizations: day 3 of ARA
standardized against day 4 of percent cover. day 5 of ARA
standardized against day 6 of percent cover. day 15 of ARA
standardized against day 16 of percent cover. day 25 against day 26
percent cover. Remaining times are dead-on.
exp2 <- read.csv("exp2.final.csv")
head(exp2)

# Subset for carbon.
ambient <- subset(exp2, exp2$carbon == "Ambient")
head(ambient)

elevated <- subset(exp2, exp2$carbon == "Elevated")
head(elevated)

# (2) Ethylene production.
par(ps=16, cex=1.3)
bargraph.CI(x.factor=time, x.cont=TRUE, group=carbon,
response=ethylene, data=exp2, col=c("peru", "lightskyblue"),
ylab="Ethylene Production (µmol/vial)", xlab="Time (Day)",
legend=TRUE, leg.lab=c(expression(paste("Ambient
CO"[2])),expression(paste("Elevated CO"[2]))), err.width=0.05, las=1,
x.leg=1, y.leg=6.1, ylim=c(0,6.5))

# (2) Ethylene production in black and white.
par(ps=16, cex=1.3)
bargraph.CI(x.factor=time, x.cont=TRUE, group=carbon,
response=ethylene, data=exp2, ylab="Ethylene Production (µmol/vial)",
xlab="Time (Day)", legend=TRUE, leg.lab=c(expression(paste("Ambient
CO"[2])),expression(paste("Elevated CO"[2]))), err.width=0.05, las=1,
x.leg=1, y.leg=6.1, ylim=c(0,6.5))

```

```

# (2) ANOVA on ethylene production.
anova.ethylene <-
aov(ethylene~factor(carbon)*factor(time)+Error(factor(vial)),
data=exp2)
summary(anova.ethylene)
quartz()
par(mfrow=c(2,2))
plot(aov(ethylene~factor(carbon)*factor(time), data=exp2))
TukeyHSD(aov(ethylene~factor(carbon)*factor(time), data=exp2))

# (2) Interaction plots for ethylene production.
par(mfrow=c(1,3))
lineplot.CI(x.factor=time, x.cont=TRUE, response=ethylene, data=exp2,
ylab="Ethylene Production (µmol/vial)", xlab="Time (Day)",
xlim=c(0,30), err.width=0.05,las=1, frame.plot=TRUE) #main effect of
time
lineplot.CI(x.factor=as.numeric(carbon), x.cont=TRUE,
response=ethylene, data=exp2, ylab="Ethylene Production (µmol/vial)",
xlab="Carbon (Ambient versus Elevated)", err.width=0.05, las=1)
#main effect of carbon
lineplot.CI(x.factor=time, x.cont=TRUE, response=ethylene,
group=carbon, data=exp2, ylab="Ethylene Production (µmol/vial)",
xlab="Time (Day)", legend=TRUE, fixed=TRUE, err.width=0.05,las=1,
frame.plot=TRUE, x.leg=2) #carbon*timepoint interaction

# (3) Ethylene production per percent cover.
par(ps=16, cex=1.5)
bargraph.CI(x.factor=time, x.cont=TRUE, group=carbon,
response=ethylene.cover, data=exp2, col=c("peru","lightskyblue"),
ylab="Ethylene Production/Percent Cover", xlab="Time (Day)",
legend=TRUE, leg.lab=c(expression("Ambient
CO"[2]),expression("Elevated CO"[2])), err.width=0.05, las=1,
x.leg=14, ylim=c(0,0.6))

# (3) Ethylene production per percent cover in black and white.
par(ps=16, cex=1.5)
bargraph.CI(x.factor=time, x.cont=TRUE, group=carbon,
response=ethylene.cover, data=exp2, ylab="Ethylene
Production/Percent Cover", xlab="Time (Day)", legend=TRUE,
leg.lab=c(expression("Ambient CO"[2]),expression("Elevated CO"[2])),
err.width=0.05, las=1, x.leg=14, ylim=c(0,0.6))

# (3) ANOVA on ethylene production standardized by percent cover.
anova.ethylene.cover <-
aov(ethylene.cover~factor(carbon)*factor(time)+Error(factor(vial)),
data=exp2)
summary(anova.ethylene.cover)
quartz()
par(mfrow=c(2,2))
plot(aov(ethylene.cover~factor(carbon)*factor(time), data=exp2))
TukeyHSD(aov(ethylene.cover~factor(carbon)*factor(time), data=exp2))

```

```

# (3) Interaction plots on ethylene production standardized by
percent cover.
par(mfrow=c(1,3))
lineplot.CI(x.factor=time, x.cont=TRUE, response=ethylene.cover,
data=exp2, ylab="Ethylene Production/Percent Cover", xlab="Time
(Day)", xlim=c(0,30), err.width=0.05, las=1, frame.plot=TRUE) #main
effect of time
lineplot.CI(x.factor=as.numeric(carbon), x.cont=TRUE,
response=ethylene.cover, data=exp2, ylab="Ethylene
Production/Percent Cover", xlab="Carbon (Ambient versus Elevated)",
err.width=0.05, las=1, frame.plot=TRUE) #main effect of carbon
lineplot.CI(x.factor=time, x.cont=TRUE, response=ethylene.cover,
group=carbon, data=exp2, ylab="Ethylene Production/Percent Cover",
xlab="Time (Day)", legend=TRUE, fixed=TRUE, err.width=0.05, las=1,
frame.plot=TRUE, x.legend=20) #carbon*timepoint interaction

#Scatterplot of ethylene production versus percent cover.
par(cex=1.5)
plot(ethylene~cover, data=ambient, col="peru")
points(ethylene~cover, data=elevated, col="lightskyblue", add=TRUE)

