THESIS

THE INFLUENCE OF OUTDOOR LIGHT ON BIOMASS, NITROGEN, AND PHYTOHORMONE CONCENTRATIONS OF A NITROGEN-FIXING ANABAENA SP. CYANOBACTERIUM

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ABSTRACT

THE INFLUENCE OF OUTDOOR LIGHT ON BIOMASS, NITROGEN, AND PHYTOHORMONE CONCENTRATIONS OF A NITROGEN-FIXING ANABAENA SP. CYANOBACTERIUM

Cultivation of a strain of freshwater heterocystous N-fixing cyanobacterium, collected in Fort Collins, CO, with 99% similarity to Anabaena cylindrica, was scaled up to raceway production volumes of 2200L for application as a fertilizer for horticultural crops. Previous research at Colorado State University showed that an Anabaena sp.-based fertilizer is comparable to organically certified sources of N, such as fish emulsion, blood meal, and feather meal. Although previous laboratory cultivations produced up to 1017 mg L^{-1} biomass and 110 mg L^{-1} N during a 14-d growth period, two week batch production in on-farm open raceways, under polyethylene covered high tunnels, and without CO_2 supplementation, achieved only 296 mg L^{-1} biomass and 30.0 mg L^{-1} N.

Furthermore, algae and cyanobacteria, including Anabaena, have been reported to produce a number of phytohormones under laboratory conditions. However, it is not known which phytohormones would be produced at detectable concentrations in an Anabaena sp. based culture under outdoor batch cultivation conditions. It is also unknown whether the resulting phytohormone concentrations would be sufficient to influence crop growth.

The primary goal of this study was to increase daily productivity (24-hr gains in biomass) and 14-d batch biomass and N concentrations of Anabaena sp. cultures under outdoor production by addressing light and CO_2 availability, the two limiting factors in cyanobacterial growth. A

secondary goal of this study was to identify phytohormone concentrations that are consistently produced in xenic Anabaena sp. outdoor cultivations and to determine whether light availability and the inoculation density of the culture could be used to influence those phytohormone concentrations.

Increasing Anabaena sp. culture outdoor batch biomass and nitrogen (N) concentrations and determining the concentrations of plant growth promoting phytohormones under outdoor production conditions could establish cyanobacteria-based fertilizers as an economical option for horticultural crop production.

All experiments were conducted at the Colorado State University Horticultural Research Farm. A two-way factorial experiment conducted under full outdoor exposure in October, 2015 included three inoculation densities and supplementation of CO_2 when pH exceeded 8.9. Anabaena sp. culture inoculation densities of 0.174 OD_{750nm} calculated to capture maximum annual photosynthetically active radiation (PAR) of 2030 µmol m^{-2} s^{-1}, and an 0.091 OD_{750nm} calculated to capture 1800 µmol m^{-2} s^{-1} PAR, experienced significantly higher photochemical efficiency of photosystem II (Fv/Fm) when exposed to sunlight than an inoculation density of 0.026 OD_{750nm}, calculated to capture approximately 1300 µmol m^{-2} s^{-1} PAR. Fv/Fm reduction and recovery was correlated to culture density. Cloudy weather and high sampling volumes limited biomass increases and allowed for measurement and comparison of Fv/Fm on four of the first six days of the 14-d batch. The design of the columns that the Anabaena sp. were cultivated in facilitated a transition after day 6 from the inoculation density treatments to treatments which allowed three levels of light transmission (10%, 25%, and 50%) through the translucent curved surface of the columns. This shading had no effect on light availability at the top of the column. The 25% Shade treatment (= to 0.091 OD_{750nm} inoculation density) with CO_2 supplementation

achieved a mean 14-d biomass of 636 +/- 99 mg L^{-1} and total N of 71.0 +/- 5.0 mg L^{-1}. These results were significantly higher than all three Shade treatments without CO_2 supplementation. The lowest density or 10% shaded cultures failed to achieve significant increases in biomass throughout the 14 d batch period. The dilution of the cultures due to Fv/Fm sampling until day 6, effectively shortened the batch period length, and the biomass and N concentrations were achieved in as little as 9 or 10 days.

In July, 2014, CO_2 was supplemented to limit Anabaena sp. culture pH to 9.5 in 9.26 m^2, 25 cm deep, 2200 L paddlewheel mixed open raceways located within polyethylene covered high tunnels. There was no significant difference between the CO_2-supplemented and control raceways after 14 d, with mean raceway biomass and N concentrations of 241 +/- 23 mg L^{-1} and 29.9 +/- 2.9 mg L^{-1}, respectively. Multiple regression analysis to predict daily productivity identified pH, light availability, and temperature as significant variables. CO_2 supplementation at pH 9.5 was insufficient to significantly increase biomass and N concentrations. However, analysis of CO_2 supplementation revealed that even during the summer months, light is a limiting factor under high tunnel raceway production.

These results suggest that it may be possible to significantly increase outdoor Anabaena sp. productivity and end of batch biomass and N concentrations through supplementation of CO_2 to limit maximum pH between 8.0 and 8.9; moving culture production out of high tunnels; and increasing Anabaena sp. culture inoculation densities to capture maximum expected PAR.

A two-factorial experiment was conducted outdoors in CO_2-supplemented 52cm x 165cm x 30cm deep troughs during the summer of 2016. The experiment compared the effect of high tunnel shading and inoculation density, calculated to capture 91% or 84% of maximum PAR on the inoculation date, on 14-d Anabaena sp. endogenous phytohormone concentrations. The

phytohormone assay detected abscisic acid (ABA), three auxins (indole-3 acetic acid (IAA), indole acetamide (IAM), indole carboxylic acid (ICA)); one cytokinin (trans Zeatin riboside (tZr)); and salicylic acid (SA). Mean phytohormone concentrations were similar among the experiments, except for SA. ICA and SA have not been reported as cyanobacterial metabolites, and may be attributable to the xenic nature of the culture. The presence of other bacteria, as well as chlorophyll-containing protozoa resembling Ochromonas sp., was observed microscopically in the cultures throughout the batch period.

Regression equations based on treatment variables and other field production conditions were used to predict phytohormone concentrations. Anabaena sp. culture inoculation density was determined to be a significant variable in predicting IAA concentrations ($R^2 = 0.52$). Light availability was included as a significant variable in all other phytohormone regression equations. SA concentrations were comparable to those reported to have beneficial effects on plant growth, while ABA concentrations were only a few orders of magnitude lower. The phytohormone concentrations reported here represent only endogenous levels in the Anabaena sp. culture biomass. An assay covering a wider range of cytokinins and inclusion of the exogenous fraction of the culture could reveal higher phytohormone concentrations.

The phytohormone study results also suggest that increased inoculation densities could increase IAA concentrations at the end of the 14-d batch. Light limitation could be used to significantly increase ABA concentrations. Conversely, maximizing light availability could significantly increase the concentration of IAM and SA. The primary goal of Anabaena sp. production is for use as an N fertilizer. The conditions most likely to maximize biomass and N concentrations are similar to those most likely to result in higher IAA, IAM and SA concentrations.

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Introduction

Background

Nitrogen-fixing cyanobacteria are attractive as a nitrogen (N) fertilizer because they are ubiquitous in nature and have minimal nutrient requirements. A locally produced, on-demand source of N could expand fertilizer options available for vegetable production. On-farm cyanobacteria-based fertilizer could be phototrophically produced under reduced land and water requirements compared with N-fixing cover crops. On-farm production of N-fixing cyanobacteria could also improve access to N for smallholder farms in regions of the world where soil N is depleted and fertilizer availability is unreliable or nonexistent. Production and application of as little as 10 to 17 kg/ha of N could provide at least 30% gains in crop yield (Twomlow et al., 2008). Small-scale production of a cyanobacteria-based fertilizer could be achieved with similar energy requirements per kg N compared to ammonia-based fertilizers (Gellings and Parmenter, 2004; Sheehan et al., 1998; Tavares et al., 2013). Small-scale batch production requires only a few kilowatt-hours per day, and meaningful N production could be realized with minimal fossil fuel inputs or low-cost solar panels.

Researchers at Colorado State University (CSU) have cultivated a local strain of Anabaena sp. that is ideally suited for fertigation. The culture does not clog irrigation drip lines and thus can be pumped directly from raceways to crops. Compared with other locally isolated N-fixing strains,

the Anabaena sp. strain was better able to acclimate to diurnal and seasonal changes in light and temperature as well as to competitive pressure from eukaryotic algae and protozoa. When applied to vegetable crops at the same N-rate, Anabaena sp. cultures have proven as effective as commonly used organically certified N-fertilizers, such as alfalfa, feather, and blood meals and fish emulsion (Sukor, 2013; Yoder, 2014).

In laboratory experiments, 0.5 L cultures of Anabaena sp. grown in N-free Allen and Arnon medium under 12h light dark periods with 140 µmol m^{-2} s^{-1} light achieve up to 30-fold gains in biomass to 730 mg L^{-1} and total N of 80 mg L^{-1} during a 14-d growth period. Under 210 µmol m^{-2} s^{-1} light up to 1017 mg L^{-1} biomass and 110 mg L^{-1} total N have been achieved. In contrast, during 14-d outdoor summer batch production, the Anabaena sp. cultures have achieved 12-fold gains to 296 mg L^{-1} biomass and 30 mg L^{-1} N.

Approaches for Increasing Microalgae Production

This research focused on bridging the biomass and total N productivity gap between laboratory and field based on the following criteria: 1) methods that could achieve the largest gains in total batch biomass and N; and 2) methods that were most likely to be adopted by end users. For these reasons, we chose to focus on outdoor raceway production of locally cultured wild-type N-fixing cyanobacteria strains. Outdoor open ponds are considered the most economical method of production (Borowitzka and Moheimani, 2013; Grobbelaar, 2010; Sheehan et al., 1998; Tredici, 2004) and thus far, have proven simple to implement on farm. Furthermore, a focus on locally cultured and adapted wild-type strains of N-fixing cyanobacteria, such as the Anabaena sp. in this study, may facilitate regional adoption, scale up, and success of cyanobacteria-based N-production (Sheehan et al., 1998).

Light

Light, CO_2, temperature, and dissolved oxygen (DO) levels are the most important parameters for phototrophic growth (Richmond, 2004; Borowitzka and Moheimani, 2013). Light is required for photosynthesis. Maximizing photosynthetic productivity in outdoor mass microalgae cultures can be challenging. Increasing solar irradiance after sunrise can quickly lead to photoinhibition, particularly in low density cultures. Photoinhibition is a reduction in quantum efficiency that may be accompanied by a decrease in maximum photosynthetic rates and long-term productivity of microalgal cultures (Osmond, 1994; Taiz and Zeiger, 2010; Vonshak and Torzillo, 2004). As photoinhibition occurs, photosystem II (PSII) reaction centers (RC) are taken off line, reducing efficiency of the culture's use of available sunlight, or the photochemical efficiency of PSII. PSII RC are continuously damaged by light at any intensity. However, the damage to PSII is also being continuously repaired. Under optimal temperature, dissolved oxygen concentrations, nutrient availability, and culture densities (with respect to light intensity), repair rates are maintained, and the loss in photosynthetic efficiency is minimal. Overall photosynthetic rates and growth may not be affected. Under suboptimal culture conditions, such as high light, low temperatures, or a build-up of reactive oxygen species (ROS), repair of PSII is inhibited (Murata et al., 2007). When rates of photodamage reach the point that repair of PSII RC cannot keep up, reductions in microalgae productivity can occur (Murata et al., 2007; Vonshak et al., 2014).

Inoculation of-farm cultures at very dilute densities, changes in the culture color shortly after inoculation, and a general difficulty successfully transitioning the culture from the laboratory to the field, suggest that photodamage may be suppressing Anabaena sp. cultures. Color changes from blue-green to olive-green were observed in outdoor Anabaena sp. within 24h of inoculation at an average optical density of 0.026 at 750nm (OD_{750nm}). When OD_{750nm} reached 0.10 after

several days of growth, the color shifted back to blue-green. The 90% dilution of an outdoor culture during inoculation, or the transfer of an undiluted culture from the laboratory to the field increases the amount of light penetrating the culture. High light can trigger an increase in carotenoid synthesis by the cyanobacteria, a photoprotective response to deal with excess energy and ROS (Mulders et al., 2014; Nichols, 1973; Schagerl and Müller, 2006; Sedoud, 2014; Wolk, 1973). Coupling the high light intensity with an N-free growth medium could magnify photodamage to PSII RC. N-limited cyanobacteria can degrade phycobilisomes to provide additional N for photoacclimation under high irradiance (Baier et al., 2014; Grossman et al., 1993; Wood and Haselkorn, 1980). Degradation of the PSII RC alters the ratio of PSII to photosystem I (PSI) since PSI is less sensitive to light intensity. This shifts carotenoids:chl-a ratios from orange or β-carotene to the more yellow myxoxanthophylls or zeaxanthophylls (Takaichi, 2011).

Variable fluorescence can be used to measure changes in the quantum yield or efficiency of electron transport through PSII of cyanobacteria. Fluorescence will be greatest (Fmaximum or Fm) when PSII RC are closed, such as when photodamaged and requiring repair. Fluorescence will be lowest (Fminimum or Fo) when all PSII RC are open – such as night time or other dark adaptation - and damaged reaction centers have, for the most part, been repaired. The ratio of variable fluorescence to maximum fluorescence (Fv/Fm, where Fv = Fm- Fo) provides a measurement of electron transport through photosystem II at any point in time (Cosgrove and Borowitzka, 2011; Masojídek, et al., 2011). By tracking Fv/Fm throughout the day, for example, we could track whether increasing light intensity may be causing photodamage beyond the culture's ability to maintain repair (Vonshak et al., 2014).

Singh et al. (2013) investigated the effect of continuous high light intensities on photosynthetic performance of Anabaena variabilis. They demonstrated that low light ($12 Wm^{-2}$

or 60 µmol m^{-2} s^{-1}) acclimated cultures with an OD_{750nm} of 0.023 exposed to high irradiance (125 Wm^{-2} or 575 µmol m^{-2} s^{-1}) had an almost complete reduction in maximal photochemical efficiency of PSII (Fv/Fm) within 10 minutes of exposure to the high light when compared to control cultures exposed to the low, 60 µmol m^{-2} s^{-1} lighting. Recovery of Fv/Fm after 4h and 8h of continuous exposure to high light intensity reached only 3.42% and 10.50%, respectively, of the Fv/Fm values of the control cultures exposed to the low light intensity. On-farm Anabaena sp. cultures are exposed to PAR exceeding 575 µmol m^{-2} s^{-1} for 8h per day throughout the April to October growing season, though an increased light path (culture depth) may help mitigate some of the excess light.

Low on-farm inoculation rates, averaging OD_{750nm} 0.026 in past field studies, have reduced the time necessary to scale up from lab cultures to on-farm raceways. However, low density cultures coupled with outdoor PAR may be subject to more severe photoinhibition and photodamage. Even if a low-density Anabaena sp. culture survives high light intensity, diverting N from growth to photoprotective functions after inoculation could limit biomass gains early in the batch period. This could extend the time required to attain maximum productivity, or daily gains in biomass. This may ultimately reduce end of batch biomass and N concentrations.

Vonshak et al. (2014) illustrated this by maintaining the cyanobacterium Arthrospira platensis in 12 cm deep outdoor ponds at two culture densities known to achieve high productivity, OD_{560nm} 0.30 and OD_{560nm} 0.50. They observed that maximal photochemical efficiency of PSII (Fv/Fm) decreased more in the lower-density, 0.30 OD_{560nm} culture in response to increasing irradiance during the day. Recovery of Fv/Fm values during the afternoon hours was more complete in the high-density cultures. The reduced photoinhibition of the OD_{560nm} 0.50 culture led to 40% more productivity compared to the culture maintained at OD_{560nm} 0.30. With

Anabaena sp. under laboratory lighting, 140 µmol m^{-2} s^{-1} PAR is sufficient to realize three-fold higher yields compared with outdoor raceway production. Outdoor light intensities up to 2030 µmol m^{-2} s^{-1} PAR< coupled with low culture densities may subject the Anabaena sp. to photodamage, reducing productivity and 14-d biomass concentrations.

