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# Nitrogen fixation by the cyanobacterium *Nostoc punctiforme* in response to variation in nitrogen availability, temperature, and atmospheric CO<sub>2</sub> concentrations

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

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NITROGEN FIXATION BY THE CYANOBACTERIUM *NOSTOC PUNCTIFORME* IN  
RESPONSE TO VARIATION IN NITROGEN AVAILABILITY, TEMPERATURE, AND  
ATMOSPHERIC CO<sub>2</sub> 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  
Master of Science

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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 N<sub>2</sub>, contribute to the soil N pool, and thereby support forest productivity. Although increases in N availability, temperature, and atmospheric CO<sub>2</sub> concentrations are expected in Canada's boreal region over the next century, little is known about the combined effects of these factors on N<sub>2</sub> fixation by axenic cyanobacteria or the associated mechanisms. I assessed changes in N<sub>2</sub> 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 N<sub>2</sub> fixation or form heterocysts. Elevated CO<sub>2</sub> stimulated growth and N<sub>2</sub> fixation, but this result was influenced by a temperature-mediated growth cycle. Overall, my findings suggest a decrease in boreal N<sub>2</sub> fixation rates in response to global environmental change.

## Keywords

atmospheric CO<sub>2</sub>, boreal forest, cyanobacteria, global environmental change, N<sub>2</sub> 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 CO<sub>2</sub> 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 (NH<sub>4</sub><sup>+</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>), 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 CO<sub>2</sub> 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 CO<sub>2</sub> concentrations.

The largest pool of unavailable N sits in Earth's atmosphere, which is composed of 78% of atmospheric N<sub>2</sub>, 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  $\text{NO}_x$  (and minimal inputs of  $\text{NH}_y$ ), 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  $41 \text{ Tg C yr}^{-1}$  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

$\text{N}_2$  fixation is the only process by which unavailable atmospheric  $\text{N}_2$  can enter the soil N pool. In terrestrial ecosystems, there are three main processes by which  $\text{N}_2$  fixation can occur: the Haber–Bosch process (anthropogenic), lightning strikes (abiotic), and biological  $\text{N}_2$  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 N<sub>2</sub> 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 N<sub>2</sub> fixation rates by bacteria symbiotically associated with tree root systems are much higher, ranging between 20 and 60 kg N ha<sup>-1</sup> yr<sup>-1</sup> (Houlton et al., 2008). N<sub>2</sub> fixation by below-ground systems such as these appears to be minimal in Northern ecosystems such as boreal forests (Vitousek et al., 2002), where N<sub>2</sub> 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<sup>-1</sup> yr<sup>-1</sup> for boreal forest ecosystems (DeLuca et al., 2002), although lower values of 0.4 to 1.6 kg N h<sup>-1</sup> yr<sup>-1</sup> have been reported (Zackrisson et al., 2004; Zackrisson et al., 2009). Much of the research to date has focused extensively on N<sub>2</sub> 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 N<sub>2</sub> fixation process is exclusively carried out by the



cyanobacterium, and many terrestrial studies fail to observe N<sub>2</sub> 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 N<sub>2</sub>. In feather mosses such as *Hylocomium splendens*, the majority of N<sub>2</sub> 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 N<sub>2</sub> in the form of NH<sub>4</sub><sup>+</sup> (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 N<sub>2</sub>. 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 N<sub>2</sub> 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 N<sub>2</sub>. 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 N<sub>2</sub> and to NH<sub>3</sub> (Dixon & Kahn, 2004), but cyanobacteria do not produce active heterocysts in conditions where available N, such as ammonium (NH<sub>4</sub><sup>+</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>), is a non-limiting resource (Meeks et al., 2002; Campbell et al., 2007); rather, N<sub>2</sub> 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 N<sub>2</sub> fixation.

Most of the known information about the collection of signals that facilitate N<sub>2</sub> fixation comes from studies on multicellular cyanobacteria such as *Nostoc punctiforme* (Meeks et al., 2001). Under N limitation, cyanobacteria receive a signal to fix N<sub>2</sub>, 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 NH<sub>4</sub><sup>+</sup> inhibits N<sub>2</sub> fixation in *Nodularia spumigena* due to decreased expression of N<sub>2</sub> 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 N<sub>2</sub> 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 N<sub>2</sub> 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 N<sub>2</sub> fixation (Campbell et al., 2007). However, little is known about how changes in global environmental change factors such as N availability, temperature, and CO<sub>2</sub> will influence the timing and amount of N<sub>2</sub> fixation in these conditions.

There are three key mechanisms by which cyanobacteria can alter N<sub>2</sub> 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 N<sub>2</sub>. Although cyanobacteria can use a variety of N sources, it is shown that cyanobacteria preferentially assimilate ammonium (NH<sub>4</sub><sup>+</sup>) (Meeks et al., 2001), suggesting that they may grow more in cultures where NH<sub>4</sub><sup>+</sup> is not the nitrogen source. Increased cyanobacterial growth and biomass can result in a net increase in N<sub>2</sub> fixation as shown during the exponential growth stage under elevated CO<sub>2</sub> conditions in *Trichodesmium* (Levitan et al., 2007). Studies on the unicellular cyanobacterium *Synechococcus* also report increased growth rates in response to elevated CO<sub>2</sub> and increased temperature (Fu et al., 2007). Secondly, the percentage of cells that are N<sub>2</sub> fixing heterocysts may increase (without an overall increase in vegetative cells which do not fix N<sub>2</sub>), although previous studies have failed to demonstrate any effect of varying CO<sub>2</sub> 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 N<sub>2</sub> fixed on a per heterocyst cell basis. However, many studies do not quantify heterocyst cell counts or standardize N<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub> (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 CO<sub>2</sub> in a factorial setting.

## 1.4 Response of N<sub>2</sub> 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 CO<sub>2</sub> concentrations, are predicted to influence N<sub>2</sub> fixation by the cyanobacteria-moss association (Lindo et al., 2013). In the case of N availability, it is known that N<sub>2</sub> 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 N<sub>2</sub> 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 N<sub>2</sub> fixation under elevated CO<sub>2</sub> (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 CO<sub>2</sub> on N<sub>2</sub> 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<sub>x</sub>, with NH<sub>3</sub> inputs to the soil N pool predominantly coming from N<sub>2</sub> fixation. With more available N present, cyanobacteria stop fixing N<sub>2</sub> in response to the nitro-stat signal, as previously described. Many N addition and natural N gradient studies corroborate a reduction or elimination of N<sub>2</sub> 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 N<sub>2</sub> when N is readily available, and this is the most likely the mechanism for reduced N<sub>2</sub> fixation under increased N availability. It is established that reductions in N<sub>2</sub> fixation are due to reduced cyanobacterial abundance, and the absence of N<sub>2</sub> fixing heterocysts. However, the effects of increases in temperature and CO<sub>2</sub> 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 N<sub>2</sub> 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). N<sub>2</sub> 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 N<sub>2</sub> fixation in boreal ecosystems. One study on cyanobacteria isolated from the moss *P. schreberi* found that N<sub>2</sub> fixation was greatest at 30 °C for *Calothrix*, with increasing temperature having a positive effect on N<sub>2</sub> fixation, whereas N<sub>2</sub> fixation was greatest at 13 °C for *Nostoc*, with an overall unimodal relationship on N<sub>2</sub> fixation (Gentili et al., 2005). Although the effects of temperature on overall N<sub>2</sub> 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 CO<sub>2</sub> concentrations in the boreal forest are expected to double by the end of the next century (IPCC, 2007). Nevertheless, the effects of CO<sub>2</sub> on terrestrial N<sub>2</sub> fixation by moss-associated cyanobacteria remain enigmatic. At present, there is only one study on the effects of CO<sub>2</sub> on N<sub>2</sub> fixation by moss-associated cyanobacteria, which found a decrease in N<sub>2</sub> fixation in response to increased CO<sub>2</sub> (Smith, 1984); however, cyanobacteria isolates were not microscopically examined, and the mechanism for decreased N<sub>2</sub> fixation in response to elevated CO<sub>2</sub> remains unknown. In marine ecosystems, the effects of CO<sub>2</sub> on N<sub>2</sub> 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 CO<sub>2</sub> was observed to increase N<sub>2</sub> 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 CO<sub>2</sub> decreased N<sub>2</sub> 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 CO<sub>2</sub> concentrations increase N<sub>2</sub> 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 CO<sub>2</sub> concentrations (Czerny et al., 2009) and overall, the mechanisms for CO<sub>2</sub> effects on N<sub>2</sub> fixation remain elusive. In general, studies on CO<sub>2</sub> effects often observe net N<sub>2</sub> 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 CO<sub>2</sub> 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 N<sub>2</sub> 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 N<sub>2</sub> fixation in cyanobacteria moss-associations, where light tends to have a stimulatory effect on N<sub>2</sub> fixation rates at low temperatures, and an inhibitory effect on N<sub>2</sub> fixation rates at high temperatures (Gundale et al., 2012). Interactive effects of temperature and CO<sub>2</sub> have also been observed in *A. fertilissima*, where elevated CO<sub>2</sub> tends to mitigate the effect of high temperatures on growth and N<sub>2</sub> fixation (Chinnasamy et al., 2009). *Synechococcus* responds positively to both temperature and CO<sub>2</sub>, 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 N<sub>2</sub> fixation and, moreover, to elucidate the mechanism(s) responsible for the observed response of axenic cyanobacteria from the moss association.