#Separate correlation for each CO2 treatment
fit <- lm(ethylene~cover, data=ambient)
anova(fit)
fit <- lm(ethylene~cover, data=elevated)
summary(fit)

#Full correlation for each CO2 treatment.
fit <- lm(ethylene~cover*carbon, data=exp2)
summary(fit)

#Full model for each CO2 treatment including time.
fit <- lm(ethylene~cover*carbon*time, data=exp2)
summary(fit)

# (4) Number of heterocysts.
quartz()
par(cex=1.5)
bargraph.CI(x.factor=time, x.cont=TRUE, group=carbon, response=het,
data=exp2, col=c("peru", "lightskyblue"), ylab="", xlab="Time (Day)",
legend=TRUE, leg.lab=c(expression(paste("Ambient
CO"[2])), expression(paste("Elevated CO"[2]))), err.width=0.05, las=1,
x.legend=0, ylim=c(0e0, 1.6e07))

# (4) Number of heterocysts in black and white.
quartz()
par(cex=1.5)
bargraph.CI(x.factor=time, x.cont=TRUE, group=carbon, response=het,
data=exp2, ylab="", xlab="Time (Day)", legend=TRUE,
leg.lab=c(expression(paste("Ambient
CO"[2])), expression(paste("Elevated CO"[2]))), err.width=0.05, las=1,
x.legend=0, ylim=c(0e0, 1.6e07))

```

```

# (4) ANOVA on number of heterocysts.
anova.het <- aov(het~factor(carbon)*factor(time)+Error(factor(vial)),
data=exp2)
summary(anova.het)
quartz()
par(mfrow=c(2,2))
plot(aov(het~factor(carbon)*factor(time), data=exp2))
TukeyHSD(aov(het~factor(carbon)*factor(time), data=exp2))

# (4) Interaction plots.
par(mfrow=c(1,3))
lineplot.CI(x.factor=time, x.cont=TRUE, response=het, data=exp2,
ylab="Growth (% Cover)", xlab="Time (Day)", xlim=c(0,30),
err.width=0.05, las=1, frame.plot=TRUE) #main effect of time
lineplot.CI(x.factor=as.numeric(carbon), x.cont=TRUE, response=het,
data=exp2, ylab="", xlab="Carbon", err.width=0.05, las=1,
frame.plot=TRUE, ylim=c(2e6,6e6)) #main effect of carbon
lineplot.CI(x.factor=time, x.cont=TRUE, response=het, group=carbon,
data=exp2, ylab="Growth (% Cover)", xlab="Carbon (Ambient versus
Elevated)", legend=TRUE, fixed=TRUE, err.width=0.05, las=1,
frame.plot=TRUE) #carbon*timepoint interaction

# (5) Number of vegetative cells.
par(cex=1.5)
bargraph.CI(x.factor=time, x.cont=TRUE, group=carbon, response=veg,
data=exp2, col=c("peru","lightskyblue"), ylab="", xlab="Time (Day)",
legend=TRUE, leg.lab=c(expression(paste("Ambient
CO"[2])),expression(paste("Elevated CO"[2]))), err.width=0.05, las=1,
x.leg=0, ylim=c(0e0,7e8), y.leg=7.1e8)

# (5) Number of vegetative cells in black and white.
par(cex=1.5)
bargraph.CI(x.factor=time, x.cont=TRUE, group=carbon, response=veg,
data=exp2, ylab="", xlab="Time (Day)", legend=TRUE,
leg.lab=c(expression(paste("Ambient
CO"[2])),expression(paste("Elevated CO"[2]))), err.width=0.05, las=1,
x.leg=0, ylim=c(0e0,7e8), y.leg=7.1e8)

# (5) ANOVA on vegetative cells.
anova.veg <- aov(veg~factor(carbon)*factor(time)+Error(factor(vial)),
data=exp2)
summary(anova.veg)
quartz()
par(mfrow=c(2,2))
plot(aov(veg~factor(carbon)*factor(time), data=exp2))
TukeyHSD(aov(veg~factor(carbon)*factor(time), data=exp2))

# (5) Interaction plots on vegetative cells.
par(mfrow=c(1,3))
lineplot.CI(x.factor=time, x.cont=TRUE, response=veg, data=exp2,
ylab="Growth (% Cover)", xlab="Time (Day)", xlim=c(0,30),
err.width=0.05, las=1, frame.plot=TRUE) #main effect of time

```

```

lineplot.CI(x.factor=as.numeric(carbon), x.cont=TRUE, response=veg,
data=exp2, ylab="", xlab="Carbon", err.width=0.05, las=1,
frame.plot=TRUE) #main effect of carbon
lineplot.CI(x.factor=time, x.cont=TRUE, response=veg, group=carbon,
data=exp2, ylab="", xlab="Carbon (Ambient versus Elevated)",
legend=TRUE, fixed=TRUE, err.width=0.05, las=1, frame.plot=TRUE)

# (6) Total cells.
quartz()
par(cex=1.5)
bargraph.CI(x.factor=time, x.cont=TRUE, group=carbon, response=total,
data=exp2, col=c("peru","lightskyblue"), ylab="", xlab="Time (Day)",
legend=TRUE, leg.lab=c(expression(paste("Ambient
CO"[2])),expression(paste("Elevated CO"[2]))), err.width=0.05, las=1,
x.leg=0, y.leg=6.1e8)

# (6) Total cells in black and white.
quartz()
par(cex=1.5)
bargraph.CI(x.factor=time, x.cont=TRUE, group=carbon, response=total,
data=exp2, ylab="", xlab="Time (Day)", legend=TRUE,
leg.lab=c(expression(paste("Ambient
CO"[2])),expression(paste("Elevated CO"[2]))), err.width=0.05, las=1,
x.leg=0, y.leg=6.1e8)