Light may also be limiting, even while photoinhibition is occurring (Richmond, 2004). Irradiance may be insufficient to maximize photosynthesis early or late in the day or on cloudy days. Under batch production, growth and increasing density also leads to self-shading of the culture. Production parameters may also contribute to light limitation. CSU's 2200L raceways have a surface area of about 9.26 m^2. Compared to 500 mL flasks under laboratory conditions, raceways have 865% of the areal volume (liters culture per square meter of culture exposed to light) and more than a threefold increase in depth, or light path. Under summer outdoor PAR averaging 1,100 µmol m^{-2} s^{-1} (2,030 µmol m^{-2} s^{-1} maximum) throughout the day, a raceway culture may be both self-shading and light limited, even at low densities. A comparison of 2L and 500mL flasks under constant PAR of 140 µmol m^{-2} s^{-1}, shows daily productivity declining in 2L volumes even before productivity is maximized in 500mL cultures (Figure 1). The 50% increase in light path length and 67% increase in the areal volume of the 2L flasks appears to limit light. It should also be noted that Anabaena sp. raceway cultures are grown under high tunnels. The plastic covering the high tunnels is rated at 91% PAR transmission (and measured at 84% due to deterioration), which may provide some photoprotection early during the batch period but may later inhibit light availability as the culture increases in density. It is likely, then, that both photoinhibition and self-shading are limiting daily productivity in raceways at any given point during the batch period.

A reduction in the maximum photosynthetic rates or maximum daily productivity would be acceptable if it ensures survival of on-farm cultures and leads to sustained increases in daily productivity compared with current field raceway productivity. Optimizing Anabaena sp. culture density to provide sufficient self-shading for photoprotection at inoculation offers a relatively easy method for offsetting high light intensities. This should lead to improved microalgal productivity, at least early in the batch period, assuming nutrients, temperature, and other production factors are not limiting (Borowitzka and Moheimani, 2013; Vonshak and Torzillo, 2004).

A cyanobacteria culture density that extinguishes or captures the maximum outdoor PAR intensity has been shown to reduce photoinhibition and associated photodamage (Richmond, 2004; Pruvost et al., 2015). Field measurements of Anabaena sp. culture OD_{750nm} and light capture provide a starting point for determining a photoprotective inoculation density (Figure 3).

At 23cm culture depths in the open raceways, an $OD_{750nm} > 0.15$ should capture all available light at peak intensity during a summer day. This may also bring field productivity closer in line with lab culture productivity much earlier in the batch period. Exploring inoculation densities down to the $0.026OD_{750nm}$ used in past field studies could identify the lowest density required to avoid photodamage and losses in productivity early in the batch. This would also minimize light limitation from self-shading as the culture density increases throughout the batch period.

CO_2

During photosynthesis, cyanobacteria synthesize carbohydrates from CO_2 to maintain and increase cyanobacterial biomass. Mass transfer of CO_2 between ambient air and the liquid culture is very poor. As light intensity increases the photosynthetic rate throughout the morning, dissolved inorganic carbon (DIC) in the culture is quickly depleted (Becker, 1994; Carvalho and Malcata,

2001; Raven and Beardall, 2014). As cyanobacteria fix carbon from HCO_3^-, OH^- is released into solution. This increases pH, which already ranges between 7.5 and 8.0 at sunrise in the unbuffered growth medium used for on-farm production. Between pH 7.5 and pH 9 HCO_3^- is the primary source of DIC in the solution. Cyanobacteria can source inorganic carbon (C_i) from the HCO_3^- available in solution through ATP-powered active transport of HCO_3^- and its carbon concentrating mechanism (CCM), but at an increased energy cost (Badger and Price, 2003; Giordano et al., 2005; Burnap et al., 2013; Raven et al., 2014). As C_i continues to be removed from solution into the early afternoon, on-farm production culture pH can exceed 10.0. Above pH 9, DIC species in solution shift from HCO_3^- to carbonate (CO_3^{2-}), which cannot be used for photosynthesis (Borowitzka and Moheimani, 2013). Upon C_i limitation, cell division rates downshift and biomass gains are reduced (Wang et al., 2004). Glycerate-3-phosphate, produced during the Calvin cycle, is required to synthesize the D1 protein. Under CO_2 limitation, D1 protein synthesis, necessary for repair of PSII, is also reduced (Takahashi and Murata, 2006). Photodamage recovery rates slow, and the duration of reduced maximum photochemical efficiency of PSII is increased.

Resolving a CO_2 deficiency could sustain more energy efficient rates of photosynthesis, improve daily productivity, and increase 14-d batch yields. When Moreno et al. (2003) and Chinnasamy et al. (2009) supplemented CO_2 to limit maximum pH between 8.0 to 9.0 in marine and freshwater Anabaena sp. cultures, they achieved biomass concentrations as much as 200 to 300% higher than the baseline 296 mg L^{-1} for CSU on-farm production.

Without CO_2 supplementation, the pH of Anabaena sp. cultures exposed to sunlight exceeds 9.0 from mid-morning until late afternoon. 500mL Anabaena sp. lab cultures achieving 1017 mg L^{-1} biomass after 14-d under 210 µmol m^{-2} s^{-1} PAR were not directly supplemented with CO_2. However, by adjusting the bubbling rates of the aquarium style aerators and silica air stones used

for mixing the flask cultures, pH could be limited to 8.0 during the 12h light period. Bubbling ambient air was sufficient to control pH in the lab at PAR intensities up to at least 210 µmol m^{-2} s^{-1} and in Erlenmeyer flask volumes up to 2L. If laboratory cultures were exposed to sunlight through a window in the laboratory (300 µmol m^{-2} s^{-1} to 500 µmol m^{-2} s^{-1} PAR), bubbling ambient air was insufficient to limit pH, which could exceed pH 10.0 in less than three hours. Under field production volumes and light intensity, paddlewheel mixing or submersible flow pumps and bubbling ambient air in Anabaena sp. cultures have been insufficient to limit pH and meet CO_2 requirements. Addition of CO_2 and limitation of pH to around 8.5 would keep DIC available during daily periods of high photosynthetic activity (Azov, 1982). If pH were to fall below 8.0, it could result in more volatile forms of CO_2, increasing the opportunity for atmospheric loss of CO_2 and decreasing the efficiency and cost effectiveness of supplementation (Putt et al., 2011). Limiting maximum pH somewhere between 8.0 and 9.0 should be enhance CO_2 availability, on-farm biomass, and total N yields of on-farm Anabaena sp.

Other Important Growth Parameters

After light and CO_2, temperature and dissolved oxygen (DO) levels are considered the next most critical control parameters for microalgal growth. Temperature would be difficult to cost effectively control in on-farm open raceways and thus will not be investigated. However, it is interesting to note that Chinnasamy et al. (2009) found that CO_2 supplementation reduced Anabaena fertilissima biomass loss under suboptimal high temperatures. DO has not exceeded 14mg L^{-1}, or 180% air saturation, through flow pump or paddlewheel agitation at the air-culture interface. Vonshak (1997) did not see negative effects on Arthrospira spirulina cultures until DO concentrations exceeded 20 mg L^{-1}. Monitoring DO concentrations will continue as increased

culture densities could lead to greater DO concentrations, however, it may not be otherwise necessary to address under outdoor raceway conditions.

This study sought to determine whether significant increases on-farm Anabaena sp. batch productivity and 14-d biomass and N concentrations could be achieved by improving management of light and CO_2. To achieve this, 1) outdoor cultures were inoculated at very low densities (0.026 OD_{750nm}) and two higher densities designed to capture maximum seasonal and annual PAR; and 2) outdoor cultures were supplemented with CO_2 in order to prevent pH from exceeding 8.9 pH. We hypothesized that:

- Cultures with inoculation densities sufficient to capture maximum available PAR will experience improved survival rates compared with lower inoculation densities.
- Inoculation density will be positively correlated with photosynthetic efficiency of photosystem II (Fv/Fm). The lowest cultures densities will experience the lowest Fv/Fm.
- Inoculation densities sufficient to capture maximum available PAR will experience greater productivity early in the batch period compared with lower inoculation densities.
- Lower inoculation densities will experience greater productivity later in the batch period, but photoinhibition early in the batch period will limit overall batch biomass and N gains compared with higher inoculation densities.
- Cultures supplemented with sufficient CO_2 to limit pH to 9.0 will see significant increases in 14-d biomass compared with non CO_2-supplemented cultures.
- CO_2 supplementation in cultures with a low, 0.026 OD_{750nm}, inoculation rate will increase survival rates under outdoor exposure compared to a culture without supplemental CO_2.

Methods

Experimental Design

The experiment was conducted outdoors at the Colorado State University (CSU) Horticultural Research Farm in Fort Collins, CO during October, 2015. The experiment was set up as a randomized compete block with three inoculation densities and two CO_2 supplementation treatments for a 3 x 2 factorial treatment design. An inoculation density and CO_2 treatment was randomly assigned to 24 columns so that each treatment combination was replicated four times. The experiment was conducted using 25 cm high, 10 cm diameter polyethylene terephthalate columns with a 2L volume.

Inoculation Densities

The high inoculation density treatment (HIGHOD) was an Anabaena sp. target OD_{750nm} sufficient to extinguish the peak annual projected PAR at a depth of 23cm. At the CSU Horticultural Research Farm in Fort Collins, this is estimated at 2030 µmol m^{-2} s^{-1}. The mid inoculation density treatment (MIDOD) was an Anabaena sp. target OD_{750nm} of half the HIGHOD density. Actual HIGHOD and MIDOD inoculation densities varied somewhat from the targets. Actual OD_{750nm} and the estimated PAR captured is included in Table 1. The low inoculation density (LOWOD) treatment was the average Anabaena sp. inoculation density of 0.026 OD_{750nm} utilized in previous CSU field experiments. 0.026 OD_{750nm} is estimated to extinguish 63% maximum annual PAR. On October 16, after the end of the 14-d batch experiment, new cultures were inoculated at the HIGHOD and MIDOD densities to measure Fv/Fm. This one-day experiment (Day 1a) adjusted the inoculation OD_{750nm} downward based on lower October 16 maximum expected PAR compared with that on October 1. PAR was

estimated for any given date using Apogee Instruments Clear Sky calculator (http://clearskycalculator.com/) for the field location (40.610917, -104.996766) with an elevation of 1523m. The maximum projected PAR for the October 1 inoculation date is projected at 1536 µmol m^{-2} s^{-1}.

Shade

Column diameters of 10cm were less than the 23cm included in light path or light capture calculations. Shading material, either a white semi-transparent label or 70% light transmission 1oz. spun bound polypropylene shade cloth (Atmore Industries, Inc., Atmore, AL USA) was wrapped around the sides of HIGHOD and MIDOD columns to offset some of the light penetration from the sides of the columns (Figure 2). Light transmission after shading was applied was measured using an Apogee MQ-306 Line Quantum with 6 Sensors Handheld Meter. LOWOD columns allowed 90% PAR to reach the culture through the sides of the cylindrical columns (10% Shade), the MIDOD columns allowed 75% PAR to reach the culture through the sides of the columns (25% Shade) and the HIGHOD columns allowed 50% PAR to reach the culture through the sides of the columns (50% Shade). 100% of the light (ignoring the angle of the fall sun) would still reach the surface of all cultures. If culture density increased over the 14-d batch period, the shading would not be correspondingly increased. For 14-d measurements such as biomass, total N or productivity, references to the three inoculation density treatments were appended to include the shading covariate.

CO_2 Supplementation

The pH of the CO_2 supplemented treatments was continuously monitored with PINPOINT pH Controllers (American Marine, Inc., Ridgefield, CT USA). When pH exceeded 8.9, a brass AC110V solenoid (Duda Energy, AL, USA. Model 2W-200-20N) allowed CO_2 to bubble through 1.3-cm x 2.5-cm aquarium air stones until pH was at a maximum of 8.7. The CO_2 control treatment received no supplemental CO_2.

Experimental Organism

A wild type heterocystous filamentous cyanobacteria, cultured from the benthic layer of Richard's Lake, Fort Collins, CO in 2010 with clones found to have a 99% similarity to Anabaena cylindrica was used for all experiments. Prior to scale up for experiments, the xenic Anabaena sp. culture was maintained phototrophically in Allen and Arnon (AA) N free medium in 500 mL glass Erlenmeyer flasks, mixed with ambient air and grown under 13W or 21W fluorescent bulbs providing 140 µmol m^{-2} s^{-1} PAR. To scale cultures up to field volumes, two week old cultures (0.60 OD_{750m} 1.00 OD_{750nm}) in 500 mL flasks were used to establish 8L to 12L volume cultures in 22.7 L glass carboys at a target density between 0.10 OD_{750nm} and 0.20 OD_{750nm}. Carboys were moved to a greenhouse where temperatures were maintained between 13ºC and 27ºC. Carboy scale cultures were mixed using one to two 2" air stones and aquarium air pumps. When culture densities reached between 0.30 OD_{750nm} and 0.40 OD_{750nm} in the greenhouse they were diluted back to between 0.15 OD_{750nm} and 0.20 OD_{750nm} by transferring to additional carboys. When greenhouse carboy culture volume and density was sufficient for column inoculation at the target densities, the culture was transferred to the field.

Culture Conditions

Columns were inoculated on the evening of September 30, 2015 with 1.8L of an Anabaena spp. culture in exponential growth phase. After culture densities were adjusted towards target inoculation rates just prior to sunset, the AA growth medium was added to the columns. Cultures were mixed by bubbling ambient air through aquarium air pumps fitted with 1-cm x 2.5-cm air stones.

Monitoring Growth and Culture Conditions

Evaporative loss was measured and replaced daily at 13:00 with tap water to bring cultures back to 23cm depth. Culture temperature, pH and dissolved oxygen (DO) were recorded daily prior to sampling for OD, between 14:00 and 15:30. Temperature, pH, and DO were measured using a Thermo Scientific™ Orion™ 5-Star Plus Dissolved Oxygen Portable Meter (Thermo Fisher Scientific Inc., Beverly, MA, USA). Solar radiation data for the nearest weather station (ARDEC) was downloaded from http://www.coagmet.colostate.edu/. Actual PAR was measured with an MQ-306 Line Quantum with 6 Sensors Handheld Meter (Apogee Instruments, Logan, UT).

4-mL of culture was sampled daily, in duplicate, between 15:00 and 15:45 on days 1, 2 and 5 (when Fv/Fm sampling was taking place) and between 14:30 and 15:30 on all other days, for measurement of OD_{750nm}. Two 50-mL samples for nutrient analysis were collected on days 0, 4, 7, 10, and 14. Volumes removed for Fv/Fm, biomass and nutrient samples were replaced with AA growth medium. OD_{750} of the culture was measured using the Hach DR3900 Spectrophotometer (Hach Company, Loveland, CO.). An OD_{750} biomass standard curve (Equation 1) was fit using linear regression of 50mL culture sampled from 2015 field and lab

cultures. The culture was filtered through 1.5 micron Whatman grade 934-AH, 47mm diameter glass microfiber binder free filters (GE Healthcare Life Sciences, USA), dried at 60^{o}C for 48 hours and weighed.

$$\text{Biomass (mg L}^{-1}) = 986.14*\text{OD 750nm} + 6.8241 \qquad \text{(Equation 1)}$$

Total N (total Kjeldahl N + NO_3-N + NO_2-N) was determined using Hach Simplified-TKN kits (TNT880, Hach Company, Loveland CO, USA.). Total N was measured from 50 mL samples drawn pre-dawn on day 1, and between 14:30 and 15:45 on days 4, 7, 10, and 14.

Health of the culture was also monitored microscopically by tracking Anabaena sp. filament length and condition and recording the presence of algae and potentially predatory protozoa. A Biological Microscope (model MT4310H, Meiji Techno Co., LTD., Japan) was used for microscopy. Relative cell counts of Anabaena sp. and frequently encountered algae such as Scenedesmus sp. were made by diluting samples so that no Anabaena sp. filaments crossed or obscured each other within a Sedgewick rafter. Cell counts were calculated by averaging counts from three 1-µL squares within a 1-mL Sedgewick rafter sample.