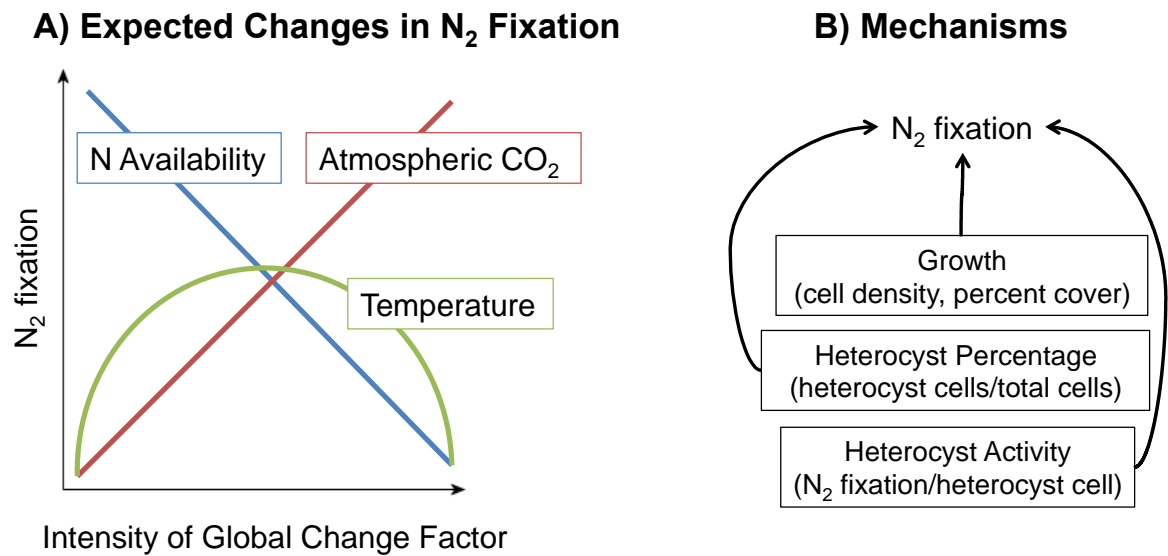
## 1.5 Objectives

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 CO<sub>2</sub> concentrations on N<sub>2</sub> 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 N<sub>2</sub> fixation activity of *N. punctiforme*, with a second objective of comparing how these patterns differ under ambient and elevated CO<sub>2</sub>. In satisfying both of these objectives, a timeline of growth and N<sub>2</sub> 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 CO<sub>2</sub> on N<sub>2</sub> 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 N<sub>2</sub> fixation rates.

## 1.6 Hypothesis & Predictions

Changes in N<sub>2</sub> 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 CO<sub>2</sub>, when increased in isolation, are known to influence the N<sub>2</sub> 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 N<sub>2</sub> fixation is an energetically costly process, I hypothesized that *N. punctiforme* would not fix N<sub>2</sub> 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 N<sub>2</sub> in conditions where N is unavailable. This would correspond with heterocyst production, and demonstrated activity of these heterocysts. Under N limitation, I predicted that N<sub>2</sub> fixation rates would increase primarily due to the stimulatory effect of CO<sub>2</sub> on vegetative cells and the greater demand for N under growth conditions. Since N<sub>2</sub> 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 N<sub>2</sub> 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 CO<sub>2</sub> were more challenging to predict, but I predicted that these two environmental change factors should have a synergistic effect on growth, with CO<sub>2</sub> and temperature being stimulatory to cyanobacterial N<sub>2</sub> fixation.



**Figure 1.1.** A schematic representation of A) expected changes in N<sub>2</sub> fixation under different global environmental change factors and B) the proposed mechanisms for the observed N<sub>2</sub> fixation response. It was expected that N availability will reduce N<sub>2</sub> fixation, whereas atmospheric CO<sub>2</sub> would stimulate N<sub>2</sub> fixation. The relationship between N<sub>2</sub> fixation and temperature was expected to have a unimodal relationship, where N<sub>2</sub> 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 N<sub>2</sub> 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-11<sub>0</sub>) 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-11<sub>0</sub> 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  $4.92 \times 10^5$  cells mL<sup>-1</sup> 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 mm<sup>2</sup>. At the beginning of the experiment, the initial surface area of the vial was 572.56 mm<sup>2</sup>; 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  $\mu$ 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  $572.56 \text{ mm}^2$  agar surface, and the corresponding 3 mm depth of cyanobacteria growth.

## 2.3 Determination of $\text{N}_2$ Fixation by the Acetylene Reduction

### Assay

The acetylene reduction assay (ARA) is a well-established technique for the quantification of  $\text{N}_2$  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 ( $\text{C}_2\text{H}_2$ ) to ethylene gas ( $\text{C}_2\text{H}_4$ ), as a proxy for the conversion of  $\text{N}_2$  to  $\text{NH}_3$  at a rate of three moles  $\text{C}_2\text{H}_4$  produced per mole of  $\text{N}_2$  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  $\text{N}_2$ . After comparing these processes stoichiometrically, the moles of  $\text{N}_2$  fixed are converted to moles of ammonia produced using a 1:2 conversion ratio, and the moles of  $\text{NH}_3$  produced are converted to the mass of ammonia produced by multiplying by the molar mass of  $\text{NH}_3$  ( $17.031 \text{ g mol}^{-1}$ ). 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  $\text{N}_2$  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 (C<sub>2</sub>H<sub>4</sub>). 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 (C<sub>2</sub>H<sub>4</sub>), which allowed me to directly obtain the output of C<sub>2</sub>H<sub>4</sub> production in μmol. I presented N<sub>2</sub> fixation measurements as C<sub>2</sub>H<sub>4</sub> produced per vial (μmol C<sub>2</sub>H<sub>4</sub> vial<sup>-1</sup>). I standardized N<sub>2</sub> fixation on a per heterocyst basis as heterocyst activity (μmol C<sub>2</sub>H<sub>4</sub> heterocyst<sup>-1</sup>), and on a surface area basis as cyanobacterial activity (μmol C<sub>2</sub>H<sub>4</sub> percent cover<sup>-1</sup>). In each case, I divided the amount of C<sub>2</sub>H<sub>4</sub> 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 CO<sub>2</sub>. 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 CO<sub>2</sub> and the



other Biome containing elevated (750 ppm) atmospheric CO<sub>2</sub> 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 CO<sub>2</sub> on growth and N<sub>2</sub> fixation by *N. punctiforme*. I inoculated six vials containing BG-11<sub>0</sub> media with 30 μL of *N. punctiforme*, and placed these vials in each of two experimental Biomes (one at ambient and one at elevated CO<sub>2</sub>). 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 CO<sub>2</sub> 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 N<sub>2</sub> 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-11<sub>0</sub> agar without acetylene, and two control vials containing both BG-11<sub>0</sub> agar and acetylene (but no cyanobacteria) to correct N<sub>2</sub> fixation measurements obtained in the ARA-GC protocol. I measured five variables during this experiment: cyanobacterial percent cover, cyanobacterial abundance, N<sub>2</sub> fixation, heterocyst activity, and cyanobacterial activity. I observed the effects of CO<sub>2</sub> on these variables, and constructed a timeline of growth and fixation events for *N. punctiforme*. I related N<sub>2</sub> 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 CO<sub>2</sub> on N<sub>2</sub> fixation by *N. punctiforme*. I filled vials with BG-

11 ( $N = 36$ ) and BG-11<sub>0</sub> ( $N = 36$ ) media and equally divided them among the six Biomes, prior to inoculating them with cyanobacteria as described above. Two Biomes (ambient and elevated CO<sub>2</sub>) 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 CO<sub>2</sub> and temperature manipulated at the Biome-level, and N availability manipulated using the media ( $2 \text{ CO}_2 \times 3 \text{ temperature} \times 2 \text{ N availability} \times 6 \text{ replicates} = 72 \text{ experimental units}$ ). Because the temperature set-points were much lower than in the growth experiment, I chose not to obtain measurements of N<sub>2</sub> 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 N<sub>2</sub> 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, CO<sub>2</sub> 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).