# (6) ANOVA on total cells.
anova.tot <-
aov(total~factor(carbon)*factor(time)+Error(factor(vial)),
data=exp2)
summary(anova.tot)
quartz()
par(mfrow=c(2,2))
plot(aov(total~factor(carbon)*factor(time), data=exp2))
TukeyHSD(aov(total~factor(carbon)*factor(time), data=exp2))

# (6) Interaction plot on total cells.
par(mfrow=c(1,3))
lineplot.CI(x.factor=time, x.cont=TRUE, response=total, data=exp2,
ylab="Growth (% Cover)", xlab="Time (Day)", xlim=c(0,30),
err.width=0.05, las=1, frame.plot=TRUE) #main effect of time
lineplot.CI(x.factor=as.numeric(carbon), x.cont=TRUE, response=total,
data=exp2, ylab="", xlab="Carbon", err.width=0.05, las=1,
frame.plot=TRUE) #main effect of carbon
lineplot.CI(x.factor=time, x.cont=TRUE, response=total, group=carbon,
data=exp2, ylab="", xlab="Carbon (Ambient versus Elevated)",
legend=TRUE, fixed=TRUE, err.width=0.05, las=1, frame.plot=TRUE)

# (7) Ethylene per heterocyst.
quartz()
par(cex=1.5)
bargraph.CI(x.factor=time, x.cont=TRUE, group=carbon,
response=(ethylene.het), data=exp2, ylab="", xlab="Time (Day)",
legend=TRUE, leg.lab=c(expression(paste("Ambient

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CO"[2])),expression(paste("Elevated CO"[2]))), err.width=0.05, las=1,
x.leg=0, col=c("peru","lightskyblue"), ylim=c(0e0,3e-06),
y.leg=3.1e-06)

# (7) Ethylene per heterocyst in black and white.
quartz()
par(cex=1.5)
bargraph.CI(x.factor=time, x.cont=TRUE, group=carbon,
response=ethylene.het), data=exp2, ylab="", xlab="Time (Day)",
legend=TRUE, leg.lab=c(expression(paste("Ambient
CO"[2])),expression(paste("Elevated CO"[2]))), err.width=0.05, las=1,
x.leg=0, ylim=c(0e0,3e-06), y.leg=3.1e-06)

# (7) ANOVA ethylene het.
anova.ethylene.het <-
aov(ethylene.het~factor(carbon)*factor(time)+Error(factor(vial)),
data=exp2)
summary(anova.ethylene.het)
quartz()
par(mfrow=c(2,2))
plot(aov(ethylene.het~factor(carbon)*factor(time), data=exp2))
TukeyHSD(aov(ethylene.het~factor(carbon)*factor(time), data=exp2))

# (7) Interaction plot on ethylene per heterocyst.
par(mfrow=c(1,3))
lineplot.CI(x.factor=time, x.cont=TRUE, response=ethylene.het,
data=exp2, ylab="Growth (% Cover)", xlab="Time (Day)", xlim=c(0,30),
err.width=0.05, las=1, frame.plot=TRUE) #main effect of time
lineplot.CI(x.factor=as.numeric(carbon), x.cont=TRUE,
response=ethylene.het, data=exp2, ylab="", xlab="Carbon",
err.width=0.05, las=1, frame.plot=TRUE) #main effect of carbon
lineplot.CI(x.factor=time, x.cont=TRUE, response=ethylene.het,
group=carbon, data=exp2, ylab="", xlab="Carbon (Ambient versus
Elevated)", legend=TRUE, fixed=TRUE, err.width=0.05, las=1,
frame.plot=TRUE)

# (8) Ethylene per total cell.
par(cex=1.5)
bargraph.CI(x.factor=time, x.cont=TRUE, group=carbon,
response=ethylene.total, data=exp2, col=c("peru","lightskyblue"),
ylab="", xlab="Time (Day)", legend=TRUE,
leg.lab=c(expression(paste("Ambient
CO"[2])),expression(paste("Elevated CO"[2]))), err.width=0.05, las=1,
x.leg=0, ylim=c(0e0,3.6e-07))

# (8) Ethylene per total cell in black and white.
par(cex=1.5)
bargraph.CI(x.factor=time, x.cont=TRUE, group=carbon,
response=ethylene.total, data=exp2, ylab="", xlab="Time (Day)",
legend=TRUE, leg.lab=c(expression(paste("Ambient
CO"[2])),expression(paste("Elevated CO"[2]))), err.width=0.05, las=1,
x.leg=0, ylim=c(0e0,3.6e-07))

```

```

# (8) ANOVA on ethylene per total.
anova.ethylene.total <-
aov(ethylene.total~factor(carbon)*factor(time)+Error(factor(vial)),
data=exp2)
summary(anova.ethylene.total)
quartz()
par(mfrow=c(2,2))
plot(aov(ethylene.total~factor(carbon)*factor(time), data=exp2))
TukeyHSD(aov(ethylene.total~factor(carbon)*factor(time), data=exp2))

# (8) Interaction plot on ethylene per total.
par(mfrow=c(1,3))
lineplot.CI(x.factor=time, x.cont=TRUE, response=ethylene.total,
data=exp2, ylab="Growth (% Cover)", xlab="Time (Day)", xlim=c(0,30),
err.width=0.05, las=1, frame.plot=TRUE) #main effect of time
lineplot.CI(x.factor=as.numeric(carbon), x.cont=TRUE,
response=ethylene.total, data=exp2, ylab="", xlab="Carbon",
err.width=0.05, las=1, frame.plot=TRUE) #main effect of carbon
lineplot.CI(x.factor=time, x.cont=TRUE, response=ethylene.total,
group=carbon, data=exp2, ylab="", xlab="Carbon (Ambient versus
Elevated)", legend=TRUE, fixed=TRUE, err.width=0.05, las=1,
frame.plot=TRUE)