Photoinhibition, Photochemical Efficiency of Photosystem II

Photoinhibition in the Anabaena sp. culture was monitored by tracking maximum photochemical efficiency (quantum yield) of open PSII reaction centers or Fv/Fm throughout the day, per Baker et al. (2001) and Cosgrove and Borowitzka (2011). On days 1, 2, and 5, 35mL of Anabaena sp. culture was sampled five times – immediately pre-dawn (06:45), midmorning (10:00), peak irradiance (13:00) mid-afternoon (16:00) and post sunset (19:00). Pre-dawn

samples were also drawn on day 6. After completion of the 14-d batch period, healthy Anabaena sp. cultures (HIGHOD no CO_2, HIGHOD with CO_2, and MIDOD with CO_2) were combined. Columns and equipment were cleaned and HIGHOD and MIDOD inoculation density treatments, with and without CO_2 supplementation, were established from the retained culture for Fv/Fm measurements on October 16 (day 1a) (Table 1). No mid-morning (10:00) Fv/Fm measurements were made on day 1a.

All samples of Anabaena sp. culture drawn for Fv/Fm analysis were dark adapted in a cooler for 75 to 90 minutes. The length of the dark period was set to allow time for transport from the field to the laboratory. 5mL of culture was filtered through a Millipore AP2501300 13-mm glass fiber pre-filter (EMD Millipore, Darmstadt, Germany) to capture the Anabaena sp. biomass. Fv/Fm of the biomass was then measured using a dual pulse amplitude modulation (PAM) fluorometer (DUAL-PAM 100, Walz, USA).

Chloramphenicol Inhibition of D1 Protein Synthesis

Chloramphenicol (CAP) can suppress protein synthesis. When added to photosynthetic organisms such as Anabaena sp., CAP inhibits the synthesis of the D1 protein, which is necessary for repair of PSII RC (Murata et al., 2007). Comparison of Fv/Fm values of Anabaena sp. samples treated and not treated with CAP at the beginning of the dark adaptation period provides support for photoinhibition-related reductions in photochemical efficiency.

Day 1a, a second set of Fv/Fm samples were drawn pre-dawn and at peak irradiance and treated with 200µg CAP mL^{-1} culture, per Rehman et al. (2016). A third set of samples drawn at 13:00 were treated with 200µg mL^{-1} CAP 30 minutes into the dark period, 45 to 60 minutes before Fv/Fm measurements.

Statistics

Statistical analysis was performed using SAS 9.4 University Edition (SAS Institute Inc., Cary, NC). Significance was defined as $\alpha = 0.05$ for all statistical analyses. Analysis of mean biomass and total N concentrations between the treatment combinations was performed using two-way ANOVA (proc mixed). Repeated measures analysis (proc mixed) was used to evaluate significant differences in Fv/Fm values among treatments for each of the four or five measurements taken throughout the day. Repeated measures analysis was also performed on productivity across days 7 through 14. Multiple linear regression, with top ten model selection based on Mallow's C(p) (proc reg), was used to identify treatment variables and covariates significant in influencing Fv/Fm or daily productivity values.

Results

Photochemical Efficiency of Photosystem II

Day 1 Fv/Fm values immediately prior to sunrise (07:00) were significantly higher in the HIGHOD treatments with and without supplemental CO_2 and MIDOD with CO_2 than both LOWOD treatments (Figure 4). CO_2 supplementation did not result in significant differences in Fv/Fm within each inoculation density at any point during day 1. By 10:15, the Fv/Fm of the HIGHOD treatments were significantly higher than the MIDOD and LOWOD treatments. PAR peaked at 1180 µmol m^{-2} s^{-1}, rather than the 1536 µmol m^{-2} s^{-1} projected for the date under clear, sunny conditions. PAR decreased after the 10:15 sampling due to increasing cloud cover. By 13:00, Fv/Fm values were significantly different between all inoculation density treatments. The three inoculation density treatments remained significantly different for the remainder of the day. LOWOD Fv/Fm values did not show recovery as PAR decreased to 925 µmol m^{-2} s^{-1} at 13:00,

60% of projected peak PAR for the day. Fv/Fm recovery was evident in LOWOD treatments by 16:00, when PAR had declined to 210 µmol m^{-2} s^{-1}.

Skies were overcast throughout day 2. Peak PAR was measured at 303µmol m^{-2} s^{-1}, 20% of the projected peak PAR. The optical density for any treatment at 13:00 on day 2 was not significantly different when compared to its inoculation density, except for HIGHOD no CO_2. HIGHOD no CO_2 had decreased significantly compared to its day 0 OD. Sampling volumes of approximately 300mL of the 1800mL total column volumes over a 24 hour period likely contributed to the dilution of any biomass gains in the cultures. Fv/Fm was significantly higher for both HIGHOD treatments compared to both LOWOD treatments until the 19:30 (sunset) measurement (Figure 5). HIGHOD with CO_2 Fv/Fm was also significantly higher than the MIDOD no CO_2 at 10:00 and 13:00 and 16:05. Fv/Fm of the HIGHOD with CO_2 treatment was significantly higher at 19:05 than at 6:45. MIDOD with CO_2 Fv/Fm was significantly higher than both LOWOD treatments at 6:45 (sunrise), 10:00, and 13:00, while MIDOD no CO_2 Fv/Fm was significantly higher than both LOWOD treatments only at10:00 and 13:00.
Fv/Fm of LOWOD no CO_2 decreased significantly between the 6:45 and the 10:00 measurement, at a PAR of 157 µmol m^{-2} s^{-1}. However, Fv/Fv of both LOWOD treatments significantly increased between 13:00 and sunset as PAR decreased from 303 µmol m^{-2} s^{-1}.

Day 5 was the first day of the batch with a forecast for clear skies, so Fv/Fm measurements were taken. Day 5 was also the first day that OD_{750nm} was significantly greater than inoculation densities for all HIGHOD and MIDOD treatments (Table 2). LOWOD treatments were not significantly different from day 0. Mean OD_{750nm} for each of the inoculation density treatments were still significantly different from each other on both days 4 and 5. There was no significant difference in OD_{750nm} between CO_2 and non CO_2 treatments within each inoculation density

treatment on day 5. Shading wrapped around the sides of the MIDOD and HIGHOD columns was not adjusted to match increases in culture density. ANCOVA analysis ($p < 0.05$) showed no significant interaction between the shading on the column sides and OD_{750nm} with respect to Fv/Fm values on day 5.

LOWOD columns had Fv/Fm values significantly higher than MIDOD columns throughout the day (Figure 6). LOWOD Fv/Fm values were also significantly higher than both the HIGHOD and MIDOD treatments at sunset. The MIDOD, no CO_2 treatment was significantly lower than the HIGHOD treatments at 10:00, and the HIGHOD, no CO_2 treatment at 16:10.

Day 5 LOWOD Fv/Fm values increased after 10:00 a.m., while MIDOD and HIGHOD cultures did not begin increasing until after 13:00. LOWOD Fv/Fm values were higher than other treatments throughout the afternoon, and significantly higher by sunset, with mean values of 0.486 ± .069. Fv/Fm values for HIGHOD treatments were 0.310 ± 0.035. Fv/Fm values for MIDOD treatments were 0.286 ± 0.45. Morning cloud cover reduced solar irradiance to about 66% of the expected intensity through 10:00. Skies were mostly sunny clear by 11:00.

Microscopic examination of LOWOD columns on day 5 appeared to show the culture shifting from Anabaena sp. to a small single cell alga, about ½ the diameter of an Anabaena sp. cell, some Scenedesmus sp. and protozoa resembling Ochromonas sp. Cell counts on day 7 showed 9.9×10^4 mL^{-1} Anabaena sp. cells, concentrated in clumps settled at the column bottoms compared with 3.59×10^5 mL^{-1} single cell algae. In comparison, cell counts on day 8 in MIDOD and HIGHOD columns detected 1.54×10^7 mL^{-1} to 2.49×10^7 mL^{-1} Anabaena sp. cells and as many as 60 mL^{-1} of the small single cell algae and 40 mL^{-1} of a Scenedesmus sp.

Day 1a (October 16)

Four Fv/Fm measurements were made throughout the day. Fv/Fm values were significantly higher in the HIGHOD columns at 6:30 (prior to sunrise) (Figure 7). Culture temperatures measured in four MIDOD columns at 6:30 averaged 0.0°C, while four HIGHOD columns averaged 0.1°C. Ice had formed around the surface edge of all MIDOD columns. No ice had formed on any HIGHOD columns. Fv/Fm values for HIGHOD with CO_2 remained significantly higher than MIDOD treatments throughout the day. Fv/Fm for the HIGHOD with CO_2 treatment was significantly higher than HIGHOD no CO_2 at 16:25 and 19:20. HIGHOD no CO_2 was significantly higher than the MIDOD treatments at 16:25 and 19:20. There was no significant difference in Fv/Fm values between MIDOD with CO_2 and MIDOD no CO_2 treatments at any of the four measurements.

Correlation between Inoculation Density and Fv/Fm values.

There was a moderate to strong correlation when inoculation density and Fv/Fm for a sampling period was compared across Fv/Fm sampling days (Table 3). LOWOD data was included on days 1 and 2. Pre-sunrise (06:00) Fv/Fm values were recorded on day 6. Fv/Fm was not measured mid-morning on day 1a.

Chloramphenicol Treatments

On day 1a (October 16) HIGHOD treatments sampled at 13:00 had significantly lower Fv/Fm values when treated with 200µg mL^{-1} chloramphenicol (CAP) at the beginning of the dark adaptation period compared to those not treated with CAP (Table 4). HIGHOD with CO_2 treatments with CAP added 30 minutes into the dark period had significantly lower Fv/Fm values

compared to samples not treated with CAP. Fv/Fm values were not significantly different between CO_2 supplemented and non-CO_2 supplemented cultures. CAP treated MIDOD Fv/Fm values were not significantly lower than non-CAP treated samples.

Multiple Regression, Photochemical Efficiency of Photosystem II

Multiple regression with model selection to explain Fv/Fm on days 1, 2, 5 and 1a included the treatment variables inoculation density (categorical), CO_2 (categorical) and the potential covariates OD_{750nm} (a continuous variable, more accurate than inoculation density) and PAR at sampling time. OD_{750nm} data was available for the 13:00 Fv/Fm measurements. Multiple regression with model selection was run on this second data set, using the inoculation density and CO_2 treatment variables, and the potential covariates column shading, pH at 13:00, OD_{750nm} at 13:00, cumulative solar irradiance sunrise to 14:00, and evaporation. Day 5 LOWOD treatment was excluded from the data sets due to algal takeover of the culture. Day 1a had no LOWOD. The lowest Mallow's C(p) for each multiple regression are presented in Table 5. All variables in the resulting models were significant at $p < 0.05$.

Biomass and Nitrogen

The shading on the sides of the columns, designed for the inoculation density, did not change as culture densities increased. Therefore, any analysis included OD_{750nm} as a potential covariate in addition to the inoculation density. The shading value is included in references to inoculation density, since light availability, rather than inoculation density may be more relevant. Significant differences in day 14 biomass were found between the MIDOD with CO_2 (25% shade) and all other treatment combinations except for HGHOD with CO_2 (50% shade) (Figure 8). LOWOD

(10% shade) showed no Anabaena sp. growth during the experiment. Batch biomass gain showed similar significance (Figure 8). Total 14-d N and N gain in the MIDOD with CO_2 (25% shade) treatment were significantly higher (Figure 9). HIGHOD with CO_2 (50% shade) was significantly higher than MIDOD no CO_2 (25% shade). LOWOD treatment total N was significantly lower than the MIDOD and HIGHOD treatments. Total N declined in the LOWOD treatments over the batch period. When averaged over the HIGHOD (50% shade) and MIDOD (25% shade) treatments, CO_2 supplementation resulted in significantly higher mean 14-d biomass, biomass gains, total N and N gains (Table 6).

Productivity

Productivity, the 24-hr change in Anabaena sp. biomass (from 14:00 to 14:00), was calculated for days 7 through 14 in HIGHOD (50% shade) and MIDOD (25% shade) columns. Sampling volumes were nearly 300 mL or 15% of the total volume of the columns on days which included Fv/Fm measurements. Column volumes were brought back up to 1.8L prior to mid-day (14:00) measurement of pH, DO, OD and temperature. This diluted cyanobacteria densities, offsetting biomass gains. Therefore, days 1 through 6, in which the 24-hr period included Fv/Fm sampling were excluded from statistical analysis of productivity. LOWOD treatments exhibited no biomass gain over the 14-d batch period and were also excluded from productivity analysis.

Repeated measures analysis showed significantly higher productivity on day 9 in MIDOD with CO_2 (25% Shade) compared with MIDOD no CO_2 (25% Shade) or HIGHOD no CO_2 (50% Shade) but not compared with HIGHOD with CO_2 (Figure 10). On day 10, productivity in MIDOD with CO_2 (25% Shade) was significantly higher than only MIDOD no CO_2 (25% Shade). On day 14, MIDOD no CO_2 (25% Shade) had significantly lower productivity than all

other treatments. On day 14, the Anabaena sp. cultures in two MIDOD no CO_2 (25% Shade) columns experienced a 35% decline in OD_{750nm} compared to day 13. Productivity for days 7, 8, 11, 12, and 13 was not significantly different among treatment groups.

Multiple regression with model selection based on Mallow's C(p) was performed for days 7 to 14 productivity with potential predictor variables including: CO_2 and inoculation OD (or Shade) treatments, 13:00 pH, DO, and temperature, OD_{750nm} at the beginning of the 24h period prior to sampling, and the 24hr total irradiance recorded by the closest CoAgMet weather station. LOWOD cultures did not grow during the batch and were omitted from the regression. Multiple regression with model selection based on Mallow's C(p) was performed for days with significant differences in productivity, day 9 and day 10. Day 14 was excluded, as the significant difference was likely due to culture collapse in the culture of two MIDOD no CO_2 (25% shade) columns LOWOD was excluded from the regression. Resulting models are included in Table 7.

Temperature

Mean temperatures recorded during daily sampling for OD_{750nm} (between 14:30 and 15:30) were compared using repeated measures analysis for days 7 through day 14, the days when culture was no longer being sampled for Fv/Fm measurements (Figure 11). Both MIDOD treatments had significantly higher temperatures than both HIGHOD treatments on days 7, 9, 10, 11, 12, and 13.

Discussion

Inoculation Density and Biomass Yields

We hypothesized that utilizing inoculation densities sufficient to capture maximum available PAR would improve Anabaena sp. culture survival rates compared with lower inoculation densities. All HIGHOD and MIDOD columns inoculated at densities estimated to capture at least 100% of the projected peak PAR for October 1 experienced positive biomass gain over the 14-d batch period. Microscopy identified Anabaena sp. filaments within clumps of biofilm and detritus at the bottom of LOWOD columns on day 14, however, there was no net biomass gain during the batch period.

Inoculation Density and Fv/Fm

We also hypothesized that the inoculation density would be positively correlated with Fv/Fm. Fv/Fm and inoculation density were positively correlated over the five days that Fv/Fm was measured. Fv/Fm was significantly higher in the HIGHOD treatments compared to the LOWOD treatments on days 1 and 2. Fv/Fm was significantly higher in the MIDOD treatments compared to the LOWOD treatments after 10:00 on day 1. MIDOD and LOWOD Fv/Fm declined to similar levels on day 1 as PAR reached 1180 µmol m^{-2} s^{-1}. However, cloud cover decreased PAR to 925 µmol m^{-2} s^{-1} by 13:00 and MIDOD Fv/Fm began to increase, while LOWOD Fv/Fm did not begin to recover until light intensity declined to between 210 µmol m^{-2} s^{-1} and 925 µmol m^{-2} s^{-1}. Under overcast conditions on day 2, LOWOD columns again experienced a significant decline in Fv/Fm between 06:45 and 10:00, when PAR was at only 157 µmol m^{-2} s^{-1}, 20% of the projected maximum for a clear sunny day. OD_{750nm} of the LOWOD cultures did not significantly change between day 1 and day 2. Low light intensity on day 2 had no significant negative impact

on MIDOD and HIGHOD Fv/Fm, and may have facilitated recovery of Fv/Fm. Depression of Fv/Fm in the 0.026 OD_{750nm} LOWOD cultures is likely a results of greater photoinhibition (Vonshak et al. 1994, Masojídek, et al., 2011).