## 3 Results

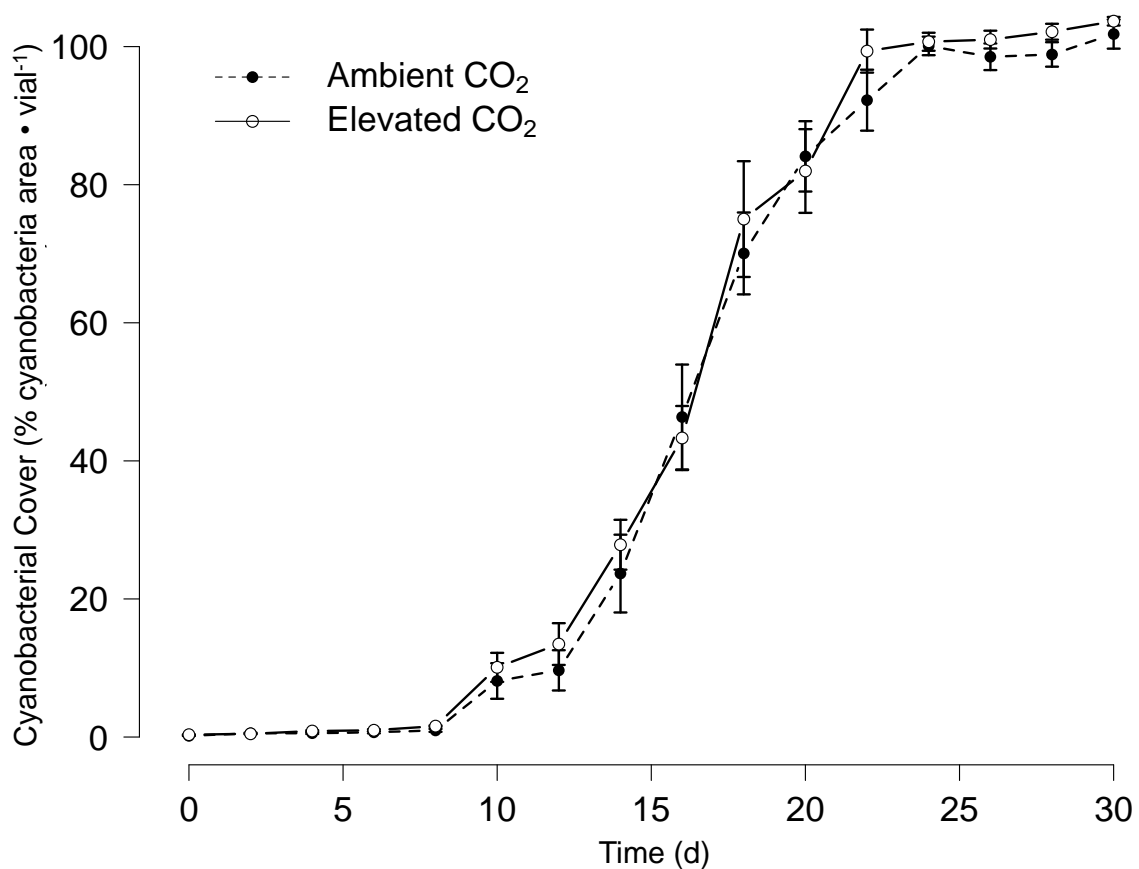
### 3.1 Growth Experiment

#### 3.1.1 Cyanobacterial Cover

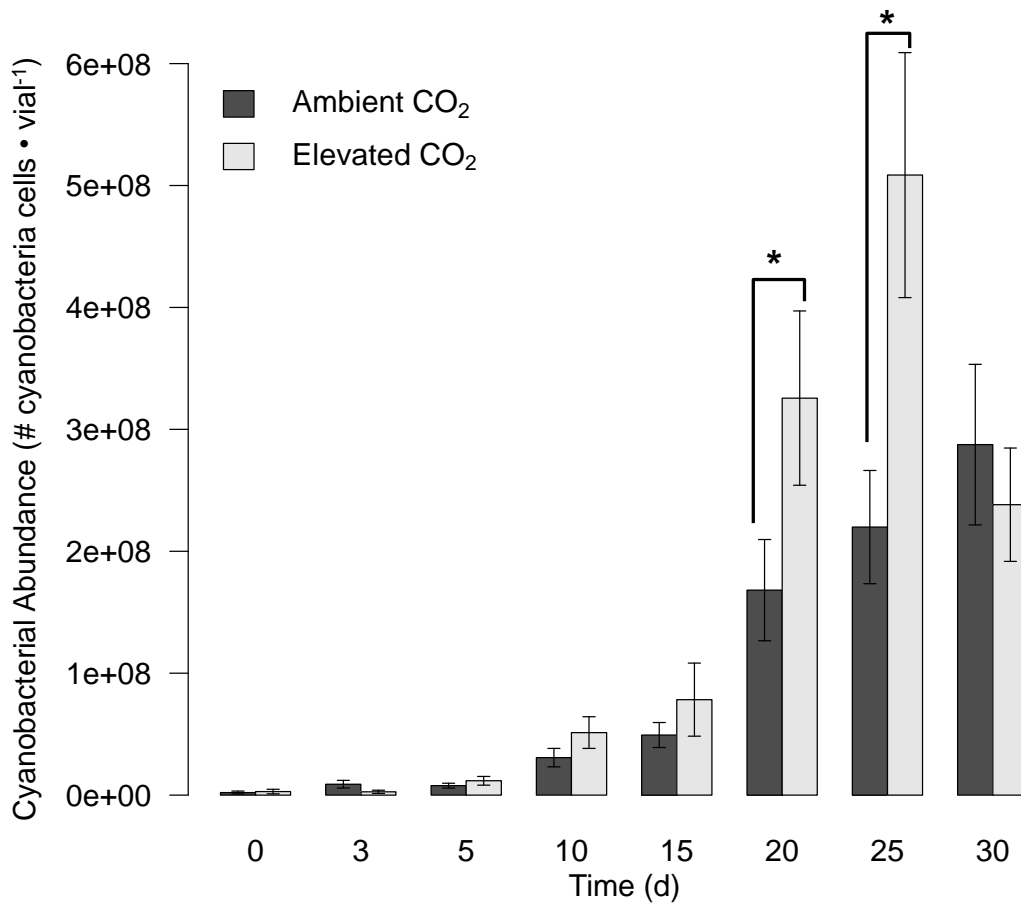
*Nostoc punctiforme* covered the entire agar surface under both ambient CO<sub>2</sub> and elevated CO<sub>2</sub> at the end of the experiment with no detectable difference in average cyanobacterial cover between ambient CO<sub>2</sub> (46%) and elevated CO<sub>2</sub> (47%) (RM-ANOVA:  $F_{1,10} = 0.37$ ,  $P = 0.56$ ; 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 CO<sub>2</sub> and elevated CO<sub>2</sub> treatments (Figure 3.1). No significant effect of CO<sub>2</sub> × time was detected (RM-ANOVA:  $F_{15,150} = 0.41$ ,  $P = 0.98$ ), indicating that the CO<sub>2</sub> 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 CO<sub>2</sub> or elevated CO<sub>2</sub>.

#### 3.1.2 Cyanobacterial Abundance

Cyanobacterial abundance (measured as the total number of cyanobacteria cells per vial) differed between ambient CO<sub>2</sub> and elevated CO<sub>2</sub> (RM-ANOVA:  $F_{1,10} = 5.44$ ,  $P = 0.04$ ), such that elevated CO<sub>2</sub> 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 CO<sub>2</sub> conditions. Ambient CO<sub>2</sub> is shown using a dotted line with a solid circle, whereas elevated CO<sub>2</sub> 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 CO<sub>2</sub> (overall N = 192).

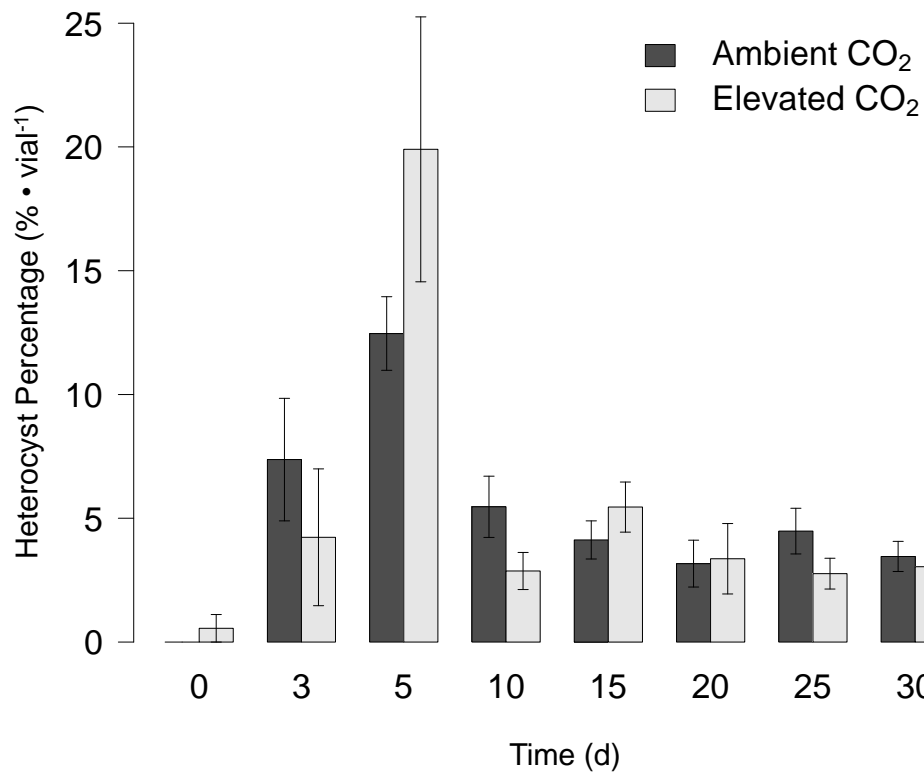


**Figure 3.2:** Cyanobacterial abundance, as measured by the total number of cyanobacterial cells, during the growth experiment. Growth of axenic *N. punctiforme* was observed on the surface of 27 mm diameter agar vials over 30 days under ambient (430 ppm) and elevated (750 ppm) atmospheric CO<sub>2</sub> conditions. Ambient CO<sub>2</sub> is shown using a dark grey bar, whereas elevated CO<sub>2</sub> is shown using a light grey bar. All data are plotted as mean  $\pm$  SD, and based upon six replicate samples per CO<sub>2</sub> 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  $\text{CO}_2$  at 30 days, and elevated  $\text{CO}_2$  at 25 days, indicating that the elevated  $\text{CO}_2$  culture was entering the decline phase at the end of the experiment, whereas the ambient  $\text{CO}_2$  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  $\text{CO}_2$  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  $\text{CO}_2$ , and 53 and 100% of the cells in each vial under elevated  $\text{CO}_2$ .

### 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  $3.63 \times 10^6$  cells vial<sup>-1</sup> under ambient  $\text{CO}_2$  and  $4.36 \times 10^6$  cells vial<sup>-1</sup> under elevated  $\text{CO}_2$ . There was no statistically significant effect of  $\text{CO}_2$  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  $\text{CO}_2$  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  $\text{CO}_2$  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  $\text{CO}_2$ .



**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 CO<sub>2</sub> conditions. Ambient CO<sub>2</sub> is shown using a dark grey bar, whereas elevated CO<sub>2</sub> is shown using a light grey bar. All data are plotted as mean  $\pm$  SD, and based upon six replicate samples per CO<sub>2</sub> treatment (overall N = 96).