# (9) percentage heterocysts.
quartz()
par(ps=16, cex=1.5)
bargraph.CI(x.factor=time, x.cont=TRUE, group=carbon,
response=percent.het*100, data=exp2, ylab="Percent Heterocysts",
xlab="Time (Day)", legend=TRUE, leg.lab=c(expression(paste("Ambient
CO"[2])),expression(paste("Elevated CO"[2]))), err.width=0.05, las=1,
x.leg=14, col=c("peru","lightskyblue"), y.leg=25.5)

# (9) percentage heterocysts in black and white.
quartz()
par(ps=16, cex=1.5)
bargraph.CI(x.factor=time, x.cont=TRUE, group=carbon,
response=percent.het*100, data=exp2, ylab="Percent Heterocysts",
xlab="Time (Day)", legend=TRUE, leg.lab=c(expression(paste("Ambient
CO"[2])),expression(paste("Elevated CO"[2]))), err.width=0.05, las=1,
x.leg=14, y.leg=25.5)

# (9) ANOVA on het percent.
anova.het.percent <-
aov(percent.het~factor(carbon)*factor(time)+Error(factor(vial)),
data=exp2)
summary(anova.het.percent)
quartz()
par(mfrow=c(2,2))
plot(aov(percent.het~factor(carbon)*factor(time), data=exp2))
TukeyHSD(aov(percent.het~factor(carbon)*factor(time), data=exp2))

# (9) Interaction plot on het percent.
par(mfrow=c(1,3))

```

```

lineplot.CI(x.factor=time, x.cont=TRUE, response=percent.het,
data=exp2, ylab="Growth (% Cover)", xlab="Time (Day)", xlim=c(0,30),
err.width=0.05, las=1, frame.plot=TRUE) #main effect of time
lineplot.CI(x.factor=as.numeric(carbon), x.cont=TRUE,
response=percent.het, data=exp2, ylab="", xlab="Carbon",
err.width=0.05, las=1, frame.plot=TRUE) #main effect of carbon
lineplot.CI(x.factor=time, x.cont=TRUE, response=percent.het,
group=carbon, data=exp2, ylab="", xlab="Carbon (Ambient versus
Elevated)", legend=TRUE, fixed=TRUE, err.width=0.05, las=1,
frame.plot=TRUE)

```

```
#####[30 DAY EXPERIMENT]#####
```

```
#####[90 DAY EXPERIMENT]#####
```

```

#Set working directory.
setwd("/Users/daniellegriffith/Google Drive/R Analysis")
getwd()

#Import data.
ara <- read.csv("ara.nov.25.csv")
head(ara)
str(ara)

#Split by nitrogen.
with.nitrogen <- subset(ara, ara$nitrogen==1)
no.nitrogen <- subset(ara, ara$nitrogen==0)

#Subset of temperature conditions for available N.
with.nitrogen.low <- subset(with.nitrogen, with.nitrogen$temperature
== 11.5)
with.nitrogen.medium <- subset(with.nitrogen,
with.nitrogen$temperature == 15.5)
with.nitrogen.high <- subset(with.nitrogen,
with.nitrogen$temperature == 19.5)

with.low.ambient <- subset(with.nitrogen.low,
with.nitrogen.low$carbon == 350)
with.medium.ambient <- subset(with.nitrogen.medium,
with.nitrogen.medium$carbon == 350)
with.high.ambient <- subset(with.nitrogen.high,
with.nitrogen.high$carbon == 350)

with.low.elevated <- subset(with.nitrogen.low,
with.nitrogen.low$carbon == 750)
with.medium.elevated <- subset(with.nitrogen.medium,
with.nitrogen.medium$carbon == 750)
with.high.elevated <- subset(with.nitrogen.high,
with.nitrogen.high$carbon == 750)

#Subset of temperature conditions for unavailable N.

```

```

no.nitrogen.low <- subset(no.nitrogen, no.nitrogen$temperature ==
11.5)
no.nitrogen.medium <- subset(no.nitrogen, no.nitrogen$temperature ==
15.5)
no.nitrogen.high <- subset(no.nitrogen, no.nitrogen$temperature ==
19.5)

no.low.ambient <- subset(no.nitrogen.low, no.nitrogen.low$carbon ==
350)
no.medium.ambient <- subset(no.nitrogen.medium,
no.nitrogen.medium$carbon == 350)
no.high.ambient <- subset(no.nitrogen.high, no.nitrogen.high$carbon
== 350)

no.low.elevated <- subset(no.nitrogen.low, no.nitrogen.low$carbon ==
750)
no.medium.elevated <- subset(no.nitrogen.medium,
no.nitrogen.medium$carbon == 750)
no.high.elevated <- subset(no.nitrogen.high, no.nitrogen.high$carbon
== 750)

#Factorial ANOVA for all combinations.
full.model <-
aov(per.vial~factor(nitrogen)*factor(carbon)*factor(temperature)*fac
tor(day)+Error(factor(vial)), data=ara)
summary(full.model)

#Subset for figures without nitrogen.
no.nitrogen.low <- subset(no.nitrogen, no.nitrogen$temperature ==
11.5)
no.nitrogen.medium <- subset(no.nitrogen, no.nitrogen$temperature ==
15.5)
no.nitrogen.high <- subset(no.nitrogen, no.nitrogen$temperature ==
19.5)