The LOWOD cultures showed additional signs of light-related stress. Cyanobacterial biomass in the LOWOD cultures sank to the bottom of the columns during day 1 and remained on the column bottoms throughout the batch period, despite mixing. This was likely an attempt to avoid high light intensities (Bebout and Garci-Pichel, 1995; Quesada and Vincent, 1997; Moon et al., 2012). Day 1 average solar irradiance was 21.8 kWm^{-2} .Partial or full cloud cover for days 2 through 6 limited solar irradiance to between 5 and 14.8 kWm^{-2}. Either day 1 photodamage to Anabaena sp. in LOWOD cultures was too severe, or the subsequent reductions in solar irradiance were insufficient to allow Anabaena sp. to recover biomass. The transition to algae around day 5 in the LOWOD cultures was also short lived, lasting until day 8 when clear skies returned. Average solar irradiance ranged from 18.3 kWm^{-2} to 23.8 kWm^{-2} from day 7 through 14. The algae, while better able to acclimate to higher light intensity than Anabaena sp., was either limited by increased photoinhibition beginning day 7 or by the net loss of N. With no Anabaena sp. biomass increase, no additional N was fixed in the columns, limiting resources for cell growth.

CO_2 Supplementation and Biomass Yields

CO_2 supplementation of the LOWOD treatment was not sufficient to increase Anabaena sp. biomass beyond the inoculation density. These results fail to support the hypothesis that CO_2 supplementation at the 0.026 OD_{750nm} inoculation rate would improve survival and productivity compared with the same culture density without supplemental CO_2. CO_2 supplementation did

result in significant improvements in 14-d biomass and total N across the HIGHOD and MIDOD treatments. However, when broken out by treatment combination, only the MIDOD (25% Shade) with CO_2 treatment 14-d biomass and total N were significantly higher than HIGHOD no CO_2 and MIDOD no CO_2 treatments. MIDOD densities were significantly lower than HIGHOD densities on day 5, however, the MIDOD with CO_2 treatment accumulated significantly higher biomass than non CO_2 supplemented treatments by day 10. Factoring in the Fv/Fm sampling volumes and cloud cover through day 6, significant improvements in biomass accumulation in CO_2 supplemented cultures could be expected as early as day 4 of the batch period.

CO_2, Light, and Fv/Fm

Multiple regression to explain Fv/Fm values using CO_2 and light availability variables determined that CO_2 supplementation, inoculation density and light intensity were significant and accounted for 0.69 of the variability in Fv/Fm results. When limited to mid-day (13:00) data, but with additional variables related to CO_2 and light availability, the model found that CO_2, inoculation density, OD_{750nm}, cumulative solar irradiance for the day, and 24h evaporation could account for 0.9258 of the variation in Fv/Fm. Both models indicate that light intensity and CO_2 availability are influencing photochemical efficiency of PSII in the outdoor Anabaena sp. cultures.

CO_2 and Culture Health

Two MIDOD no CO_2 columns in the current study experienced a 35% decline in OD_{750nm} between day 13 and 14. Declines of this magnitude are indicative of a complete collapse of the Anabaena sp. culture. On days 7 through 13, mid-day temperatures in the MIDOD cultures were

significantly higher than the HIGHOD cultures, likely due to the increased shading on the HIGHOD column sides. Mean temperature for MIDOD exceeded 30°C by14:30 on day 10 and day 13. Chinnasamy et al. (2009) reported that CO_2 mitigated some of the negative effect of high temperature on the productivity and chlorophyll-a content of Anabaena fetilissima. The supplemental CO_2 compensates for the reduced efficiency of Ribulose-1,5-bisphosphate carboxylase/oxygenase at high temperatures (Badger et al., 2005). While the maximum pH of CO_2 supplemented cultures was limited to 8.9, the pH of the MIDOD no CO_2 and HIGHOD no CO_2 cultures averaged 10.67 and 10.64, respectively, at the daily measurement time from day 7 to 14. This degree of CO_2 limitation would require additional energy to obtain C_i via the carbon concentrating mechanism, diverting metabolic resources for growth (Raven et al., 2014). Furthermore, limited CO_2 and N in the medium may have encouraged predation of cyanobacteria by mixotrophic protozoa, such as the Ochromonas sp. observed in the culture. With sufficient C, from supplemental CO_2, Ochromonas sp. could sustain growth through photosynthesis. Ochromonas sp. are also regularly observed feeding on biofilms or exopolysaccharides in both outdoor and laboratory xenic Anabaena sp. cultures. If C_i becomes limited, and Anabaena sp. exopolysaccharide exudates decrease, Anabaena sp. cells may become a more attractive food source for the phagotrophic protozoa (Porter, 1988; Singh et al., 2016; Vincenzini et al., 1990; Wilken et al., 2014; Zhang et al., 2016).

CO_2 supplementation may have played role in recovery of Fv/Fm at the higher densities. On day 2, when overcast skies limited PAR to 303 µmol $m^{-2}s^{-1}$, Fv/Fm increased significantly between sunrise (06:45) and sunset (19:30) in HIGHOD with CO_2 treatment. Day 1a also saw faster recovery of Fv/Fm after 13:00 in the HIGHOD with CO_2 treatment. Significant differences between HIGHOD no CO_2 and both MIDOD treatments after 13:00 suggest that CO_2 is more

effective at Fv/Fm recovery at higher densities, presumably when repair rates are already better able to keep up with photodamage.

Day 1a CAP treatments of mid-day Fv/Fm samples prevented PSII repair during the dark adaptation period. There was no significant difference in Fv/Fm between the HIGHOD, MIDOD or CO_2 treatment combinations when treated with CAP. The difference in Fv/Fm between HIGHOD with CO_2 treatment and MIDOD treatments arose during dark adaptation. Fv/Fm of HIGHOD with CO_2 was not significantly different compared to HIGHOD without CO_2 during the dark adaptation period. The results tend to agree with Takahashi and Murata (2006), who saw reduced rates of D1 synthesis under CO_2 limitation.

The apparent lack of recovery during the dark adaptation period by either MIDOD treatment on day 1a, as well as the $R^2 = 0.69$ resulting from multiple regression of Fv/Fm with only light and CO_2 related variables may indicate other variables at play in photoinhibition. Repair of PSII in the MIDOD treatments may be inhibited by other culture conditions, such as temperature or salt stress, which in turn could contribute to depression of Fv/Fm values (Murata et al., 2007). Salt concentrations in freshwater Anabaena sp. cultures using an N-free AA growth medium are between 3 to 4 mM. Salt stress studies on Anabaena sp. utilize concentrations between 62mM and 150mM, so salt stress seems unlikely (Yoshida et al., 2004; Srivastava et al., 2010; Rai et al., 2014). There were significant differences in temperatures recorded at 13:00 among most treatments on days 1 to 6 and day 1a. Temperature data was not collected at each Fv/Fm measurement point, except for day 1a. However, on days when Fv/FM measurements were made, temperatures had not exceeded 28^{o}C by the 13:00 measurement. It is possible that culture temperatures increased for another hour or two after this measurement, but it is not clear if this might exhibit negative effects on growth. Temperature measured at 13:00 was also not selected

as a significant variable from multiple regression with model selection of Fv/Fm at 13:00 against all available culture conditions. More data is needed to determine whether significant differences in Anabaena sp. culture temperatures negatively influence the ability to recover Fv/Fm.

Low temperatures may be more likely stressors during early and late season production. Overnight temperatures regularly fall below 10^{o}C at the beginning and end of the outdoor cyanobacteria production season. Pre-sunrise air temperatures on day 1 and day 1a (October 1 and 16) were 9.9^{o}C and 0.7^{o}C, respectively. Culture temperatures were not measured at that time on day 1, but averaged 0.1^{o}C on day 1a. Allakhverdiev and Murata (2004), saw D1 and other protein synthesis rates decrease in Synechocystis sp. as temperature declined from 34^{o}C, with no synthesis occurring at 10^{o}C. They also found that light availability determined rates of protein synthesis. When light decreased to 70 µmol m^{-2} s^{1} PAR, protein synthesis was 50% of maximum rates. In the current study, LOWOD Fv/Fm values were significantly lower than HIGHOD at sunset on days 1, 2 and 5, and MIDOD was significantly lower than HIGOD cultures on day 1a. Rates of photoinhibition and required PSII repairs will be higher and extend later into the afternoon for lower density cultures. Repeated measures analysis of Fv/Fm between day 1 post-sunset and day 2 pre-sunrise, and day 5 post-sunset and day 6 pre-sunrise, showed no significant changes in overnight Fv/Fm for any inoculation density treatment. If repairs have not been made by sunset, the overnight dark period and low temperatures could delay repair until shortly before or after sunrise (Saha et al., 2016).

Fv/Fm and Carotenoids

Cyanobacteria pigment production could also influence Fv/Fm. Orange carotenoid proteins (OCP) can absorb and dissipate excess energy received by the phycobilisomes in cyanobacteria

(Bissati et al., 2000; Wilson et al., 2006; Muramatsu and Hihara, 2011). Under high light conditions, OCP production is increased in cyanobacteria (Hihara et al., 2001; Huang et al., 2002; Tu et al., 2004, Singh et al., 2008). On a clear, sunny day, high light conditions will be reached more quickly in the more dilute, LOWOD or MIDOD cultures than in the HIGHOD cultures, and carotenoid accumulation could be greater (Schagerl and Müller, 2006). Under PAM fluorescence of cyanobacteria, orange carotenoid proteins continue to act as electron receptors (Acuña et al., 2015). This would reduce maximal fluorescence (Fm) measurements, depressing Fv/Fm values.

Day 1 pre-sunrise Fv/Fm values were not significantly different between HIGHOD and MIDOD cultures. Day 1a pre-sunrise Fv/Fm, however, was significantly different between the two inoculation densities. Dilution to inoculation densities for day 1 cultures occurred between 16:30 and 20:30 of the previous day. Day 1a dilution from 0.60 OD_{750nm} to target inoculation densities occurred over two days, with an initial dilution to 0.20 OD_{750nm} and 0.10 OD_{750nm} for HIGHOD and MIDOD treatments, respectively, occurring two evenings prior to Fv/Fm measurements. A smaller adjustment to the target inoculation density was made the evening before. The day 1 MIDOD would have had carotenoid concentrations similar to the HIGHOD treatments. The day 1a MIDOD cultures, on the other hand, would have had a day to acclimate to increased light availability and accumulate carotenoids. Day 2, 5, 6 and 1a differences in Fv/Fm may be partially attributable, then, to differences in carotenoid accumulation rather than photoinhibition.

Other cyanobacterial photosynthetic processes can also influence PAM Fv/Fm measurements. When compared with algae, cyanobacteria PSI to PSII ratios are higher and the primary quinone of PSII does not become fully oxidized during dark adaptation (Schuurmans et al., 2014). This

increases Fo after dark adaptation when compared with algae (Shuurmans et al., 2015). 60% or more of Fo may be attributable to PSI and phycobilisomes (Ogawa and Sonoike, 2016). These increases to Fo coupled with the reductions in Fm from electron absorption by carotenoids decrease the overall PAM estimations of photochemical efficiency of PSII. PAM Fv/Fm values in healthy eukaryotic algae might be expected to reach 0.80, while values in cyanobacteria would be closer to 0.60 (Campbell et al., 1998; Masojídek et al., 2011; Schuurmans et al., 2015). The increase in day 5 LOWOD treatment Fv/Fm values beyond peak Fv/Fm value of 0.36 observed in heathy Anabaena sp. MIDOD or HIGHOD cultures throughout the experiment, may be attributable to the observed shift from Anabaena sp. to algae. Comparison across multiple days (or even between morning and afternoon) of Fv/Fm between cultures densities near or below that required to capture maximum PAR would also need to estimate or account for differences in OCP.

Daily Productivity

Productivity was significantly different among treatments for only two of the eight days after Fv/Fm sampling. Attempts to explain productivity using multiple regression with model selection resulted in a weak model that included only the density of the culture and the pH at sampling time (14:30 to 15:30) as significant variables. Multiple regression to explain productivity of MIDOD and HIGHOD treatments on days 9 and 10, when significant differences were evident, identified a stronger model ($R^2 = 0.40$) with shading of the column sides and pH as significant variables ($p < 0.05$). The MIDOD with CO_2 treatment may have reached the optimal cell density for productivity at a 23cm depth on days 9 and 10, when OD_{750nm} was between 0.28 and 0.37. From the productivity multiple regression, neither light nor CO_2 appear to be

significant variables, so other culture conditions may be limiting productivity (Richmond, 2004). While early afternoon temperature, DO and pH data is available, more measurements throughout the day may be required to definitively include or exclude these variables from productivity models. Biological factors in the xenic culture could also be at play. Predation by large protozoa or competition with protozoa and bacteria for nutrients could constrain biomass gain by the cyanobacteria.

High sampling volume prevented accurate determination of daily growth for the first six days. Further work needs to be done to establish whether the differences in Fv/Fm values between the HIGHOD and MIDOD inoculation densities translate to significant differences in Anabaena sp. productivity as Vonshak et al. (2014) saw with A. spirulina. Changes in Fv/Fm or photoinhibition don't necessarily lead to loss of photosynthetic productivity, since light intensity and availability can make up for loss of PSII efficiency (Cosgrove and Borowitzka, 2011). Loss of daily productivity related to photoinhibition will be more important under semi-continuous or continuous harvesting. During the current 14-d batch study, the significant difference in culture density between the HIGHOD and MIDOD treatments disappeared within a day or two. MIDOD with CO_2 was significantly higher than the non CO_2 treatments by day 10. While there was never any significant separation in biomass between the HIGHOD with CO_2 and MIDOD with CO_2 treatments, there was significantly higher total N in the MIDOD with CO_2 treatment by day 14. This agrees with Oh et al. (1991) who showed that both short- and long-term light availability influenced N-fixation in Anabaena sp.

Under batch production at 23cm depths, differences in light availability related to culture density or shading are soon offset by biomass increases under similar light conditions, particularly when the culture density is sufficient to capture maximum PAR. Even at the

minimum photoprotected density with supplemental CO_2, productivity is likely to be reduced by the end of the first week due to self-shading. The onset of self-shading may be earlier and more limiting under raceway conditions. Outdoor columns achieved two- to three- fold higher daily productivity than typical summer production raceways (see Appendix I for raceway production data). Linear regression of OD_{750nm} and daily productivity illustrates the potential for the CO_2 supplementation and improved light availability to close the gap between field and laboratory 14-d batch (Figure 12). Actual average hourly solar irradiance (CoAgMet ARDEC station data) for late July 2014, reduced to 84% to account for reduced light transmission under high tunnels, was 22.7 kWm^{-2} over 15h, while the average from October 7 to 14, 2015, in the current experiment, was 18.6 kWm^{-2} over 11h. Supplementation of CO_2 to limit pH to 8.9 should improve DIC availability and bridge some of the productivity gaps between field columns and on-farm raceways. However, the culture surface area (exposed to light) to volume ratio of 1.8L columns is .0249 $m^2 L^{-1}$. The same ratio in 2200L on farm raceways with 9.26 m^2 surface area is 0.0042 $m^2 L^{-1}$, 17% that of the columns. Despite the questions remaining on productivity under field conditions, improving CO_2 and light availability should help close the gap between field and laboratory 14-d batch biomass, even at the end of the growing season.

Conclusions

Anabaena sp. cultures at 0.026 OD_{750nm} in an N-free growth medium are not likely to survive one day outdoor exposure to sunlight, even at half the maximum PAR expected during the summer growing seasons. CO_2 does not improve survival of such a low density culture. Photodamage, or the inability of the low density Anabaena sp. culture to repair the damage high

light intensities is likely responsible for the failure to thrive. The damage is extensive enough that the culture is unable to recover during subsequent days of cloudy weather and reduced PAR.

Culture density is the primary factor in Anabaena sp. photochemical efficiency of PSII Fv/Fm, with higher cultures densities capturing more available energy and reducing the opportunity for photodamage. CO_2 supplementation was more effective in facilitating repair of PSII and restoring photosynthetic efficiency in the highest density cultures. However, it also appeared to assist Anabaena sp. maintain resilience under temperature extremes and xenic conditions at the tail end of the production season. CO_2 supplementation should also be able to mitigate negative effects of summer production season stressors such as high daytime temps, and higher, more sustained maximal light intensity.

On-farm Anabaena sp. inoculation densities for batch production should capture the maximum expected PAR on the inoculation date to buffer outdoor production stresses and ensure both survival and economical productivity. Pruvost et al. (2012) saw no significant gains in annual A. spirulina productivity when adjusting harvest densities for seasonal changes in light availability. While a static inoculation density based on the peak estimated annual PAR is more appropriate to semi-continuous production, establishing a single inoculation rate for batch production may improve the ease of on-farm management.