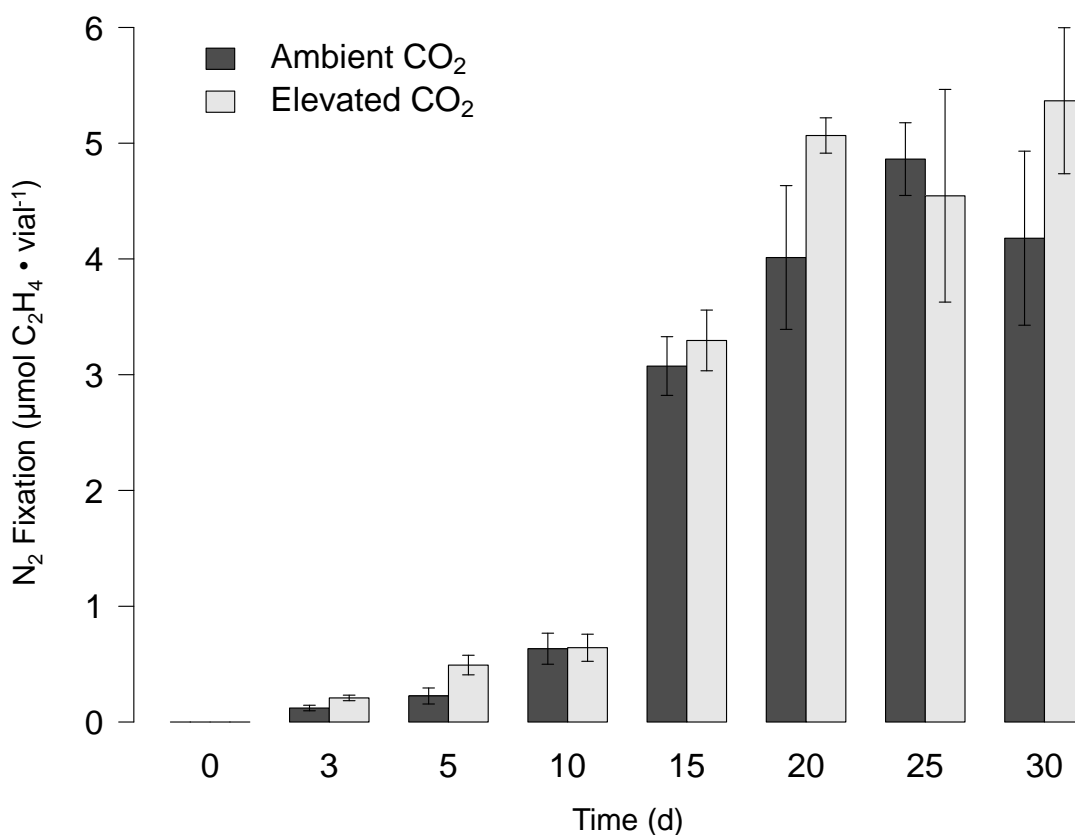


### 3.1.4 N<sub>2</sub> Fixation

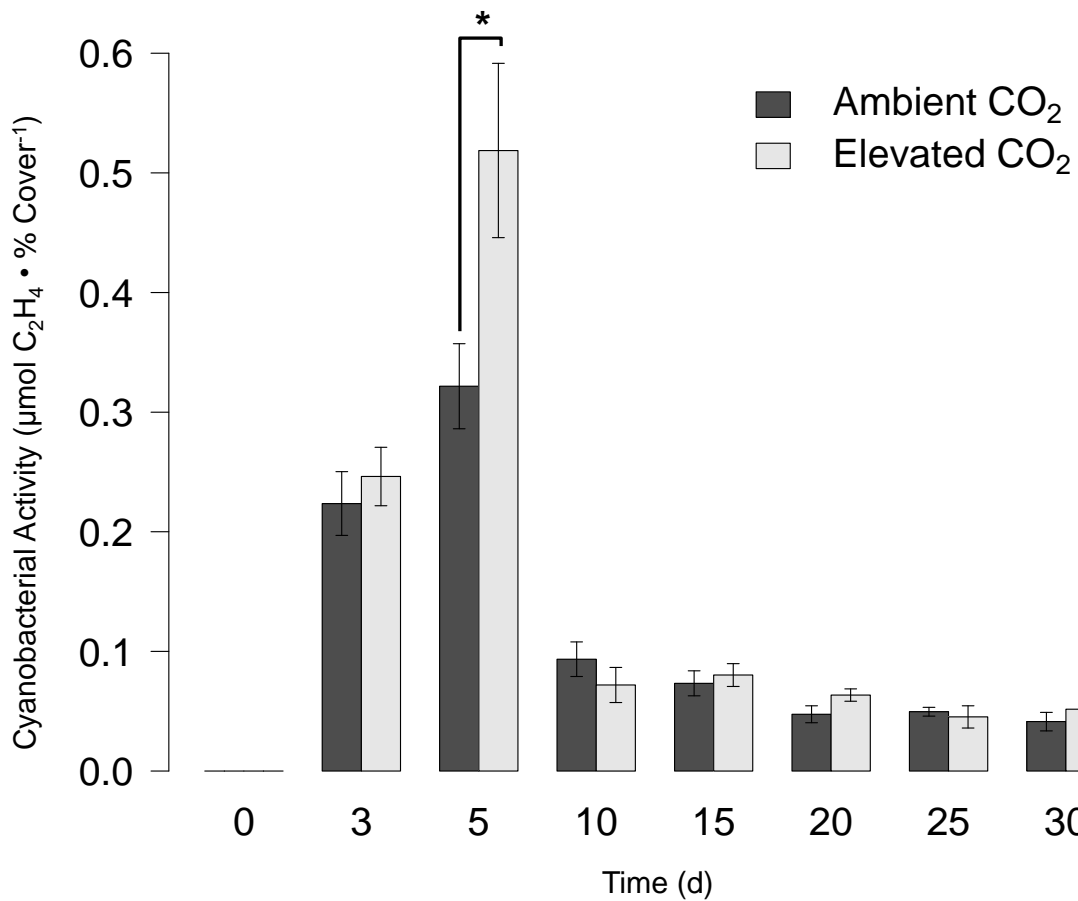
N<sub>2</sub> fixation, as measured by ethylene production per vial, did not differ between ambient CO<sub>2</sub> and elevated CO<sub>2</sub> treatments (RM-ANOVA:  $F_{1,10} = 2.04$ ,  $P < 0.18$ ; Figure 3.4), although N<sub>2</sub> fixation increased throughout time (RM-ANOVA:  $F_{7,70} = 64.74$ ,  $P < 0.001$ ). At ambient CO<sub>2</sub>, average N<sub>2</sub> fixation was 2.14  $\mu\text{mol vial}^{-1}$ , versus 2.45  $\mu\text{mol vial}^{-1}$  under elevated CO<sub>2</sub> conditions. No significant interaction effect of CO<sub>2</sub> and time was detected (RM-ANOVA:  $F_{7,70} = 0.93$ ,  $P = 0.48$ ). At all sampling times between 0 and 10 d, average N<sub>2</sub> 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). N<sub>2</sub> 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 N<sub>2</sub> fixation divided by percent cover) was influenced by CO<sub>2</sub>, such that elevated CO<sub>2</sub> increased cyanobacterial activity relative to ambient CO<sub>2</sub> (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 CO<sub>2</sub> 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:** N<sub>2</sub> fixation of axenic *N. punctiforme* as measured by the ethylene (C<sub>2</sub>H<sub>4</sub>) 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 CO<sub>2</sub> conditions. Ambient CO<sub>2</sub> is shown using a dark grey bar, whereas elevated CO<sub>2</sub> is shown using a light grey bar. All data are plotted as mean ± SD, and based upon six replicate samples per CO<sub>2</sub> treatment (overall N = 96).



**Figure 3.5:** Cyanobacterial activity of axenic *N. punctiforme* as measured by the ethylene (C<sub>2</sub>H<sub>4</sub>) 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 CO<sub>2</sub> conditions. Ambient CO<sub>2</sub> is shown using a dark grey bar, whereas elevated CO<sub>2</sub> is shown using a light grey bar. All data are plotted as mean ± SD, and based upon six replicate samples per CO<sub>2</sub> treatment (overall N = 96). Pairwise differences are shown using a symbol (\*) with a bar.