#Subsets for figures with nitrogen.
with.nitrogen.low <- subset(with.nitrogen, with.nitrogen$temperature
== 11.5)
with.nitrogen.medium <- subset(with.nitrogen,
with.nitrogen$temperature == 15.5)
with.nitrogen.high <- subset(with.nitrogen,
with.nitrogen$temperature == 19.5)

#Split by carbon.
ambient <- subset(ara, ara$carbon==350)
elevated <- subset(ara, ara$carbon==750)

#Subsets for CO2 at each temperature.
low.ambient <- subset(no.nitrogen.low, no.nitrogen.low$carbon ==350)
low.elevated <- subset(no.nitrogen.low, no.nitrogen.low$carbon
==750)

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```

medium.ambient <- subset(no.nitrogen.medium,
no.nitrogen.medium$carbon ==350)
medium.elevated <- subset(no.nitrogen.medium,
no.nitrogen.medium$carbon ==750)

high.ambient <- subset(no.nitrogen.high, no.nitrogen.high$carbon
==350)
high.elevated <- subset(no.nitrogen.high, no.nitrogen.high$carbon
==750)

#Subsets for day.
thirty <- subset(no.nitrogen, no.nitrogen$day ==30)
forty <- subset(no.nitrogen, no.nitrogen$day ==40)
fifty <- subset(no.nitrogen, no.nitrogen$day ==50)
sixty <- subset(no.nitrogen, no.nitrogen$day ==60)
seventy <- subset(no.nitrogen, no.nitrogen$day ==70)
eighty <- subset(no.nitrogen, no.nitrogen$day ==80)
ninety <- subset(no.nitrogen, no.nitrogen$day ==90)

# (10) Ethylene production for vials without nitrogen.
quartz(pointsize=14)
par(mfrow=c(1,3), las=1, cex=1.5)
bargraph.CI(x.factor=day, response=per.vial, group=carbon,
data=no.nitrogen.low, legend=TRUE, fixed=TRUE, x.leg=1, y.leg=8,
leg.lab=c(expression("Ambient CO"[2]),expression("Elevated CO"[2])),
ylim=c(0,8), tck=0.01, ylab="Ethylene production (µmol/vial)",
axes=FALSE, err.width=0.05, col=c("peru","lightskyblue"))
axis(2, at=c(0:8),
labels=c("0.0","1.0","2.0","3.0","4.0","5.0","6.0","7.0","8.0"))
bargraph.CI(x.factor=day, response=per.vial, group=carbon,
data=no.nitrogen.medium, tck=0.01, ylim=c(0,8), xlab="Time (Day)",
axes=FALSE, err.width=0.05, col=c("peru","lightskyblue"))
axis(2, at=c(0:8),
labels=c("0.0","1.0","2.0","3.0","4.0","5.0","6.0","7.0","8.0"))
bargraph.CI(x.factor=day, response=per.vial, group=carbon,
data=no.nitrogen.high,tck=0.01, ylim=c(0,8), axes=FALSE,
err.width=0.05, col=c("peru","lightskyblue"))
axis(2, at=c(0:8),
labels=c("0.0","1.0","2.0","3.0","4.0","5.0","6.0","7.0","8.0"))

# (10) Ethylene production for vials without nitrogen in black and
white.
quartz(pointsize=14)
par(mfrow=c(1,3), las=1, cex=1.5)
bargraph.CI(x.factor=day, response=per.vial, group=carbon,
data=no.nitrogen.low, legend=TRUE, fixed=TRUE, x.leg=1, y.leg=8,
leg.lab=c(expression("Ambient CO"[2]),expression("Elevated CO"[2])),
ylim=c(0,8), tck=0.01, ylab="Ethylene production (µmol/vial)",
axes=FALSE, err.width=0.05)
axis(2, at=c(0:8),
labels=c("0.0","1.0","2.0","3.0","4.0","5.0","6.0","7.0","8.0"))

```

```

bargraph.CI(x.factor=day, response=per.vial, group=carbon,
data=no.nitrogen.medium, tck=0.01, ylim=c(0,8), xlab="Time (Day)",
axes=FALSE, err.width=0.05)
axis(2, at=c(0:8),
labels=c("0.0", "1.0", "2.0", "3.0", "4.0", "5.0", "6.0", "7.0", "8.0"))
bargraph.CI(x.factor=day, response=per.vial, group=carbon,
data=no.nitrogen.high, tck=0.01, ylim=c(0,8), axes=FALSE,
err.width=0.05)
axis(2, at=c(0:8),
labels=c("0.0", "1.0", "2.0", "3.0", "4.0", "5.0", "6.0", "7.0", "8.0"))

# (10) ANOVA on Ethylene production for vials without nitrogen.
ethylene.per.vial <-
aov(per.vial~factor(carbon)*factor(temperature)*factor(day)+Error(fa
ctor(vial)), data=no.nitrogen)
summary(ethylene.per.vial)
par(mfrow=c(2,2))
plot(aov(per.vial~factor(carbon)*factor(temperature)*factor(day),
data=no.nitrogen))

#TukeyHSD on model, but removes vial as the random and considers
day as a fixed effect.
TukeyHSD(aov(no.nitrogen$per.vial~factor(no.nitrogen$carbon)*factor(
no.nitrogen$temperature)*factor(no.nitrogen$day)))

# (10) Interaction plots.
par(mfrow=c(1,3))
lineplot.CI(x.factor=carbon, response=per.vial, data=no.nitrogen,
xlab="Carbon", ylab="Ethylene per vial")
lineplot.CI(x.factor=day, group=temperature, response=per.vial,
data=no.nitrogen, xlab="Carbon", ylab="Ethylene per vial")
lineplot.CI(x.factor=day, group=carbon, response=per.vial,
data=no.nitrogen, xlab="Carbon", ylab="Ethylene per vial")