Additional data from similar studies could tighten field production parameters including:

- More complete data, including OD_{750nm}, dissolved oxygen, and temperature during all Fv/Fm sampling times to strengthen explanatory models of Fv/Fm and productivity.
- Accounting for differences in orange carotenoid protein and phycobilisome concentrations when comparing Fv/Fm measurements from different treatment densities.

The results of this study suggest that it should be possible to significantly improve outdoor Anabaena sp. productivity and yields under batch production through:

- Inoculation at the minimum density that will capture maximum PAR on day 1.
- Supplementation of CO_2 to limit maximum pH between 8.0 and 8.75.
- Placement of raceways outside of high tunnels.
- Minimization of evaporation effects on culture density by replenishing near sunrise rather than mid-day, or multiple times over the day.
- Semi-continuous production at the optimal cell density (OD_{750nm} 0.30 to 0.40) to sustain higher productivity over 14-d and increase yields compared with batch production.
- Five- to seven- day batch production periods with inoculation densities that capture maximum PAR and harvest at culture densities of 0.45 OD_{750nm} to offset low productivity towards the end of 14-d batch production.

Tables

Table 1 Fall 2015 Inoculation Densities and estimated PAR capture.

Inoculation Density Treatment and Date	Culture Depth (cm)	Optical Density (750nm) (Target)	Optical Density (750nm) (Actual)	PAR Captured (µmol m^{-2} s^{-1})	% Maximum Annual PAR Captured	Maximum PAR Inoculation date (µmol m^{-2} s^{-1})
HIGH Oct 1	23	0.174	0.174	2030	100	1536
MID Oct 1	23	0.087	0.092	1791	88	1536
LOW Oct 1	23	0.026	0.027	1286	63	1536
HIGH Oct 16	23	0.120	0.121	1906	94	1375
MIDOD Oct 16	23	0.075	0.081	1711	84	1375

Table 2. Comparison of days 0, 2 and 5 optical density (OD) by treatment. HIGHOD is OD at 750nm sufficient to capture 2030µmol m^{-2} s^{-1} PAR at 23cm depth on day 0. MIDOD is an OD sufficient to capture 1791µmol m^{-2} s^{-1} PAR. LOWOD is the average field inoculation density which captures 1286µmol m^{-2} s^{-1} PAR. "+CO2" cultures are CO_2 supplemented to maintain maximum pH 8.9. Different letters within a treatment have significantly different OD_{750nm} ($p < 0.05$)

Treatment	n	Day 0 OD_{750nm}	Day 2 OD_{750nm} (13:00)	Day 4 OD_{750nm} (13:00)	Day 5 OD_{750nm} (13:00)
LOWOD	4	0.026 ± 0.006	0.007 ± 0.005	0.018 ± 0.009	0.021 ± 0.010
LOWOD+CO_2	4	0.028 ± 0.002a	0.008 ± 0.005a	0.013 ±0.007b	0.018 ± 0.010a
MIDOD	4	0.086 ± 0.002b	0.070 ± 0.035c	0.110 ± 0.018b	0.162 ± 0.007a
MIDOD+CO_2	4	0.097 ± 0.009c	0.070 ± 0.024c	0.108 ± 0.010b	0.152 ± 0.020a
HIGHOD	4	0.174 ± 0.006b	0.130 ± 0.060c	0.182 ± 0.005b	0.234 ± 0.020a
HIGHOD+CO_2	4	0.173 ± 0.003c	0.152 ± 0.019c	0.190 ± 0.015b	0.233 ± 0.032a

Table 3. Correlation between inoculation density and Fv/Fm within each Fv/Fm sampling period across Fv/Fm sampling days. HIGHOD, an optical density at 750nm (OD_{750nm}) sufficient to capture 2030 or 1906 (day 1a only) µmol m^{-2} s^{-1} photosynthetically active radiation (PAR) at 23cm depth. MIDOD is OD_{750nm} sufficient to capture 1791 or 1711 (day 1a only) µmol m^{-2} s^{-1} PAR. LOWOD is the average raceway inoculation density 0.026 OD_{750nm}, which captures 1286 µmol m^{-2} s^{-1} PAR. LOWOD data included on days 1 and 2. No Fv/Fm data for 10:00 on day 1a.

Fv/Fm Sampling Time	Days	n	Pearson Correlation Coefficient	p-value
06:00 to 07:00	1, 2, 5, 6, 1a	104	0.57809	$p < 0.001$
09:40 to 10:15	1, 2, 5	64	0.74334	$p < 0.001$
13:00 to 13:20	1, 2, 5, 1a	76	0.74560	$p < 0.001$
16:00 to 16:30	1, 2, 5, 1a	80	0.68120	$p < 0.001$
19:00 to 19:30	1, 2, 5, 1a	80	0.65880	$p < 0.001$

Table 4. Effect of 200µg mL^{-1} chloramphenicol (CAP) on Fv/Fm recovery of Anabaena sp. when added for full 75m or 45m into 75m dark adaptation period after exposure to 1400 µmol m^{-2} s^{-1} PAR. HIGHOD is an optical density at 750nm (OD_{750nm}) sufficient to capture 1906 µmol m^{-2} s^{-1} PAR at 23cm depth. MIDOD is OD_{750nm} sufficient to capture 1711 µmol m^{-2} s^{-1} PAR. "+CO2" cultures are CO_2 supplemented to limit maximum pH to 8.9. Different letters have significantly different Fv/Fm values ($p < 0.05$).

Inoculation Density	n	CO_2	No CAP	CAP 75 min	CAP 45 min
MIDOD	3	+	0.118 ± 0.018	0.111 ± 0.007	N/A
MIDOD	3	-	0.121 ± 0.017	0.112 ± 0.003	0.118 ± 0.009
HIGHOD	3	+	0.198 ± 0.023a	0.145 ± 0.002b	0.162 ± 0.013b
HIGHOD	3	-	0.165 ± 0.029a	0.131 ± 0.017b	N/A

Table 5. Multiple regression with model selection based on Mallow's C(p). Full day data set includes all Fv/Fm data points. 13:00 data set includes Fv/Fm multiple regression using only 13:00 Fv/Fm readings. Day 5 LOWOD excluded from both data sets. Day 1a did not include LOWOD treatment. HIGHOD is an optical density at 750nm (OD_{750nm}) sufficient to capture 2030 or 1906 (day 1a) µmol m^{-2} s^{-1} photosynthetically active radiation (PAR) at 23cm depth. MIDOD is OD_{750nm} sufficient to capture 1791 or 1711 (day 1a) µmol m^{-2} s^{-1} PAR. LOWOD is the average field inoculation density of 0.026, which captures 1286 µmol m^{-2} s^{-1} PAR. "+CO2" cultures are CO_2 supplemented to maintain maximum pH 8.9.

Model	n	R^2	F value	p value	Notes
Fv/Fm (Full Day) = 0.3122 + 0.00933 * CO_2 supplementation - 0.1292 * LOWOD (10% Shade) - 0.0636 * MIDOD (25% Shade) - 0.00009248 * PAR (µmol m^{-2} s^{-1})	377	0.69	F (4, 372) = 203.96	< 0.0001	
Fv/Fm (13:00) = 0.2384 + 0.0130 * CO_2 supplementation - 0.115 * LOWOD (10% shade) - 0.0469 * MIDOD (25% shade) + 0.253 * Optical Density 750nm - 0.000449 * Solar irradiance to 14:00 (kWm^{-2}) + 0.00022* 24h Evaporation (mL)	76	0.93	F (6, 69) = 143.56	< 0.0001	pH at 13:00 removed, $p > 0.05$.

Table 6. 14-d biomass and nitrogen totals and gains by CO_2 treatment, averaged over HIGHOD and MIDOD inoculation treatments. HIGHOD is optical density at 750nm (OD_{750nm}) sufficient to capture 2030 µmol m^{-2} s^{-1} PAR at 23cm depth, column sides shaded to reduce light transmission by 50%. MIDOD is an OD_{750nm} sufficient to capture 1791 µmol m^{-2} s^{-1} PAR, column sides shaded to reduce light transmission by 25%. "+CO2" cultures are CO_2 supplemented to maintain maximum pH 8.9. Different letters have significantly different nitrogen values ($p<0.05$).

CO_2 Treatment, averaged over inoculation density (shade) treatments	14-d Biomass (mg L^{-1})	14-d Biomass Gain (mg L^{-1})	14-d Nitrogen (mg L^{-1})	14-d Nitrogen Gain (mg L^{-1})
CO_2	570 ± 116a	430 ± 143a	64.0 ± 10.8a	53.7 ± 11.8a
No CO_2	386 ± 88.9b	251 ± 55.3b	39.9 ± 6.8b	29.5 ± 5.4b

Table 7. Multiple regression with model selection based on Mallow's C(p). LOWOD inoculation density (10% Shade) treatment excluded. HIGHOD is an optical density at 750nm (OD_{750nm}) sufficient to capture 2030 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR) at 23cm depth. MIDOD is OD_{750nm} sufficient to capture 1791 µmol m^{-2} s^{-1} PAR.

Model	n	R^2	F-value	p-value	Notes
Productivity, Day 7 to 14 = 211.31 - 74.28 * OD_{750nm}, start of 24h period - 15.32 * pH at 13:00	128	0.1423	F(2,127) = 10.37	p < 0.0001	Dissolved oxygen (p=0.582) removed from initial model
Productivity, Days 9 and 10 = 236.99 - 21.15* pH + 20.95 * MIDOD (25% shade) - 0.0 * HIGHOD (50% shade)	32	0.4426	F(2, 29) = 11.51	p < 0.0002	Solar Irradiance (p=.0952), OD_{750nm} at start of 24hr period (p = 0.1330) removed from the initial model.

Figures

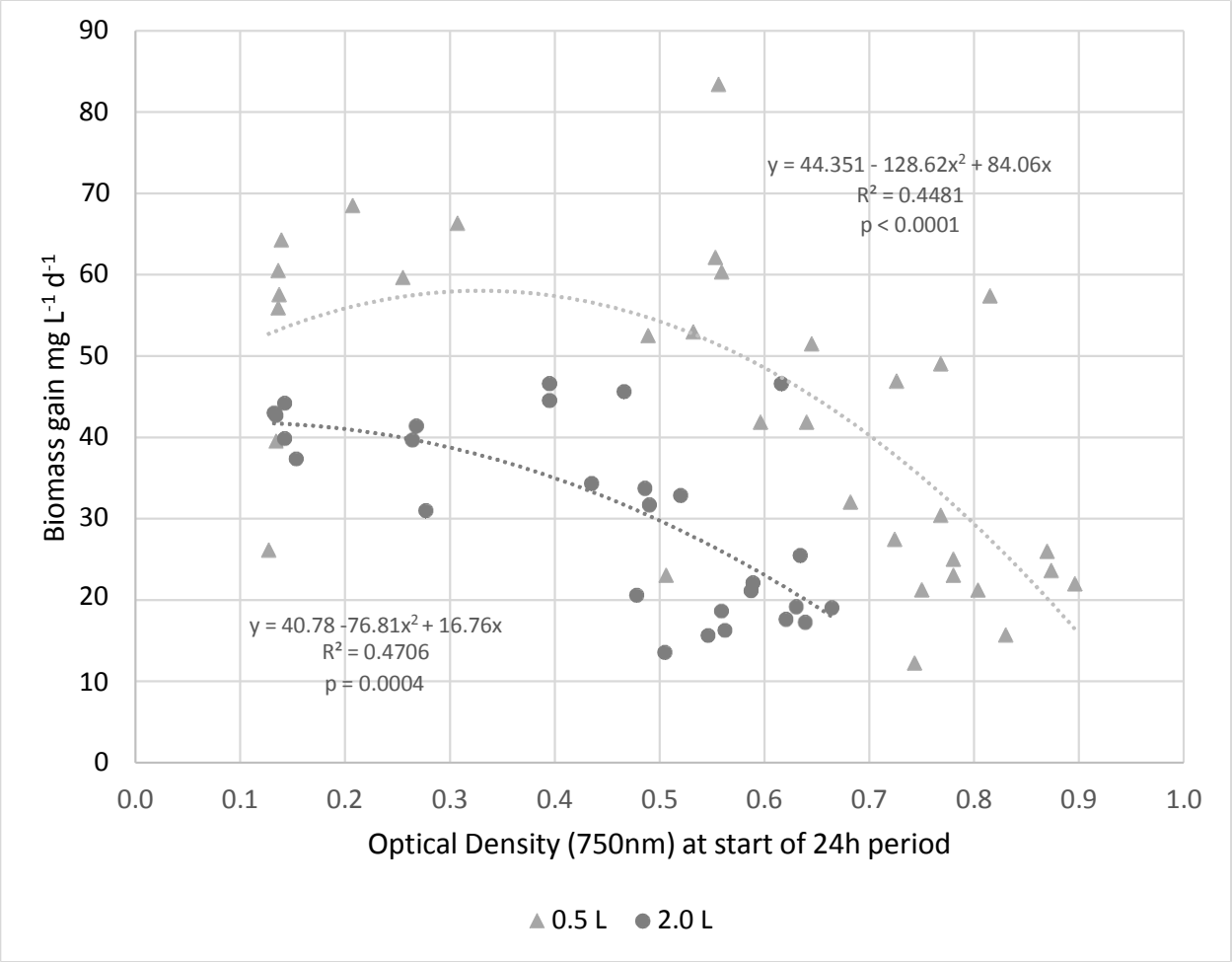

Figure 1. Productivity vs. OD_{750nm} of Anabaena sp. in Allen and Arnon N-free growth medium in 500mL and 2.0L flasks under 12h light at 140 µmol m^{-2} s^{-1} : 12h dark. Light path is 7.5 cm for 500mL flasks and 11.5cm for 2.0L flasks. Surface area to culture volume of 2.0L flasks is 67% of 500mL flasks. Results are from two 14-d experiments. 500mL flasks n = 6; 2L flasks n = 5

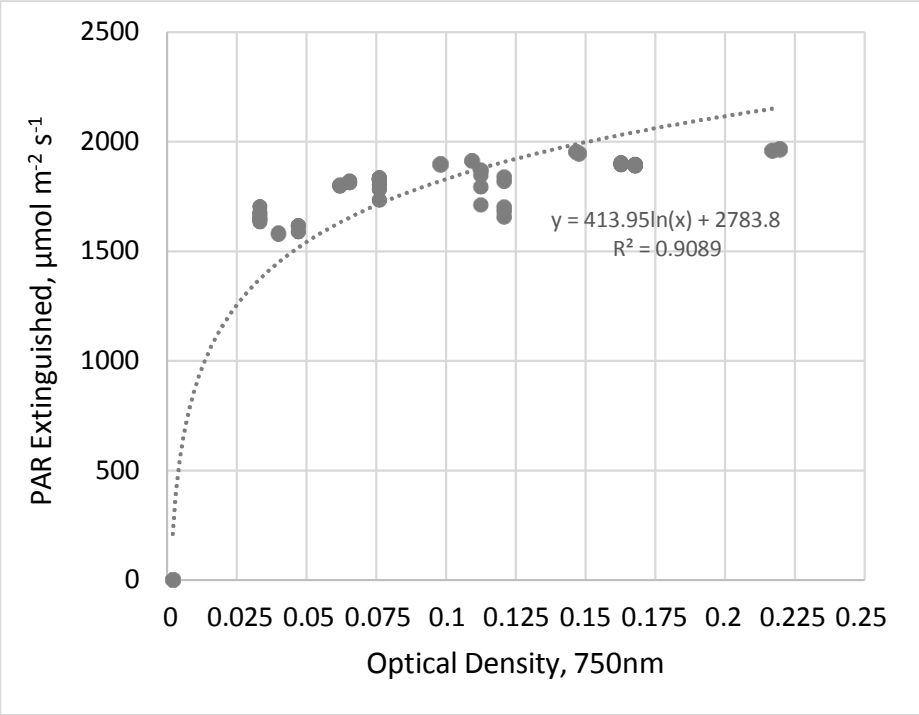

Figure 3. Anabaena sp. culture optical density, 750nm vs photosynthetically active radiation (µmol m-2 s-1) captured at 23cm. OD750nm < 0.15 may result in excess PAR and photoinhibition in culture during the day.