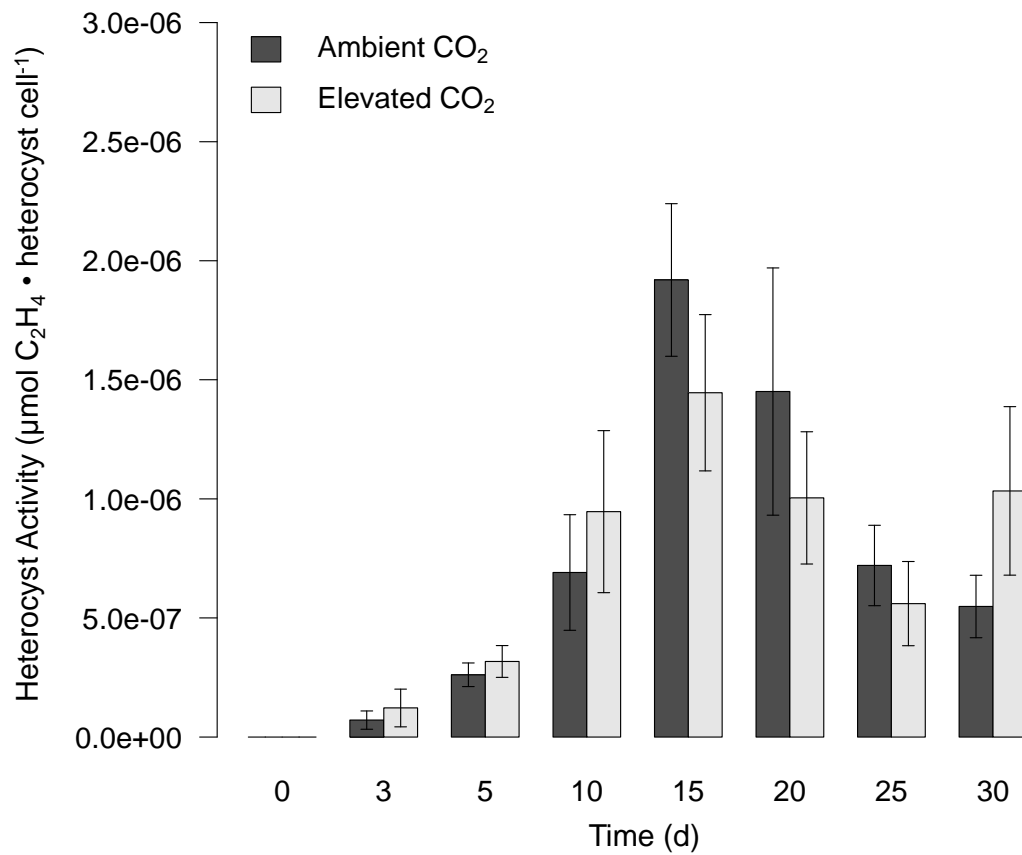
### 3.1.6 Heterocyst Activity

Heterocyst activity (calculated as N<sub>2</sub> fixation per heterocyst cell) between ambient CO<sub>2</sub> and elevated CO<sub>2</sub> was not statistically different (RM-ANOVA:  $F_{1,10} = 0.05$ ,  $P = 0.82$ ; Figure 3.6), with average values of  $7.08 \times 10^{-7}$   $\mu\text{mol C}_2\text{H}_4$  heterocyst<sup>-1</sup> and  $6.78 \times 10^{-7}$   $\mu\text{mol C}_2\text{H}_4$  heterocyst<sup>-1</sup> for each CO<sub>2</sub> 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 CO<sub>2</sub> and elevated CO<sub>2</sub> treatments at values of  $1.92 \times 10^{-6}$  and  $1.45 \times 10^{-6}$   $\mu\text{mol C}_2\text{H}_4$  heterocyst<sup>-1</sup> (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 CO<sub>2</sub>  $\times$  time was observed (RM-ANOVA:  $F_{7,70} = 0.91$ ,  $P = 0.50$ ).

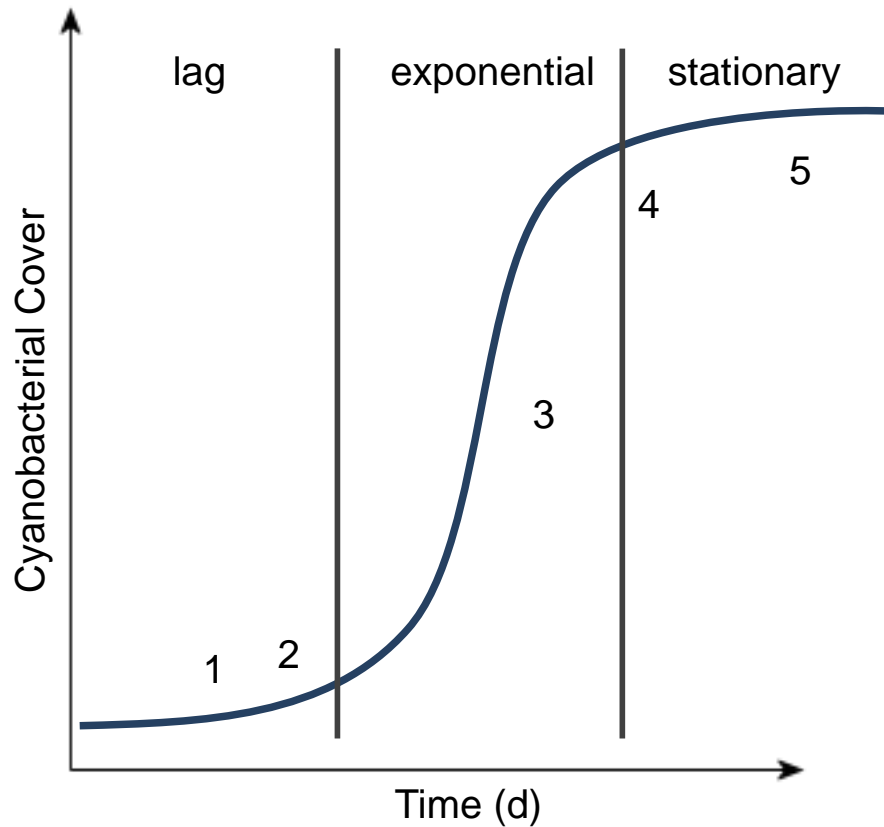
### 3.1.7 Timeline of Cyanobacterial Growth & N<sub>2</sub> Fixation

#### 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 N<sub>2</sub> 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 (C<sub>2</sub>H<sub>4</sub>) 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 CO<sub>2</sub> conditions. Ambient CO<sub>2</sub> is shown using a dark grey bar, whereas elevated CO<sub>2</sub> is shown using a light grey bar. All data are plotted as mean ± SD, and based upon six replicate samples per CO<sub>2</sub> treatment (overall N = 96).



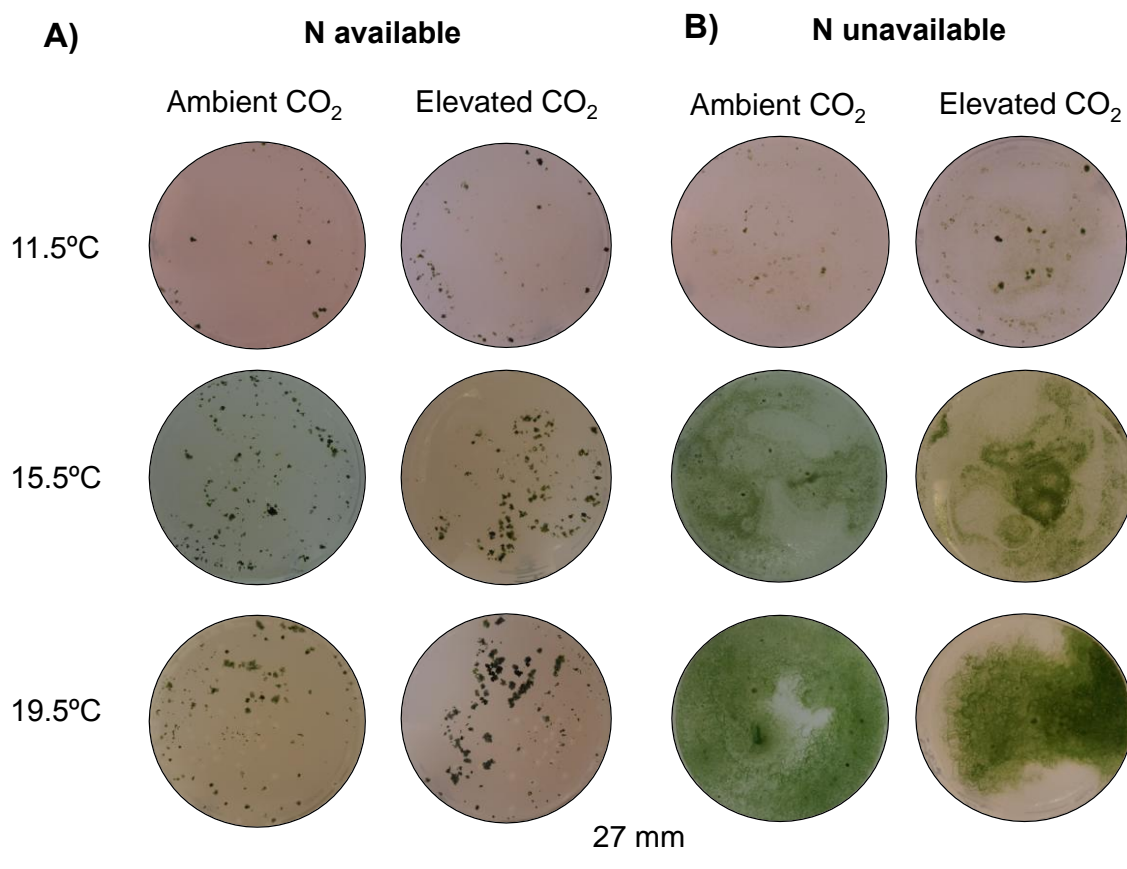
**Figure 3.7:** Timeline of growth and N<sub>2</sub> fixation events for the cyanobacterium *N. punctiforme*. Samples were grown on 27 mm agar vials during the 30 day growth experiment. In this experiment, CO<sub>2</sub> had a statistically significant effect on cyanobacterial activity, and N<sub>2</sub> fixation and growth followed the same pattern. The numbers shown on the curve represent the timing of critical growth and N<sub>2</sub> fixation events: 1) first heterocysts observed, 2) maximum cyanobacterial activity and maximum heterocyst percentage, 3) maximum heterocyst activity and growth rate, 4) maximum N<sub>2</sub> 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 CO<sub>2</sub> treatments, as described above with measurements obtained during a 90 day period. However, due to the absence of N<sub>2</sub> 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 CO<sub>2</sub> and temperature on N<sub>2</sub> fixation (Figure 3.8).