# (10) Interaction plots for each temperature treatment.
par(mfrow=c(1,3))
lineplot.CI(x.factor=day, group=carbon, response=per.vial,
data=no.nitrogen.low, xlab="Day", ylab="Ethylene per vial",
main="low")
lineplot.CI(x.factor=day, group=carbon, response=per.vial,
data=no.nitrogen.medium, xlab="Day", ylab="Ethylene per vial",
main="medium")
lineplot.CI(x.factor=day, group=carbon, response=per.vial,
data=no.nitrogen.high, xlab="Day", ylab="Ethylene per vial",
main="high")

# (11) Ethylene production in vials with nitrogen.
quartz(pointsize=14)
par(mfrow=c(1,3), las=1, cex=1.5)
bargraph.CI(x.factor=day, response=per.vial, group=carbon,
data=with.nitrogen.low, ylab="Ethylene Production (µmol/vial)",
legend=TRUE, fixed=TRUE, x.legend=1, legend.lab=c(expression("Ambient

```

```

CO"[2]),expression("Elevated CO"[2])), ylim=c(0,0.05),
col=c("peru","lightskyblue"))
bargraph.CI(x.factor=day, response=per.vial, group=carbon,
data=with.nitrogen.medium, ylim=c(0,0.05), xlab="Time (Day)",
col=c("peru","lightskyblue"))
bargraph.CI(x.factor=day, response=per.vial, group=carbon,
data=with.nitrogen.high,
ylim=c(0,0.05),col=c("peru","lightskyblue"))

# (11) Ethylene production in vials with nitrogen in black and white.
quartz(pointsize=14)
par(mfrow=c(1,3), las=1, cex=1.5)
bargraph.CI(x.factor=day, response=per.vial, group=carbon,
data=with.nitrogen.low, ylab="Ethylene Production (µmol/vial)",
legend=TRUE, fixed=TRUE, x.legend=1, legend.lab=c(expression("Ambient
CO"[2]),expression("Elevated CO"[2])), ylim=c(0,0.05))
bargraph.CI(x.factor=day, response=per.vial, group=carbon,
data=with.nitrogen.medium, ylim=c(0,0.05), xlab="Time (Day)")
bargraph.CI(x.factor=day, response=per.vial, group=carbon,
data=with.nitrogen.high, ylim=c(0,0.05))

# (11) Ethylene production in vials with nitrogen.
ethylene.per.vial <-
aov(per.vial~factor(carbon)*factor(temperature)*factor(day)+Error(fa
ctor(vial)), data=with.nitrogen)
summary(ethylene.per.vial)
TukeyHSD(aov(with.nitrogen$per.vial~factor(with.nitrogen$carbon)*fac
tor(with.nitrogen$temperature)*factor(with.nitrogen$day)))

# (11) Checking assumptions.
par(mfrow=c(2,2))
plot(aov(with.nitrogen$per.vial~factor(with.nitrogen$carbon)*factor(
with.nitrogen$temperature)*factor(with.nitrogen$day)))

#Interaction plot
par(mfrow=c(1,3))
lineplot.CI(x.factor=carbon, response=per.vial, data=with.nitrogen,
xlab="Carbon", ylab="Ethylene per vial")
lineplot.CI(x.factor=day, group=temperature, response=per.vial,
data=with.nitrogen, xlab="Carbon", ylab="Ethylene per vial")
lineplot.CI(x.factor=day, group=carbon, response=per.vial,
data=with.nitrogen, xlab="Carbon", ylab="Ethylene per vial")

# (10) Interaction plots for each temperature treatment.
par(mfrow=c(1,3))
lineplot.CI(x.factor=day, group=carbon, response=per.vial,
data=with.nitrogen.low, xlab="Day", ylab="Ethylene per vial",
main="low")
lineplot.CI(x.factor=day, group=carbon, response=per.vial,
data=with.nitrogen.medium, xlab="Day", ylab="Ethylene per vial",
main="medium")

```

```

lineplot.CI(x.factor=day, group=carbon, response=per.vial,
data=with.nitrogen.high, xlab="Day", ylab="Ethylene per vial",
main="high")

#ANALYSIS FOR DATA COLLECTED AT DAY 90--includes cell densities, etc.
#Import data.
endpoint <- read.csv("endpoint.nov.25.csv")
with.nitrogen <- subset(endpoint, endpoint$nitrogen==1)
no.nitrogen <- subset(endpoint, endpoint$nitrogen==0)

ambient <- subset(no.nitrogen, no.nitrogen$carbon == 350)
elevated <- subset(no.nitrogen, no.nitrogen$carbon == 750)

# (12) Densities of vegetative and heterocyst cells in vials without
nitrogen.
quartz(pointsize=16)
par(mfrow=c(1,2), las=1, xpd=TRUE)
bargraph.CI(x.factor=carbon, response=veg.per.vial,
group=temperature, data=no.nitrogen, ylab="", ylim=c(0,6e8))
bargraph.CI(x.factor=carbon, response=het.per.vial,
group=temperature, data=no.nitrogen, ylab="",
xlab=expression("CO"[2]~"concentration (ppm)"), legend=TRUE,
fixed=TRUE, x.legend=4.3, legend.lab=c("11.5°C", "15.5°C", "19.5°C"),
ylim=c(0,6e8))

# (12) Densities of vegetative and heterocyst cells in vials without
nitrogen in black and white.
quartz(pointsize=16)
par(mfrow=c(1,2), las=1, xpd=TRUE)
bargraph.CI(x.factor=carbon, response=veg.per.vial,
group=temperature, data=no.nitrogen, ylab="", ylim=c(0,6e8),
col=c("peru", "lightskyblue", "olivedrab4"))
bargraph.CI(x.factor=carbon, response=het.per.vial,
group=temperature, data=no.nitrogen, ylab="",
xlab=expression("CO"[2]~"concentration (ppm)"), legend=TRUE,
fixed=TRUE, x.legend=4.3, legend.lab=c("11.5°C", "15.5°C", "19.5°C"),
ylim=c(0,6e8), col=c("peru", "lightskyblue", "olivedrab4"))