Figure 2. Columns experimental design and setup.

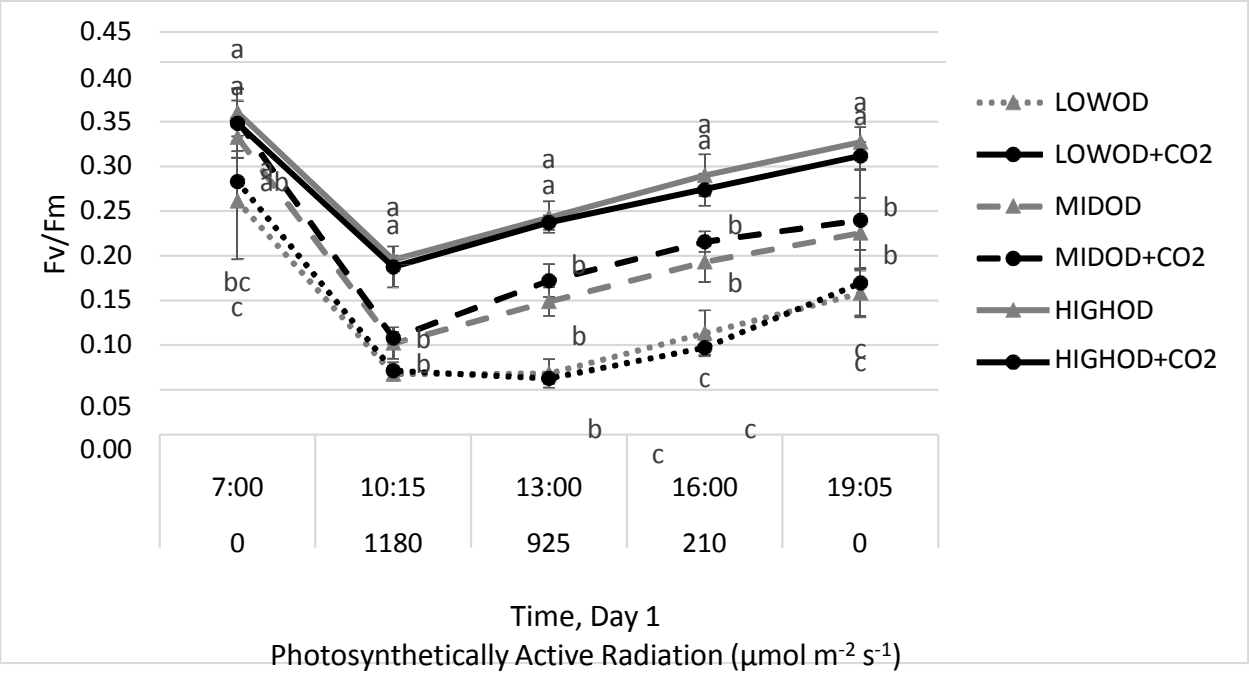

Figure 4. Photochemical efficiency of photosystem II (Fv/Fm), day 1. HIGHOD is optical density at 750nm sufficient to capture 2030 µmol m^{-2} s^{-1} PAR at 23cm depth. MIDOD is an optical density sufficient to capture 1791 µmol m^{-2} s^{-1} PAR. LOWOD is the average field inoculation density of 0.026, which captures 1286 µmol m^{-2} s^{-1} PAR. "+CO2"cultures are CO2 supplemented to maintain maximum pH 8.9. Different letters have significantly different Fv/Fm values ($p<0.05$).

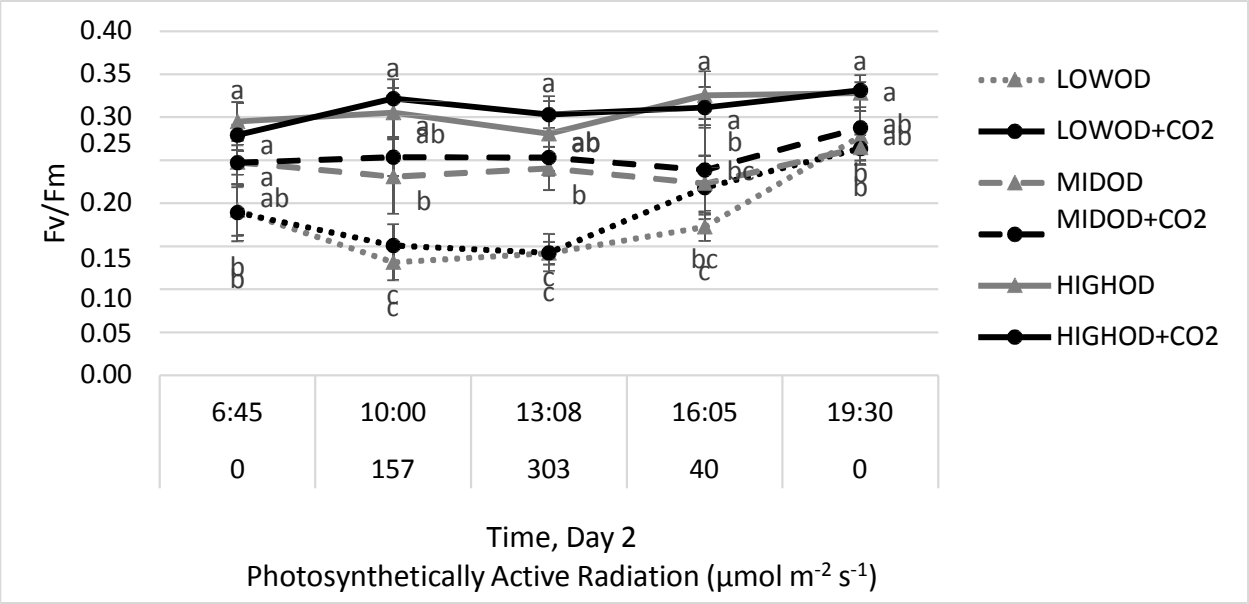

Figure 5. Photochemical efficiency of photosystem II (Fv/Fm), day 2. HIGHOD is optical density at 750nm (OD_{750nm}) sufficient to capture 2030 µmol m^{-2} s^{-1} PAR at 23cm depth. MIDOD is an OD_{750nm} sufficient to capture 1791 µmol m^{-2} s^{-1} PAR. LOWOD is the average field inoculation density of 0.026, which captures 1286 µmol m^{-2} s^{-1} PAR. "+CO2" cultures are CO_2 supplemented to maintain maximum pH 8.9. Different letters have significantly different Fv/Fm values ($p<0.05$)

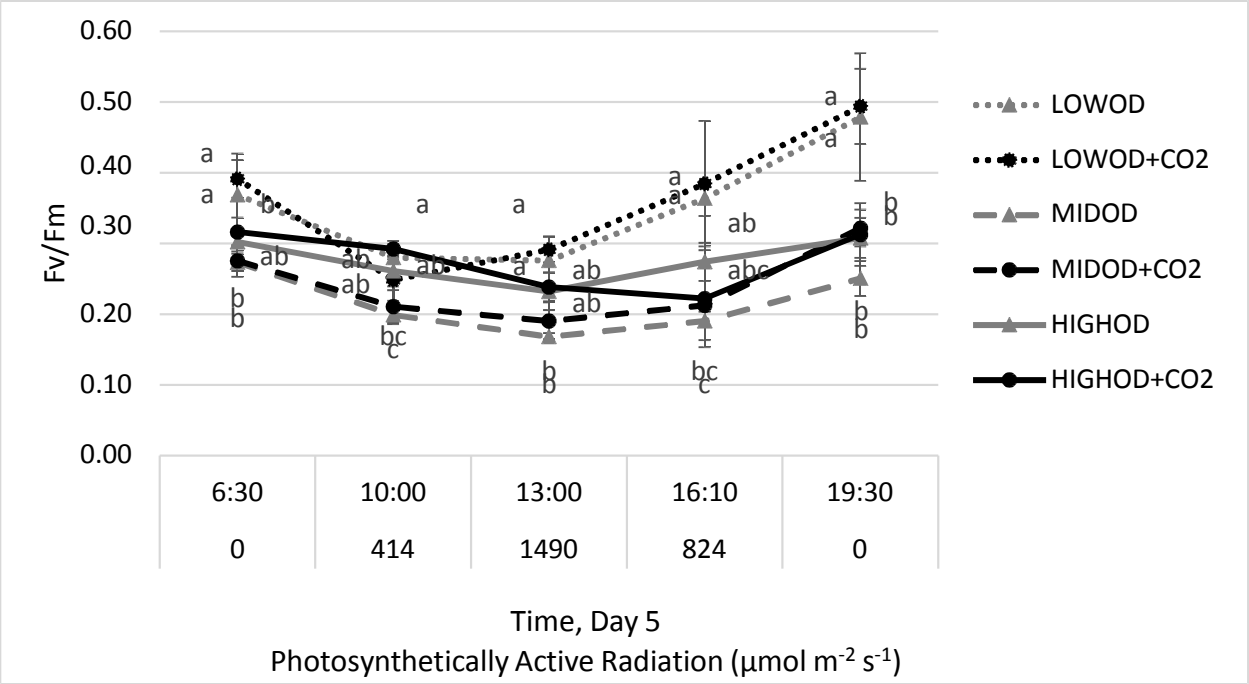

Figure 6. Photochemical efficiency of photosystem II (Fv/Fm), day 5. HIGHOD is optical density at 750nm (OD_{750nm}) sufficient to capture 2030 µmol m^{-2} s^{-1} PAR at 23cm depth. MIDOD is an OD_{750nm} sufficient to capture 1791 µmol m^{-2} s^{-1} PAR. LOWOD is the average field inoculation density of 0.026, which captures 1286 µmol m^{-2} s^{-1} PAR. "+CO2" cultures are CO_2 supplemented to maintain maximum pH 8.9. Different letters have significantly different Fv/Fm values ($p<0.05$).

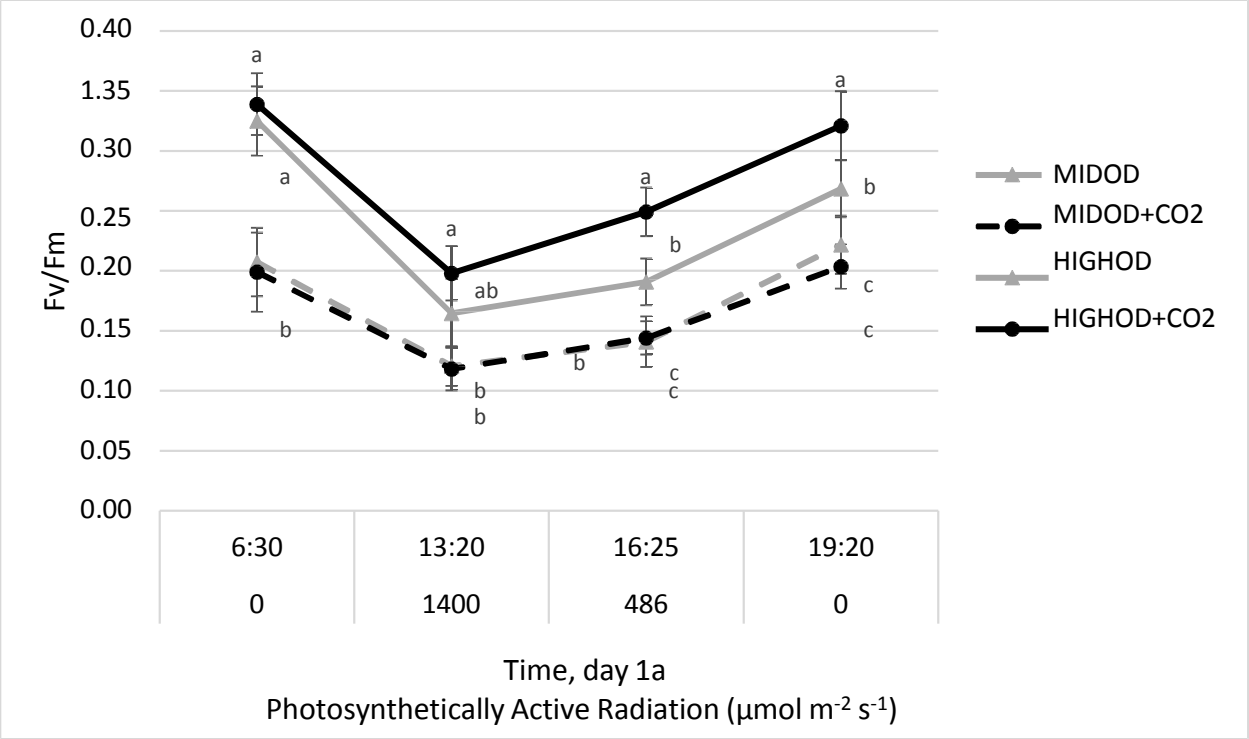

Figure 7. Photochemical efficiency of photosystem II (Fv/Fm), day 1a (October 16). HIGHOD is optical density at 750nm (OD_{750nm}) sufficient to capture 1906 µmol m^{-2} s^{-1} PAR at 23cm depth. MIDOD is an OD_{750nm} sufficient to capture 1711 µmol m^{-2} s^{-1} PAR. "+CO2" cultures are CO_2 supplemented to maintain maximum pH 8.9. Different letters have significantly different Fv/Fm values.

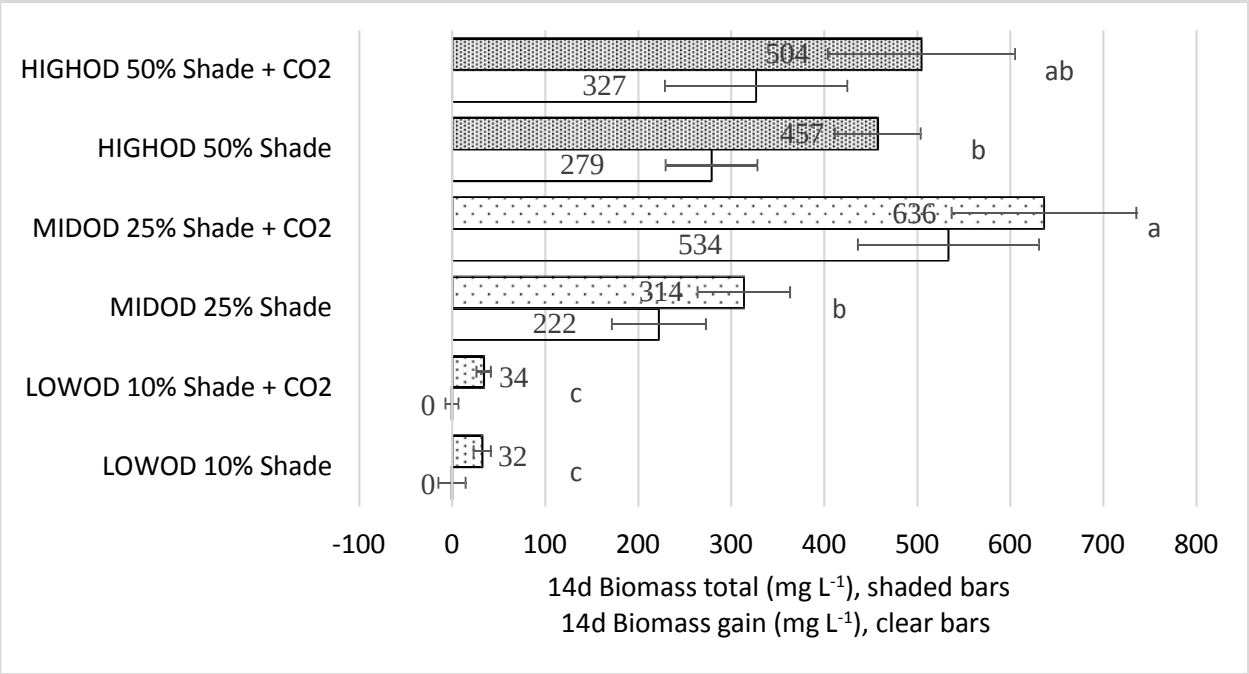

Figure 8. 14-d total Anabaena sp. biomass (top, shaded bar) and 14-d biomass gain (bottom, clear bar) by treatment. HIGHOD is optical density at 750nm (OD_{750nm}) sufficient to capture 2030 µmol m^{-2} s^{-1} PAR at 23cm depth, column sides shaded to reduce light transmission by 50%. MIDOD is an OD_{750nm} sufficient to capture 1791 µmol m^{-2} s^{-1} PAR, column sides shaded to reduce light transmission by 25%. LOWOD is the average field inoculation density of 0.026, which captures 1286 µmol m^{-2} s^{-1} PAR, column sides effectively reduce light transmission by 10%. "+CO2" cultures are CO_2 supplemented to maintain maximum pH 8.9. Different letters have significantly different biomass values ($p<0.05$).