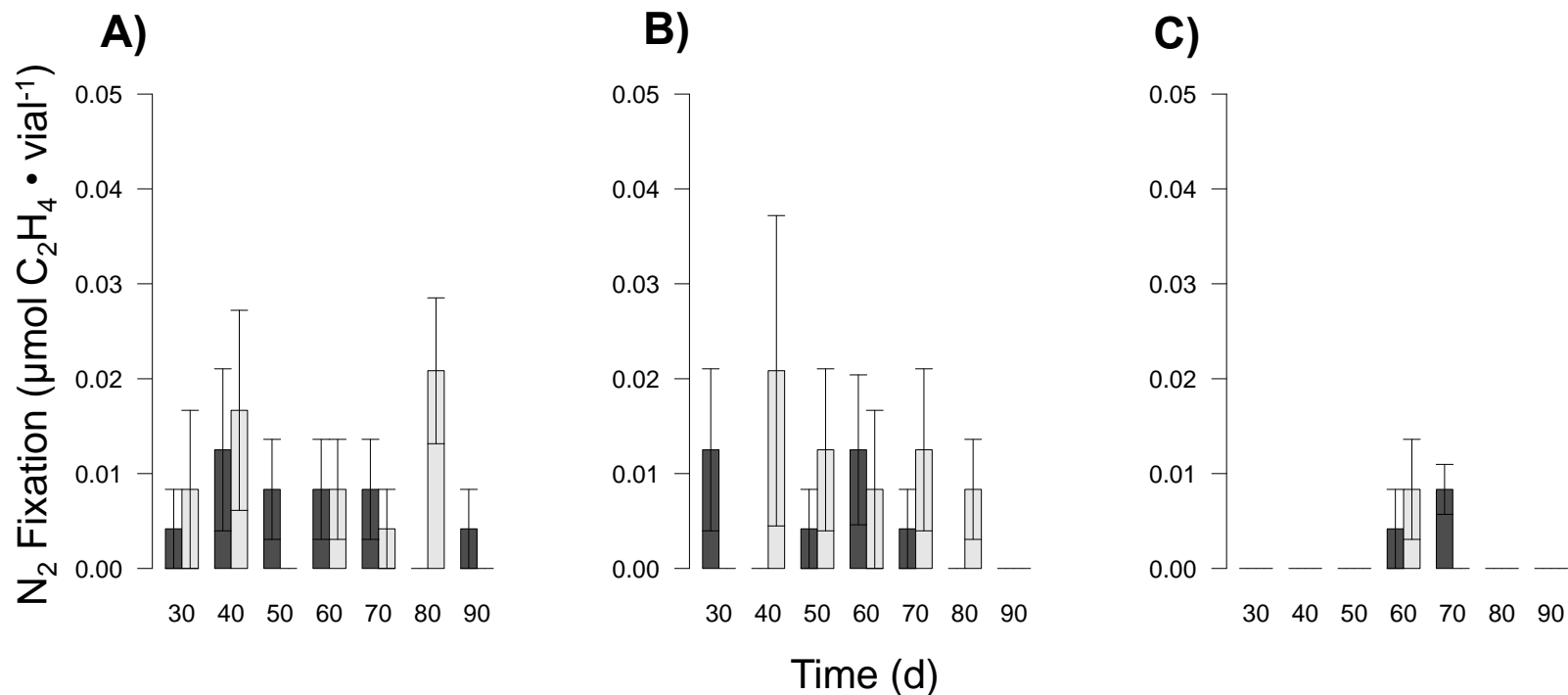
### 3.2.1 N<sub>2</sub> Fixation and Cyanobacterial Abundance Under N Available Conditions

N<sub>2</sub> fixation was virtually absent in conditions with N available, with observed values consistently less than 0.04  $\mu\text{mol vial}^{-1}$  (Figure 3.9). The factors CO<sub>2</sub> (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 N<sub>2</sub> fixation under N available conditions. Moreover, no significant effects of CO<sub>2</sub>  $\times$  time (RM-ANOVA:  $F_{6,180} = 1.70$ ,  $P = 0.12$ ), temperature  $\times$  time (RM-ANOVA:  $F_{12,180} = 0.73$ ,  $P = 0.72$ ), or CO<sub>2</sub>  $\times$  temperature  $\times$  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).



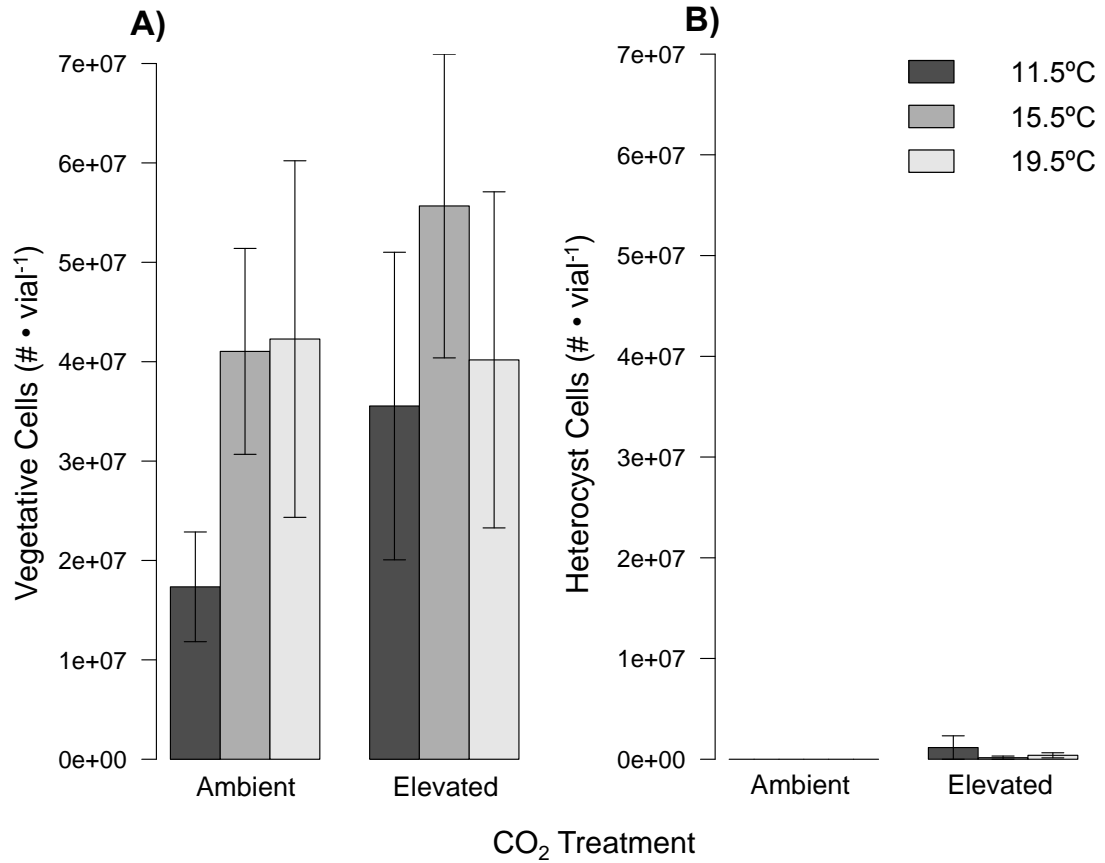


**Figure 3.9:**  $N_2$  fixation of axenic *N. punctiforme* as measured by the ethylene ( $C_2H_4$ ) produced per 27 mm diameter agar vials over 90 days. Cultures were grown at A) 11.5 °C, B) 15.5 °C, and C) 19.5 °C on BG-11 media. The conversion from ethylene production to  $N_2$  fixation relies upon 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).

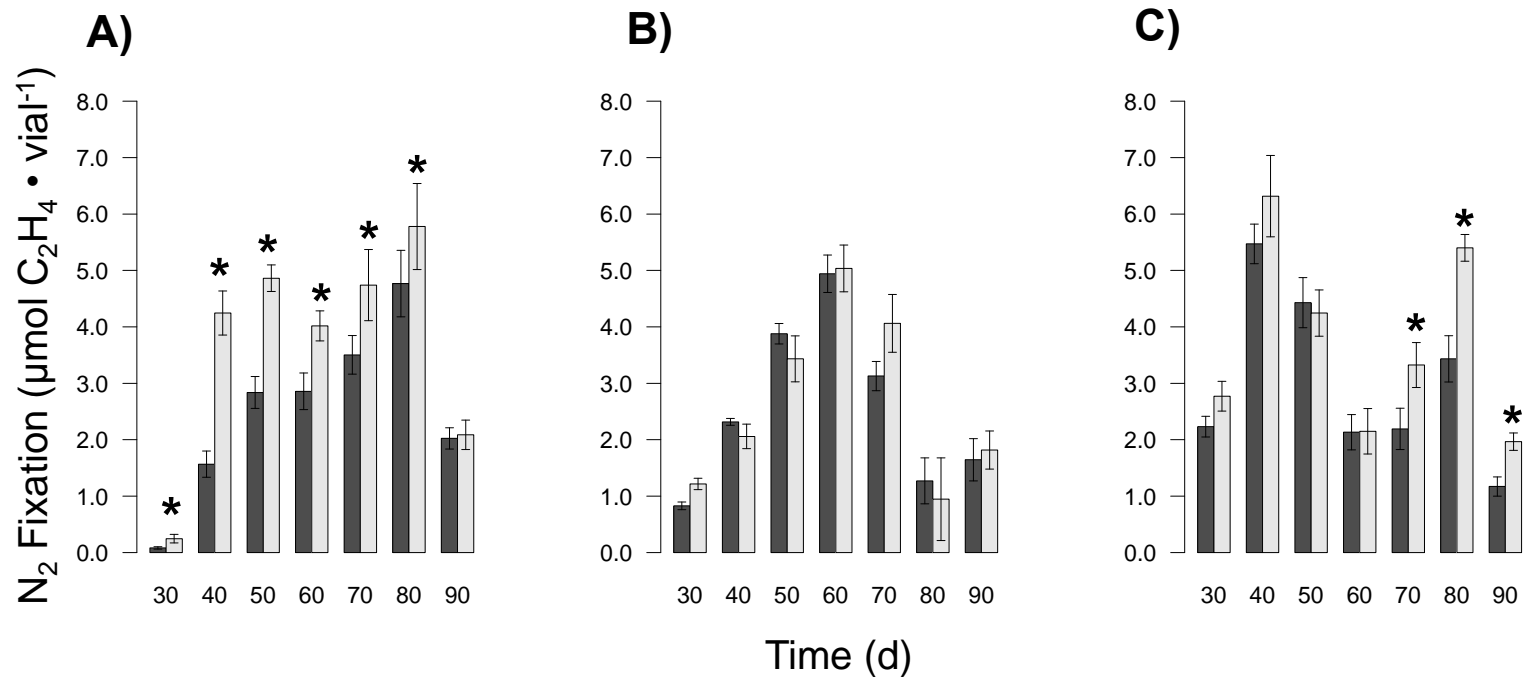
Likewise, there were no significant differences in cyanobacterial abundance present between CO<sub>2</sub> treatments (RM-ANOVA:  $F_{1,30} = 0.84$ ,  $P = 0.37$ ), or temperature treatments (RM-ANOVA:  $F_{2,30} = 1.14$ ,  $P = 0.34$ ) under N available conditions (Figure 3.1). Further, there was no significant effect of CO<sub>2</sub> × 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 N<sub>2</sub> Fixation and Cyanobacteria Abundance Under N Limitation Conditions