# (12) Percent heterocysts in vials without nitrogen.
par(ps=16, cex=1.5, las=1)
bargraph.CI(x.factor=carbon,
response=het.per.vial/total.per.vial*100, group=temperature,
data=no.nitrogen, ylab="", tck=0.01, ylim=c(0,8), axes=FALSE,
col=c("peru", "lightskyblue", "olivedrab4"), legend=TRUE, x.legend=6,
legend.lab=c("11.5°C", "15.5°C", "19.5°C"), y.legend=8)
axis(2, at=c(0:8),
labels=c("0.0", "1.0", "2.0", "3.0", "4.0", "5.0", "6.0", "7.0", "8.0"))

# (12) Percent heterocysts in vials without nitrogen in black and
white.
par(ps=16, cex=1.5, las=1)

```

```

bargraph.CI(x.factor=carbon,
response=het.per.vial/total.per.vial*100, group=temperature,
data=no.nitrogen, ylab="", tck=0.01, ylim=c(0,8), axes=FALSE,
legend=TRUE, x.leg=6, leg.lab=c("11.5°C", "15.5°C", "19.5°C"),
y.leg=8)
axis(2, at=c(0:8),
labels=c("0.0", "1.0", "2.0", "3.0", "4.0", "5.0", "6.0", "7.0", "8.0"))

#MANOVA on het and veg.
manova <-
manova(cbind(veg.per.vial,het.per.vial)~factor(carbon)*factor(temper
ature),data=no.nitrogen)
summary(manova, test="Wilks")

#ANOVA on heterocyst percent.
percent.anova <-
aov((het.per.vial/total.per.vial*100)~factor(carbon)*factor(temperat
ure), data=no.nitrogen)
summary(percent.anova)
TukeyHSD(percent.anova)
par(mfrow=c(2,2))
plot(aov((het.per.vial/total.per.vial*100)~factor(carbon)*factor(tem
perature), data=no.nitrogen))

#Interaction plot.
par(mfrow=c(1,3))
lineplot.CI(x.factor=temperature, group=carbon,
response=veg.per.vial, data=no.nitrogen, xlab="Day", ylab="Ethylene
per vial")
lineplot.CI(x.factor=temperature, group=carbon,
response=het.per.vial, data=no.nitrogen, xlab="Day", ylab="Ethylene
per vial")
lineplot.CI(x.factor=temperature, group=carbon,
response=het.per.vial/total.per.vial*100, data=no.nitrogen,
xlab="Day",)

# (13) Cell densities in vials with nitrogen.
quartz(pointsize=16)
par(mfrow=c(1,2), las=1)
bargraph.CI(x.factor=carbon, response=veg.per.vial,
group=temperature, data=with.nitrogen, ylab="",
col=c("peru", "lightskyblue", "olivedrab4"))
bargraph.CI(x.factor=carbon, response=het.per.vial,
group=temperature, data=with.nitrogen, xlab="Time (Day)",
col=c("peru", "lightskyblue", "olivedrab4"), legend=TRUE, fixed=TRUE,
x.leg=1, leg.lab=c(expression("Ambient CO"[2]),expression("Elevated
CO"[2])), ylim=c(0, 7e7))

# (13) Cell densities in vials with nitrogen in black and white.
quartz(pointsize=16)
par(mfrow=c(1,2), las=1)
bargraph.CI(x.factor=carbon, response=veg.per.vial,
group=temperature, data=with.nitrogen, ylab="")

```

```

bargraph.CI(x.factor=carbon, response=het.per.vial,
group=temperature, data=with.nitrogen, xlab="Time (Day)",
legend=TRUE, fixed=TRUE, x.leg=1, leg.lab=c(expression("Ambient
CO"[2]),expression("Elevated CO"[2])), ylim=c(0, 7e7))

# (13) Heterocyst percentage in vials with nitrogen.
quartz(pointsize=16)
bargraph.CI(x.factor=carbon,
response=het.per.vial/total.per.vial*100, group=temperature,
data=with.nitrogen, tck=0.01,
col=c("peru", "lightskyblue", "olivedrab4"), legend=TRUE)

# (13) Heterocyst percentage in black and white.
quartz(pointsize=16)
bargraph.CI(x.factor=carbon,
response=het.per.vial/total.per.vial*100, group=temperature,
data=with.nitrogen, tck=0.01, legend=TRUE)

#ANOVA on heterocyst percent in vials with nitrogen.
percent.anova <-
aov((het.per.vial/total.per.vial*100)~factor(carbon)*factor(temperat
ure), data=with.nitrogen)
summary(percent.anova)
par(mfrow=c(2,2))
plot(aov((het.per.vial/total.per.vial*100)~factor(carbon)*factor(tem
perature), data=with.nitrogen))

#Interaction plot.
par(mfrow=c(1,3))
lineplot.CI(x.factor=temperature, group=carbon,
response=veg.per.vial, data=with.nitrogen, xlab="Day",
ylab="Ethylene per vial", main="low")
lineplot.CI(x.factor=temperature, group=carbon,
response=het.per.vial, data=with.nitrogen, xlab="Day",
ylab="Ethylene per vial", main="medium")
lineplot.CI(x.factor=temperature, group=carbon,
response=het.per.vial/total.per.vial*100, data=with.nitrogen,
xlab="Day", ylab="Ethylene per vial", main="high")