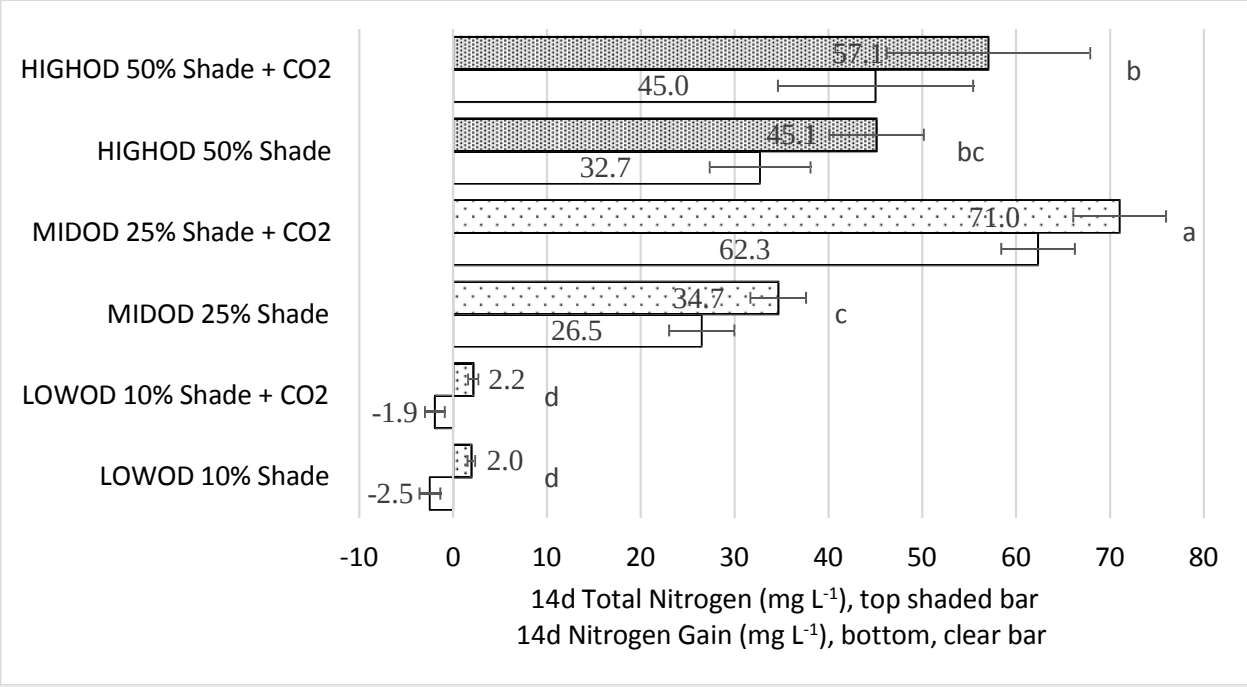

Figure 9. 14-d Total Anabaena sp. nitrogen (top, shaded bar) and 14-d nitrogen gain (bottom, clear bar) by treatment. HIGHOD is optical density at 750nm (OD_{750nm}) sufficient to capture 2030 µmol m^{-2} s^{-1} PAR at 23cm depth, column sides shaded to reduce light transmission by 50%. MIDOD is an OD_{750nm} sufficient to capture 1791 µmol m^{-2} s^{-1} PAR, column sides shaded to reduce light transmission by 25%. LOWOD is the average field inoculation density of 0.026, which captures 1286 µmol m^{-2} s^{-1} PAR, column sides effectively reduce light transmission by 10%. "+CO2" cultures are CO_2 supplemented to maintain maximum pH 8.9. Different letters have significantly different nitrogen values ($p<0.05$).

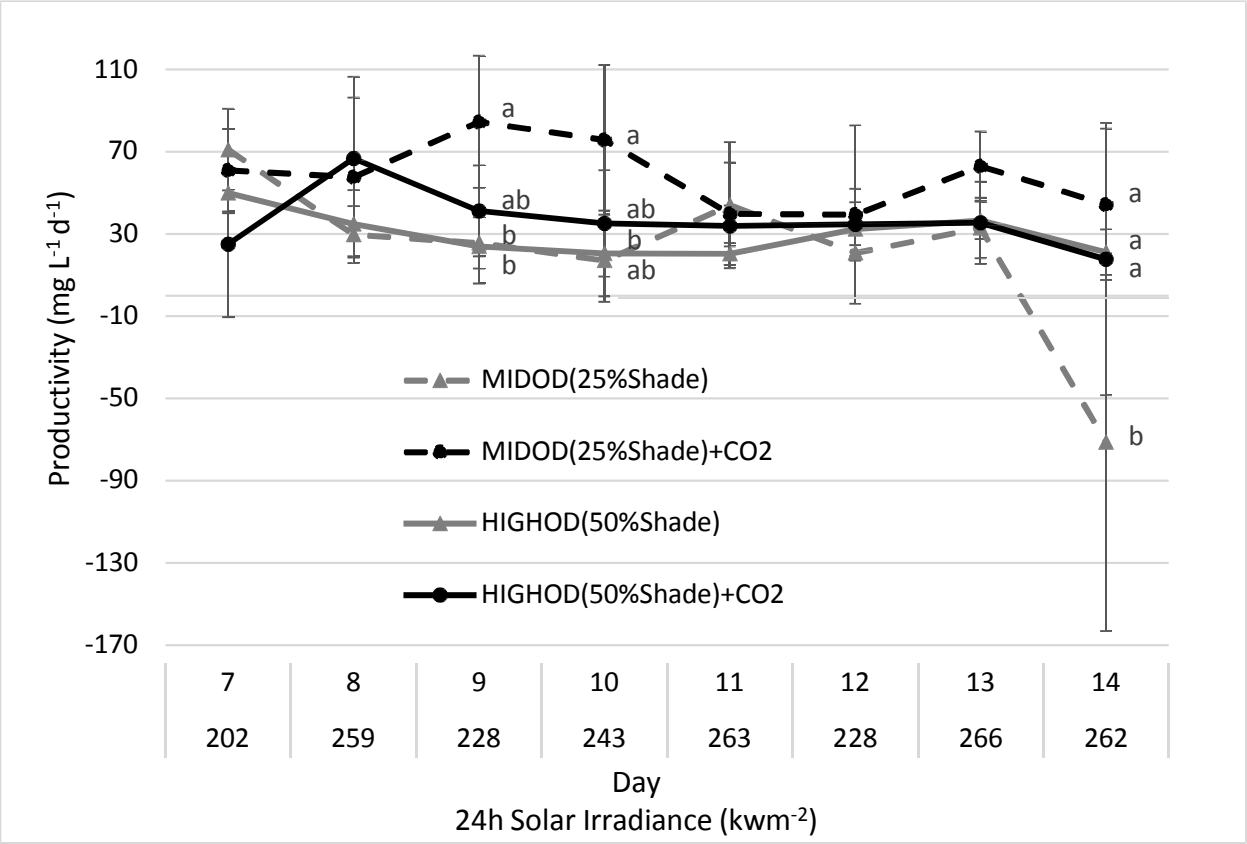

Figure 10. Anabaena sp. daily productivity days 7 through 14 of 14-d batch in 2L columns. HIGHOD is optical density at 750nm (OD_{750nm}) sufficient to capture 2030 µmol m^{-2} s^{-1} PAR at 23cm depth, column sides shaded to reduce light transmission by 50%. MIDOD is an OD_{750nm} sufficient to capture 1791 µmol m^{-2} s^{-1} PAR, column sides shaded to reduce light transmission by 25%. "+CO2" cultures are CO_2 supplemented to maintain maximum pH 8.9. Different letters have significantly different daily productivity, from repeated measures analysis ($p<0.05$).

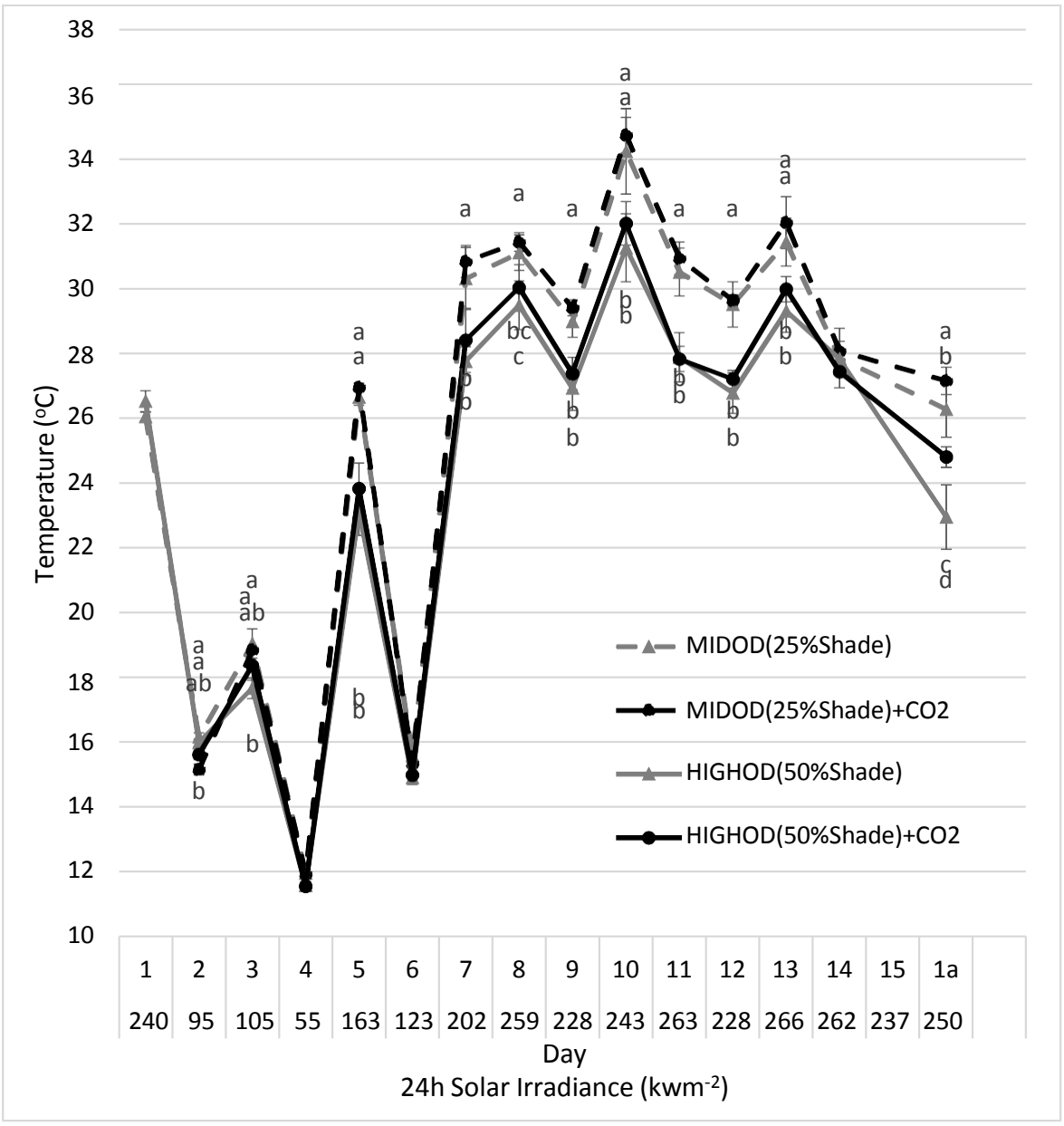

Figure 11. Mean temperature recorded between 14:00 and 15:30, October 1 to 16 (day 1a). HIGHOD is an optical density at 750nm (OD_{750nm}) sufficient to capture 2030 or 1906 (day 1a) µmol m^{-2} s^{-1} photosynthetically active radiation (PAR) at 23cm depth. MIDOD is OD_{750nm} sufficient to capture 1791 or 1711 (day 1a) µmol m^{-2} s^{-1} PAR. "+CO2" cultures are CO_2 supplemented to maintain maximum pH 8.9. HIGHOD column sides shaded to reduce light penetration by 50%. MIDOD column sides shaded to reduce light penetration by 25%. Different letters represent significant differences ($p < 0.05$).

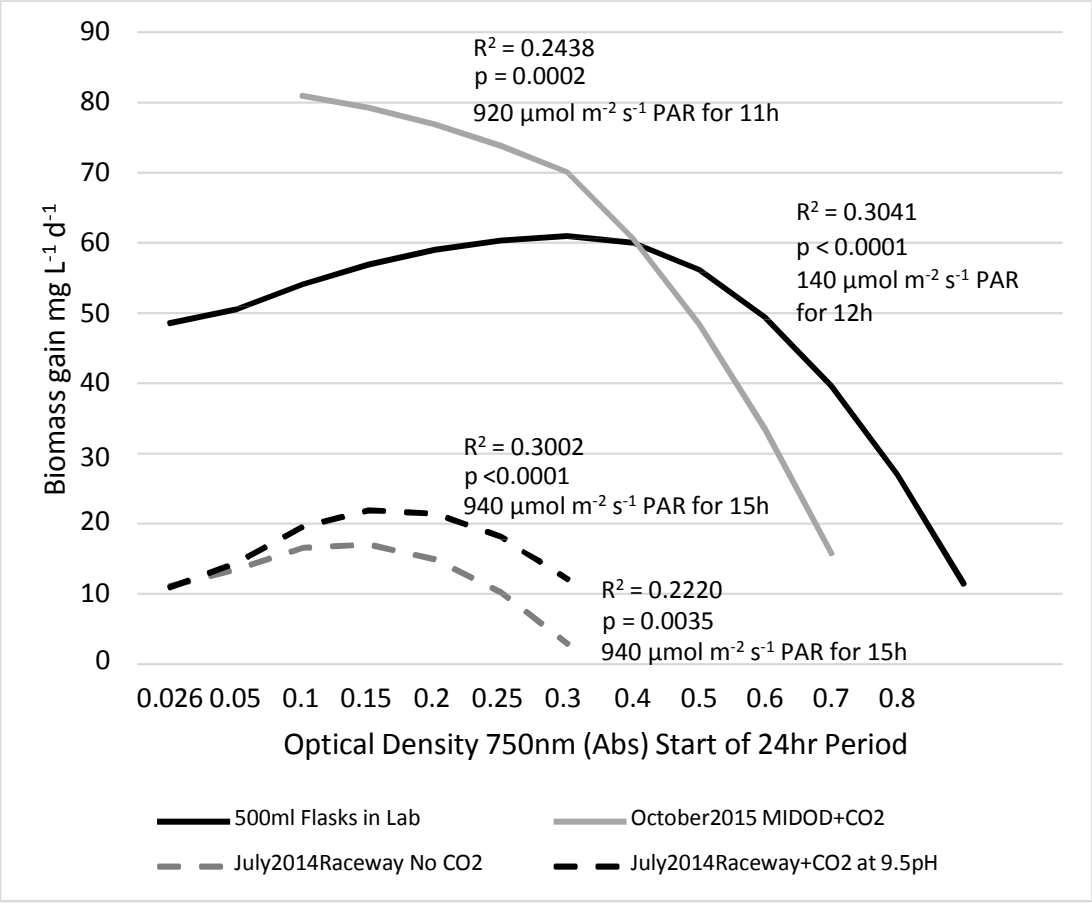

Figure 12. Linear regression of productivity and Anabaena sp. optical density (750nm) at the start of the 24hr period under different production conditions. MIDOD column width and column side shade cover allow up to 25% supplemental light penetration. Average hourly light intensity estimated at 920 µmol m^{-2} s^{-1} PAR for 11h. CO_2 supplemented to maintain maximum pH 8.9. 500mL Flasks in lab were grown under 12h 140 µmol m^{-2} s^{-1} light:dark period. Inoculation between 0.030 and 0.17 OD_{750nm}, pH did not exceed 8.4. July 2014 Raceways were 9.26 m^2 open raceways, inoculated at 0.011 OD_{750nm}, mixed using paddlewheels and grown under high tunnels. CO_2 supplemented raceways received CO_2 when pH exceeded 9.25. Estimated average hourly light intensity for outdoor raceways was 1120 µmol m^{-2} s^{-1} PAR x 84% high tunnel light transmission for 15h. R^2 and p-value of the model is included.