Under conditions where N was unavailable, N<sub>2</sub> fixation was influenced by CO<sub>2</sub>, such that overall N<sub>2</sub> fixation rates were higher for ambient CO<sub>2</sub> (1.35 μmol vial<sup>-1</sup>) than elevated CO<sub>2</sub> (1.69 μmol vial<sup>-1</sup>) (RM-ANOVA:  $F_{1,30} = 21.77$ ,  $P < 0.001$ ; Figure 3.1). Temperature also influenced N<sub>2</sub> fixation (RM-ANOVA:  $F_{2,30} = 9.77$ ,  $P < 0.001$ ), with overall higher N<sub>2</sub> 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 CO<sub>2</sub> × 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 CO<sub>2</sub> effect at 11.5 °C (2.52 μmol vial<sup>-1</sup> versus 3.71 μmol vial<sup>-1</sup>), but no CO<sub>2</sub> effect at 15.5 °C, and a transient positive CO<sub>2</sub> effect at 19.5 °C between days 70 and 90 (Figure 3.1). No significant effect of CO<sub>2</sub> × time was present (RM-ANOVA:  $F_{6,180} = 1.41$ ,  $P = 0.22$ ). Time also had a statistically significant effect on N<sub>2</sub> 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 CO<sub>2</sub> 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.



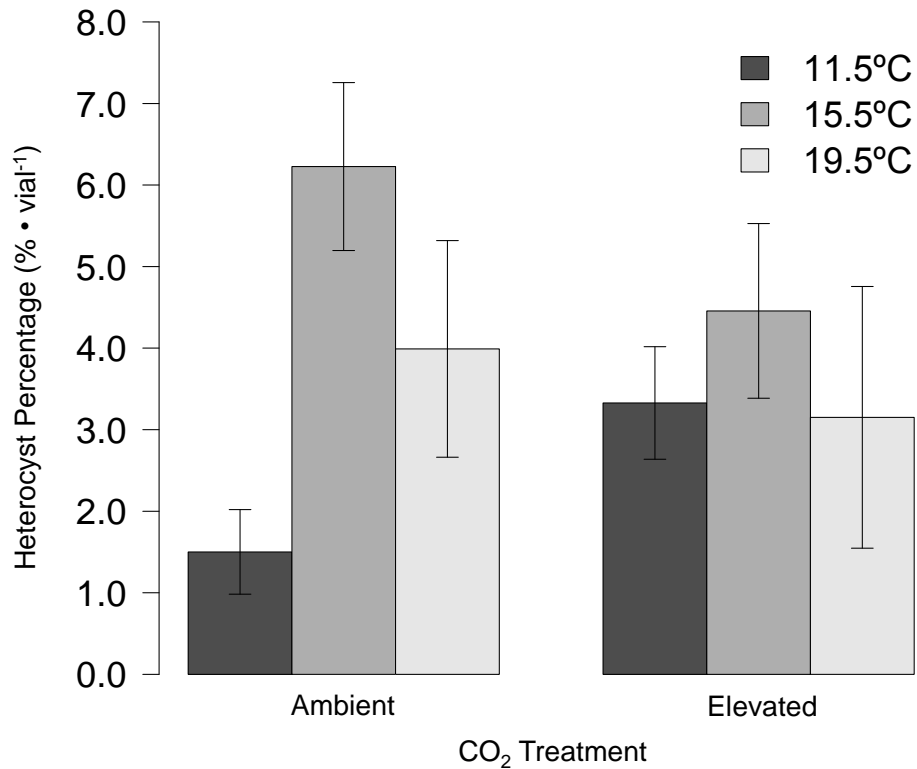
**Figure 3.11:**  $N_2$  fixation of axenic *N. punctiforme* as measured by the ethylene ( $C_2H_4$ ) produced per 27 mm diameter agar vial over 90 days during the global environmental change experiment. Vials were grown at A) 11.5 °C, B) 15.5°C, and C) 19.5°C. The conversion from ethylene production 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:  $F_{12,180} = 2.51$ ,  $P < 0.01$ ).

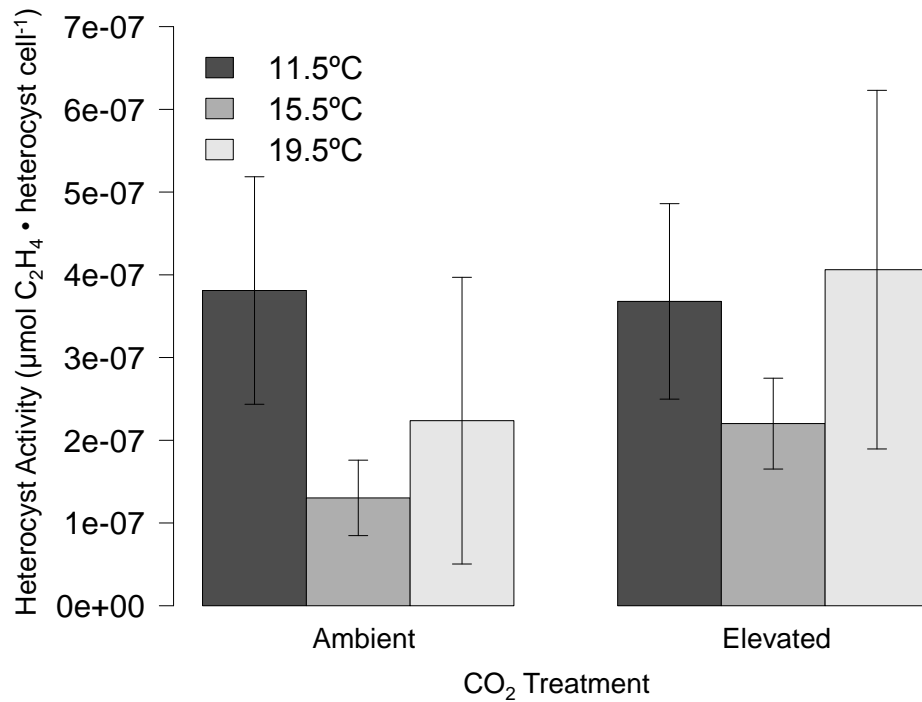
There was no significant effect of any of the treatment variables on cyanobacterial abundance at the end of 90 days in vials under N free conditions using RM-ANOVA ( $CO_2$ :  $F_{1,30} = 0.08$ ,  $P = 0.79$ ; temperature:  $F_{2,30} = 0.88$ ,  $P = 0.43$ ;  $CO_2 \times$  temperature:  $F_{2,30} = 1.08$ ,  $P = 0.35$ ). 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 CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub> 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 N<sub>2</sub> 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 CO<sub>2</sub> on growth and N<sub>2</sub> fixation activity in axenic cultures of *N. punctiforme*. This experiment was performed to gain mechanistic insights into N<sub>2</sub> fixation patterns with respect to growth, and the timing of developmental events for cyanobacteria in N-limited settings. This experiment revealed that N<sub>2</sub> fixation and growth are strongly correlated, and that N<sub>2</sub> fixation rates are highly dependent upon the growth stage of the culture. I observed a stimulatory effect of CO<sub>2</sub> 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 N<sub>2</sub> 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 N<sub>2</sub> fixation assay, and as a result would not demonstrate N<sub>2</sub> fixation activity.

Growth by cyanobacterial cover and N<sub>2</sub> 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 CO<sub>2</sub> conditions, did not translate to greater N<sub>2</sub> fixation rates. This is likely because vegetative cells are photosynthetic but not N<sub>2</sub> 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 N<sub>2</sub> fixation; as such, I use this to form the relationship between growth and N<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub>, such that elevated CO<sub>2</sub> cultures appeared to be entering a decline phase by the end of the experiment, whereas ambient CO<sub>2</sub> cultures did not; this highlights growth as a confounding factor in studies on cyanobacterial N<sub>2</sub> 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 N<sub>2</sub> fixation focus on axenic cultures of cyanobacteria, rather than moss systems.