#MANOVA on het and veg.
manova <-
manova(cbind(veg.per.vial,het.per.vial)~factor(carbon)*factor(temper
ature),data=with.nitrogen)
summary(manova, test="Wilks")

# (14) Ethylene production per heterocyst.
par(ps=16,cex=1.5, las=1)
bargraph.CI(x.factor=carbon, response=ara.per.het, group=temperature,
data=no.nitrogen, col=c("peru", "lightskyblue", "olivedrab4"),
legend=TRUE, x.leg=0.7, ylim=c(0e0,7e-07),
leg.lab=c("11.5°C", "15.5°C", "19.5°C"), y.leg=7e-7)

# (14) Ethylene production per heterocyst in black and white.

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```

par(ps=16,cex=1.5, las=1)
bargraph.CI(x.factor=carbon, response=ara.per.het, group=temperature,
data=no.nitrogen, legend=TRUE, x.leg=0.7, ylim=c(0e0,7e-07),
leg.lab=c("11.5°C","15.5°C","19.5°C"), y.leg=7e-7)

#interaction plots.
par(mfrow=c(1,3))
lineplot.CI(x.factor=temperature, response=ara.per.veg,
data=no.nitrogen, xlab="Temperature", ylab="Het Percent")
lineplot.CI(x.factor=temperature, group=carbon, response=ara.per.het,
data=no.nitrogen, xlab="Temperature", ylab="Het Percent")
lineplot.CI(x.factor=temperature, group=carbon,
response=ara.per.total, data=no.nitrogen, xlab="Temperature",
ylab="Het Percent")

# (15) Ethylene production per total in vials without nitrogen.
quartz(pointsize=16)
par(las=1)
bargraph.CI(x.factor=carbon, response=ara.per.total,
group=temperature, data=no.nitrogen,
col=c("peru","lightskyblue","olivedrab4"), ylim=c(0,6e-8))

# (15) GRAYSCALE Ethylene production per total in vials without
nitrogen.
quartz(pointsize=16)
par(las=1)
bargraph.CI(x.factor=carbon, response=ara.per.total,
group=temperature, data=no.nitrogen, ylim=c(0,6e-8))

#ANOVA on ARA/heterocyst in vials without nitrogen.
ara.het.anova <-aov((ara.per.het)~factor(carbon)*factor(temperature),
data=no.nitrogen)
summary(ara.het.anova)
par(mfrow=c(2,2))
plot(aov((ara.per.het)~factor(carbon)*factor(temperature),
data=no.nitrogen))

#ANOVA on ARA/veg in vials without nitrogen.
ara.veg.anova <-aov((ara.per.veg)~factor(carbon)*factor(temperature),
data=no.nitrogen)
summary(ara.veg.anova)
par(mfrow=c(2,2))
plot(aov((ara.per.veg)~factor(carbon)*factor(temperature),
data=no.nitrogen))

#ANOVA on ARA/tot in vials without nitrogen.
ara.tot.anova <-
aov((ara.per.total)~factor(carbon)*factor(temperature),
data=no.nitrogen)
summary(ara.tot.anova)
par(mfrow=c(2,2))
plot(aov((ara.per.total)~factor(carbon)*factor(temperature),
data=no.nitrogen))

```

```
#ANOVA on total cells per vial in vials with nitrogen.
tot.anova <-aov((total.per.vial)~factor(carbon)*factor(temperature),
data=with.nitrogen)
summary(tot.anova)
par(mfrow=c(2,2))
plot(aov((total.per.vial)~factor(carbon)*factor(temperature),
data=with.nitrogen))
```

```
#ANOVA on total cells per vial in vials without nitrogen.
tot.anova <-aov((total.per.vial)~factor(carbon)*factor(temperature),
data=no.nitrogen)
summary(tot.anova)
par(mfrow=c(2,2))
plot(aov((total.per.vial)~factor(carbon)*factor(temperature),
data=no.nitrogen))
```

```
#####[90 DAY EXPERIMENT]#####
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Curriculum Vitae

Name: Danielle Alyce Griffith

Post-secondary Education and Degrees: University of Western Ontario
London, Ontario, Canada
2008-2012 H.B.Sc. Biology

The University of Western Ontario
London, Ontario, Canada
2012-2014 M.Sc. Biology

Research Experience:

- 2012 – 2014 Graduate Research
Dr. Zoë Lindo
The University of Western Ontario
Thesis Title: Nitrogen fixation by the cyanobacterium *Nostoc punctiforme* in response to variation in nitrogen availability, temperature, and atmospheric CO₂ concentrations
- 2012 Summer Research Assistant
Dr. Yolanda Morbey
The University of Western Ontario
- 2011 – 2012 Undergraduate Research
Dr. Yolanda Morbey
The University of Western Ontario
Thesis Title: Phenotypic plasticity of body size in Chinook salmon (*Oncorhynchus tshawytscha*) in response to different pre-hatch rearing temperatures

Teaching Experience:

- 2012 – present Biology/Statistics 2244A/B
The University of Western Ontario

Presentations:

Thorn MW, Griffith DA, Morbey YE. A comparison of Chinook salmon (*Oncorhynchus tshawytscha*) developmental traits between the Credit and Sydenham Rivers. First Joint Congress on Evolutionary Biology. Ottawa, ON. June 6-10, 2012.

Griffith DA, Lindo Z. Combined effects of altered nitrogen availability, temperature, and CO₂ on nitrogen fixation by the cyanobacterium *Nostoc punctiforme*. BGRF. London, ON. October 19, 2013.

Anticipated Publications:

Griffith DA, Lindo Z. Growth demands under elevated CO₂ stimulate N fixation activity in the terrestrial cyanobacterium *Nostoc punctiforme*. *In Submission*.