Elevated CO<sub>2</sub> 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, CO<sub>2</sub> concentration had a unimodal effect on growth in the cyanobacterium *A. fertilissima*, with a decline in growth when culture were incubated at CO<sub>2</sub> concentrations above 6% in the headspace (Chinnasamy et al., 2009). I suggest that the CO<sub>2</sub> effect on growth depends upon the growth stage of the culture, similar to Levitan et al. (2007), who demonstrated that the CO<sub>2</sub> effect on N<sub>2</sub> fixation was growth stage dependent. I posit that the discrepancy in results obtained for heterocystous cyanobacteria for the effects of CO<sub>2</sub> within the literature are likely due to growth as a confounding factor on N<sub>2</sub> fixation rates. My study suggests that CO<sub>2</sub> stimulation of growth in *N. punctiforme* is transient, and further that this transient effect will not result in increased N<sub>2</sub> 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 N<sub>2</sub> 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 N<sub>2</sub>) 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 N<sub>2</sub> fixation (Dixon & Kahn, 2004), I focus my mechanistic interpretation of N<sub>2</sub> 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 N<sub>2</sub> fixation under elevated CO<sub>2</sub> (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 N<sub>2</sub> 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 N<sub>2</sub> 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 N<sub>2</sub> 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 CO<sub>2</sub> on N<sub>2</sub> fixation in *Nostoc punctiforme*. I correctly predicted that N<sub>2</sub> fixation would be minimal in conditions with available N present, with heterocysts and their N<sub>2</sub> fixation activity essentially absent. My corresponding prediction of reduced vegetative cell growth was also correct. Likewise, I predicted and observed that biologically relevant N<sub>2</sub> fixation occurs in conditions where fixed N<sub>2</sub> is unavailable, with more heterocysts and their N<sub>2</sub> fixation activity in this N availability

scenario. I found an overall stimulatory effect of CO<sub>2</sub> on N<sub>2</sub> fixation that was temperature dependent, whereby increased N<sub>2</sub> fixation was observed at 11.5 °C under elevated CO<sub>2</sub>, although we failed to observe this effect at 15.5 °C, and observed a transient stimulation at 19.5 °C. Although I predicted that CO<sub>2</sub> and temperature would have a synergistic effect on N<sub>2</sub> fixation rates, this effect was antagonistic instead. Together, my findings suggest that the mechanism for increased N<sub>2</sub> fixation under elevated CO<sub>2</sub> 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 CO<sub>2</sub> on cyanobacterial growth

In previous studies, CO<sub>2</sub> 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 CO<sub>2</sub> 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% CO<sub>2</sub> concentration (Chinnasamy et al., 2009). The overall stimulatory effect of CO<sub>2</sub> observed in the global environmental change experiment did not translate to a great discrepancy in N<sub>2</sub> fixation rates or cyanobacterial abundance, because the strength of the CO<sub>2</sub> 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 CO<sub>2</sub> effect on N<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub> effects on N<sub>2</sub> fixation in mosses did not quantify cyanobacterial abundance (Smith, 1984), leaving the effects of CO<sub>2</sub> on the cyanobacterial communities enigmatic. Since my study demonstrates that the CO<sub>2</sub> 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 CO<sub>2</sub> experiment in an *in situ* ecosystem setting would be an ideal next step in the mechanistic interpretation of CO<sub>2</sub> effects on N<sub>2</sub> 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 CO<sub>2</sub> 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.

N<sub>2</sub> 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-11<sub>0</sub> 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 N<sub>2</sub> 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 (NH<sub>4</sub><sup>+</sup>) over nitrate (NO<sub>3</sub><sup>-</sup>), suggesting that the type of available N may also have differing effects on cyanobacterial abundance (Liu et al., 2013).

#### 4.2.4 N<sub>2</sub> fixation of axenic cultures of *Nostoc punctiforme*

Previous studies have measured the abundance of cyanobacteria cells on moss shoots to understand N<sub>2</sub> 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 N<sub>2</sub> fixation, my interpretation of the results can be extended to the cyanobacteria in association with mosses. Given that cyanobacterial cover and N<sub>2</sub> fixation were closely related, it suggests that an increase in growth due to vegetative cell production was not the mechanism for increased N<sub>2</sub> fixation under elevated CO<sub>2</sub> 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 N<sub>2</sub> 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 N<sub>2</sub> fixation rates.

In the growth experiment, N<sub>2</sub> fixation accelerated rapidly at day 15, which corresponded to the middle of the exponential growth cycle. Hence, it appears that cyanobacteria produce fixed N<sub>2</sub> 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 N<sub>2</sub> fixation, since available N production must occur before this increase in growth. Indeed, it has previously been showed that N<sub>2</sub> 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 N<sub>2</sub> fixation precedes rapid vegetative cell production.

### 4.3 N<sub>2</sub> Fixation of Axenic Cultures of *Nostoc punctiforme* In Response to Variation In Global Environmental Change Factors

#### 4.3.1 Effects of elevated atmospheric CO<sub>2</sub> conditions on N<sub>2</sub> fixation

No effect of CO<sub>2</sub> on N<sub>2</sub> 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 N<sub>2</sub> 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 N<sub>2</sub> was fixed. However, stimulatory effects of CO<sub>2</sub> on cyanobacterial abundance did not translate to increased N<sub>2</sub> fixation at days 20 and 25, therefore I postulate that the CO<sub>2</sub> effect was either absent or temperature dependent on N<sub>2</sub> fixation rates. My observation of a transient and stage dependent effect on cyanobacterial abundance suggests that the decreased N<sub>2</sub> fixation previously observed in moss systems under elevated CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub> effects on N<sub>2</sub> fixation in cyanobacteria from the moss association, it is unknown how species differences between cyanobacterial communities will contribute to the observed effects of CO<sub>2</sub> on N<sub>2</sub> 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 CO<sub>2</sub> effect on N<sub>2</sub> fixation varies between species. Given that *Calothrix* and *Nostoc* possess different temperature maxima for N<sub>2</sub> 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 CO<sub>2</sub> effects is obtained.

At the conclusion of the global environmental change experiment, heterocyst percentage appeared to be similar across all CO<sub>2</sub> 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 N<sub>2</sub> fixation

The growth experiment demonstrated that N<sub>2</sub> 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 N<sub>2</sub> fixation to growth rate and stage in the cyanobacterial culture. I found that CO<sub>2</sub> had a stimulatory effect on N<sub>2</sub> fixation, but that the extent of this was temperature dependent. My study is the first to demonstrate an interactive effect of CO<sub>2</sub> and temperature on a terrestrial cyanobacterium from the moss association. Previous studies demonstrated that CO<sub>2</sub> had a beneficial effect on N<sub>2</sub> fixation at elevated temperature for marine species (Chinnasamy et al., 2009), but I observed that CO<sub>2</sub> stimulates N<sub>2</sub> 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 CO<sub>2</sub> treatments at each temperature, suggesting that vegetative cell production was not the mechanism for increased N<sub>2</sub> fixation. However, N<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub> concentrations are both expected to increase in boreal ecosystems (IPCC, 2007), I suggest that rising temperatures will likely negate the beneficial effect of CO<sub>2</sub> on N<sub>2</sub> fixation rates. However, it cannot be excluded that differences

in cyanobacterial community composition will contribute to the response to increases in temperature and atmospheric CO<sub>2</sub> concentrations. For example, *Calothrix* experiences increased N<sub>2</sub> 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 N<sub>2</sub> fixation

N<sub>2</sub> fixation did not occur to any biologically relevant extent in conditions where available N was present, with no associated effects of temperature or CO<sub>2</sub>. Previous literature supports that N<sub>2</sub> 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 N<sub>2</sub> fixation responds to temperature changes in minutes (Jean et al., 2012), suggesting the effects of N<sub>2</sub> fixation to other global environmental change factors could also be instantaneous. In previous studies on the cyanobacteria-moss association, N<sub>2</sub> fixation rates were often not correlated with cyanobacterial abundance (Smith, 1984), leaving the mechanism responsible for reductions in N<sub>2</sub> fixation in response to available N unknown. Here, I demonstrated that N<sub>2</sub> fixation decreases in response to available N, and reveal that reduced heterocyst abundance and activity can be attributed to this decrease.

N<sub>2</sub> 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 N<sub>2</sub>. 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 N<sub>2</sub> 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 N<sub>2</sub> 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 CO<sub>2</sub> 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 N<sub>2</sub> fixation rates. Further, I demonstrated that a decrease in cyanobacterial abundance is likely the mechanism for the observed decrease in N<sub>2</sub> 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  $\text{NO}_x$  will result in decrease of cyanobacterial abundance and their  $\text{N}_2$  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  $\text{N}_2$ , or display cyanobacterial activity to any significant extent. These findings are consistent with genetic studies on  $\text{N}_2$  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  $\text{N}_2$  fixation rates. My research adds to the field, as I am able to demonstrate that the decrease in  $\text{N}_2$  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 ( $\text{NH}_3$ ) produced on average under each combination of temperature and  $\text{CO}_2$  suggests that increases in  $\text{N}_2$  fixation due to elevated  $\text{CO}_2$  are greatest under ambient temperature. Given that the  $\text{CO}_2$  stimulation was absent at 15.5 °C, and minimal at 19.5 °C, this suggests that increases in temperature and  $\text{CO}_2$  will result in reduced  $\text{N}_2$  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 CO<sub>2</sub> and elevated CO<sub>2</sub> growth curves for a cyanobacterium that is terrestrial, axenic, and symbiotically competent. This is the first study to demonstrate that elevated CO<sub>2</sub> is stimulatory on N<sub>2</sub> fixation in a terrestrial cyanobacterium. In doing so, I demonstrated that N<sub>2</sub> fixation and growth closely follow each other. I also show that there is a statistically significant effect of CO<sub>2</sub> 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 N<sub>2</sub> fixation, which did not occur until 20 days after vial inoculation. I suggest that this is due to an increased growth demand under elevated CO<sub>2</sub> 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 CO<sub>2</sub> effects previously documented in the literature. Collectively, these findings demonstrated that N<sub>2</sub> fixation in cyanobacteria is dependent upon both growth rate and growth stage. The results gained in this thesis present a mechanistic understanding of N<sub>2</sub> 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 CO<sub>2</sub> on *N. punctiforme*. In doing so, I demonstrated that N<sub>2</sub> 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 N<sub>2</sub> fixation occurs in conditions where available N is absent, and demonstrated that CO<sub>2</sub> stimulation of N<sub>2</sub> fixation occurs at boreal ambient temperature (11.5°C). It is possible that overall reductions in N<sub>2</sub> fixation might be observed, both through influxes of available N and the combined effect of temperature moderating cyanobacterial growth under elevated CO<sub>2</sub>. Overall, this collection of findings suggests that any increases in N<sub>2</sub> fixation under elevated CO<sub>2</sub> 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

```

```

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)

```

```

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.

```

```

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]#####
```

# 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 CO<sub>2</sub> 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 CO<sub>2</sub> on nitrogen fixation by the cyanobacterium *Nostoc punctiforme*. BGRF. London, ON. October 19, 2013.

## Anticipated Publications:

Griffith DA, Lindo Z. Growth demands under elevated CO<sub>2</sub> stimulate N fixation activity in the terrestrial cyanobacterium *Nostoc punctiforme*. *In Submission*.