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CHAPTER 2: INFLUENCE OF LIGHT ON ENDOGENOUS PHYTOHORMONE CONCENTRATIONS OF A NITROGEN-FIXING ANABAENA SP. CYANOBACTERIUM CULTURE IN OPEN RACEWAYS FOR USE A FERTILIZER FOR HORTICULTURAL CROPS

Introduction

Background

Nitrogen-fixing cyanobacteria are attractive as a nitrogen (N) fertilizer because they are ubiquitous in nature and have minimal nutrient requirements. A locally produced, on-demand source of N could expand fertilizer options available for vegetable production. On-farm cyanobacteria-based fertilizer could be phototrophically produced under reduced land and water requirements compared with N-fixing cover crops. On-farm production of N-fixing cyanobacteria could also improve access to N for smallholder farmers in regions of the world where soil N is depleted and fertilizer availability is unreliable or nonexistent. Production and application of as little as 10 to 17 kg N ha^{-1} could provide at least 30% gains in crop yield (Twomlow et al., 2008). Small-scale production of a cyanobacteria-based fertilizer could be achieved with similar energy requirements per kg N compared to ammonia-based fertilizers (Gellings and Parmenter, 2004; Sheehan et al., 1998; Tavares et al., 2013). Small-scale batch production requires only a few kilowatt-hours per day, and meaningful N production could be realized with minimal fossil fuel inputs or low-cost solar panels.

Researchers at Colorado State University (CSU) have cultivated a local strain of Anabaena sp. that is ideally suited for fertigation. The culture does not clog irrigation drip lines and thus can be pumped directly from raceways to crops. Compared with other locally isolated N-fixing

strains, the Anabaena sp. strain was better able to acclimate to diurnal and seasonal changes in light and temperature as well as to competitive pressure from eukaryotic algae and protozoa. When applied to vegetable crops at the same N-rate, Anabaena sp. cultures have proven as effective as commonly used organically certified N-fertilizers, such as fish emulsion, and alfalfa, feather, and blood meals (Sukor, 2013; Yoder, 2014).

In addition to their use as an N fertilizer, filamentous N-fixing freshwater cyanobacteria produce a variety of phytohormones. When applied to plant roots, cyanobacterial-derived auxins, abscisic acid, and cytokinins have been shown to alleviate iron (Fe) deficiencies, promote plant growth, and improve yields (Hussain and Hasnain, 2011; Lei et al., 2013; Mazhar et al., 2013; Shariatmadari et al., 2015). Indole 3-acetic acid (IAA) is the most frequently encountered phytohormones in Anabaena, with concentrations varying between species (Hussain et al., 2010; Prasanna et al., 2011; Hashtroudi et al., 2012; Shariatmadari et al., 2015). Cytokinins (CK) and abscisic acid (ABA) have also been detected in Anabaena sp. (Zahradníčková et al., 1991; Stirk et al., 2002; Hussain and Hasnain 2011). Gheda and Ahmed (2015) detected gibberellins in Anabaena cylindrica. Wickham (2016) and Sukor (2016) detected IAA and salicylic acid (SA) in a low density Anabaena sp. culture (< 300 mg L^{-1}) grown in high tunnels at the Colorado State University Horticultural Research Farm.

On-farm production of Anabaena sp. cultures by the CSU research team has taken place inside polyethylene covered high tunnels to date. Shifting production out of high tunnels could increase biomass concentrations by increasing available sunlight or photosynthetically active radiation (PAR). Higher culture densities may also facilitate detection of phytohormones (Stirk et al., 2013).

In higher plants, salt and oxidative stresses can lead to increased ABA production (Taiz and Zeiger, 2010). This has been shown more tenuously in algae and cyanobacteria (Maršálek et al., 1992; Yoshida et al., 2004). A combination of higher evaporation rates, photosynthetic activity, and its accompanying reactive oxygen species (ROS) might be sufficient to enhance ABA production in fully exposed outdoor cultures compared to those grown under high tunnels. On the other hand, Pouneva (2006) and Stirk et al. (2014) showed that energy stress may trigger ABA synthesis, so a more light-limited culture could lead to higher ABA concentrations.

Prasanna et al. (2010) found that light stimulates IAA production in cyanobacteria and that auxin concentrations accumulate over time. Bacterial production of IAA appears to be related to potential stresses encountered in the plant rhizosphere, such as C limitation and low solute potential (Spaepen et al., 2007). Cyanobacteria synthesize scytonemins and mycosporine-like amino acids (MAA) in response to exposure to UV-A and UV-B (Balskus and Walsh, 2010; Rastogi et al., 2010). Indole-3 pyruvic acid is a scytonemin precursor. Increased light availability, UV stress, greater variability in culture temperature, solute potential, and pH might all be expected to increase concentrations of auxins in cultures located outside when compared to those inside high tunnels.

Cytokinin concentrations have been shown to fluctuate with light/dark cycles in Chlorella minutissema cultures and accumulated as the culture aged (Stirk et al., 2014). Improved light availability outside the high tunnel may increase cell division rates, stimulating production and accumulation of cytokinins (Burkiewicz, 1987; Selivankina et al., 2006).

It is less clear whether other phytohormones, such as giberrellins (GA), brassinosteroids (BR), and salicylic acid (SA) would accumulate sufficiently to be detected at the end of a 14-d cyanobacterial batch culture in stationary growth (Stirk et al., 2014). GA have been detected in in

one strain of cyanobacteria, while BR have not. Klejdus et al. (2009) found benzoic acid (BA) derivatives in four different cyanobacteria, including Anabaena doliolum. SA, which has not been specifically detected as a cyanobacterial metabolite, was found in an initial CSU on-farm xenic Anabaena sp. culture. Tillberg (1970) showed that SA may be produced by some eukaryotic algae. Islam et al. (2013) detected SA in 10 N fixing non-photoautotrophic bacteria sampled from rice paddy fields. A variety of both algae and bacteria have been observed microscopically in field production cultures.

Plants may synthesize moderate amounts of SA during light acclimation, which in turn induces synthesis of alternative oxidase, a supplemental electron acceptor, reducing buildup of ROS (Muhlebock et al., 2008; Chang et al., 2009). Similarly, SA could induce synthesis of plastoquinol terminal oxidase, or some other antioxidant, in cyanobacteria and algae (McDonald and Vanlerberghe, 2006; Vicente and Plasencia, 2011; Yu et al., 2015). However, higher concentrations of SA in plants may actually cause a buildup of ROS and lead to cell death (Samuilov et al., 2001; Overmyer et al., 2003; Rajjou, et al., 2006; Horváth et al., 2007). Raman and Ravi (2010) saw concentration dependent effects of SA on the growth of the microalgae Haematococcus pluvialis, with higher concentrations causing cell bleaching and death. Park et al. (2006) found SA and BA to be anti-cyanobacterial when applied to Microcystis aeruginosa culture, while Rice et al. (1980) found concentration-dependent positive and negative effects of p-hydroxybenzoic acid on growth of Anabaena cylindrica. Kepekçi and Saygideger (2011) were able to increase overall concentrations of phenolic compounds by increasing light intensity available to 6-day old Spirulina platensis cultures. While average light availability per cell decreases over the batch production period, a light-dependent effect on SA and BA in Anabaena sp. cultures might still be expected under the high irradiance of outdoor production.

Studies on microalgal phytohormone production in response to light have been conducted at low light intensities typically ranging from 40 to 300 µmol m^{-2} s^{-1}, while on-farm intensities reach 2000 µmol m^{-2} s^{-1} daily throughout the summer growing season. Furthermore, cyanobacterial cultures produce 11% N, dry weight biomass. The volumes applied to achieve on-farm N fertilization goals of 56 kg ha^{-1} will magnify slight differences in concentrations, possibly improving phytohormone availability in the soil, or increasing efficacy where phytohormones may have a cumulative effect. The objective of this study was to assess Anabaena sp. phytohormone production under outdoor, open pond conditions. To achieve this, we attempted to establish whether consistent phytohormone concentrations could be expected in on-farm Anabaena sp. cultures at the end of 14-d batch production periods. The study also evaluated the effect of light availability and other culture conditions on 14-d open pond phytohormone concentrations to identify potential avenues for manipulating phytohormone production.

Methods

Experimental Design

The experiments were conducted outdoors at the Colorado State University Horticultural Research Farm in Fort Collins, CO, May through August, 2016. The experiment was set up as a split plot with two inoculation densities and two light exposure treatments for a 2 x 2 factorial treatment design. An inoculation density was randomly assigned to six ponds under each of the two levels of light exposure so that each treatment combination was replicated three times. Three experiments (Experiment 1, Experiment 2, and Experiment 3) were conducted during the summer 2016 season. Culture depth and associated densities were adjusted after Experiment 1 to

improve productivity. Differences in Experiment 1 methods are addressed at the end of the section.

Inoculation Densities

The high inoculation density treatment (HD) was an Anabaena sp. target OD_{750nm} sufficient to extinguish 91% of peak projected photosynthetically active radiation (PAR) at a depth of 23cm on May 27, and a depth of 13cm on July 20 and August 9, 2016, the dates of batch period inoculation (Table 8). The low inoculation density treatment (LD) was an Anabaena sp. target OD_{750nm} measured to extinguish 84% of the peak PAR for the inoculation date at a 23cm or 13cm depth. PAR was estimated with Apogee Instruments Clear Sky calculator (http://clearskycalculator.com/) for the field location (40.610917, -104.996766) with an elevation of 1523m.

Light Exposure

Six 52cm by 165cm, 30cm deep galvanized troughs lined with 6 mil recycled polyethethylene (Home Depot) were oriented north-south in the center of a 6.1 x 15.2 meter, east-west oriented high tunnel covered with 6 mil UV resistant polyethylene plastic (AT Films, Edmonton, AB Canada). The plastic is rated to allow 91% light transmission, however actual PAR transmission within the high tunnel was measured at 84%. Six additional troughs were placed under full outdoor exposure to sunlight, oriented north-south and positioned 0.6m south of the high tunnel. The metal interior of some of the troughs used in prior year experiments had been coated with a red or white sealant to prevent zinc contamination of cultures. An attempt was made to distribute troughs with red, white, or no (the original metal) coating so that each treatment group

had the same number of a given color or coating underneath the plastic liner. Coating color was included as a potential variable during statistical analysis.

Outdoor Pond Conditions

To scale cultures up to field volumes, two week old Anabaena sp. cultures (0.60 OD_{750m} to 1.00 OD_{750nm}) in 500 mL flasks grown phototrophically under 140 µmol m^{-2} s^{-1} fluorescent lighting were used to establish 8L to 12L volume cultures in 22.7 L glass carboys at a target density between 0.10 OD_{750nm} and 0.20 OD_{750nm}. Carboys were moved to a greenhouse where temperatures were maintained between 13°C and 27°C. Carboy-scale cultures were mixed using one to two 2" air stones and aquarium air pumps. When culture densities reached between 0.30 OD_{750nm} and 0.40 OD_{750nm} in the greenhouse they were diluted back to between 0.15 OD_{750nm} and 0.40 OD_{750nm} by transferring to additional carboys. When greenhouse carboy culture volume and density was sufficient to inoculate at least two 23cm deep troughs at a minimum 0.10 OD_{750nm} the culture was transferred to the CSU Horticultural Research Farm. Trough-scale cultures were grown for approximately two weeks in AA growth medium and supplemental CO_2 troughs until sufficient volume and density was available to inoculate 12 troughs at the target densities.

Troughs were inoculated with Anabaena sp. culture in exponential growth phase. Tap water was added to adjust the culture to the target inoculation density at a final depth of 13cm and a final volume of 52L. Total N concentrations in the tap water at inoculation were 0.45 mg L^{-1}. After the target density was achieved, Allen and Arnon N-free growth medium (AA) at one-fourth agar concentration was added. Cultures were mixed using a SEIO 1500 submersible flow pump (Technological Aquatic Associated Manufacturing, CA, USA) and by bubbling ambient air through 1.3 x 3.8-cm Sweetwater silica air diffusers (Southern Aquaculture Supply, Lake

Village, AR, USA). Evaporative loss was replaced daily at 13:00 with tap water to maintain a 13cm culture depth. CO_2 was supplemented to the cultures when pH exceeded 8.9. pH was continuously monitored with a PINPOINT pH Controller (American Marine, Inc., Ridgefield, CT USA). A brass AC110V solenoid (Duda Energy, AL, USA. Model 2W-200-20N) attached to CO_2 pressure regulators allowed CO_2 to bubble through 1.3-cm x 2.5-cm aquarium air stones until pH was at a maximum of 8.7.

Monitoring Growth and Culture Conditions

Culture temperature, pH and dissolved oxygen (DO) were recorded daily prior to sampling for optical density (OD), between 13:45 and 14:30. pH was measured using an Oakton Waterproof pH Testr 30 Pocket Tester (Vernon Hills, Il USA). Temperature and DO were measured using a Thermo Scientific™ Orion™ 3-Star Plus Dissolved Oxygen Portable Meter (Thermo Fisher Scientific Inc., Beverly, MA, USA). Air temperature and solar radiation data for the nearest weather station (ARDEC) was downloaded from http://www.coagmet.colostate.edu/. Actual PAR was measured with an MQ-306 Line Quantum with 6 Sensors Handheld Meter (Apogee Instruments, Logan, UT).

50mL of culture was sampled daily, between 13:45 and 14:30, in duplicate for OD measurements and biomass estimations. Initial biomass was calculated from samples collected prior to sunrise on day 1. OD_{750} of the culture was measured using the Hach DR3900 Spectrophotometer (Hach Company, Loveland, CO). An OD_{750} biomass standard curve (Equation 1) was fit using linear regression of 50mL culture sampled from summer 2016 field and lab studies. The culture was filtered through 1.5 micron Whatman grade 934-AH, 47mm

diameter glass microfiber binder free filters (GE Healthcare Life Sciences, USA), dried at 60ºC for 48 hours and weighed.

$$\text{Biomass (mg L}^{-1}) = 981.5 * OD_{750nm} - 3.3245 \quad \text{(Equation 1)}$$

Total N was determined using Hach Simplified-TKN kits (TNT880, Hach Company, Loveland CO, USA.). Total N was measured from 50 mL samples drawn pre-dawn on day 1, and between 14:00 and 14:30 on days 4, 7, 10, and 14.

Health of the culture was also monitored microscopically by tracking Anabaena sp. filament length and the presence of algae and potentially predatory protozoa. A biological microscope (model MT4310H, Meiji Techno Co., LTD., Japan) was used for microscopy.

Phytohormone Analysis

At day 14, 50mL of culture was sampled from each trough and filtered through 1.5 micron Whatman grade 934-AH, 47mm diameter glass microfiber binder free filters (GE Healthcare Life Sciences, USA). 2mL of the filtrate was then filtered through 25mm, 0.2 micrometer pore size nylon syringe filters (Fisherbrand, Fisher Scientific, USA). Samples were cryopreserved at -80ºC until extraction by the Colorado State University Proteomics and Metabolomics Facility (CSU-PMF). Filters were transferred to 8 mL glass vials, and 6 mL 100% met-tert-butyl ether, chilled at -20C was added. Samples were extracted at 4° C for 1 hour with mild shaking, followed by centrifugation at 3500 x g at 4° C. The supernatant (6 mL) was transferred to a new vial and concentrated to dryness under N_2 (g) stream at room temperature. Samples were resuspended in 1mL of 100% methanol and stored at -80ºC until analysis by LC-MS/MS. Seven

deuterated internal standards were used as either a direct internal standard or as a surrogate internal standard: Salicylic acid-d4, phaesic acid-d3, diphaesic acidd3, jasmonic acid-d5, indole acetic acid-d5, abscisic acid-d6, and gibberellin-3-d2. One 15N isotopic standard, transzeatin-15N, was used for transzeatin and transzeatin riboside. LC-MS/MS analysis was performed on a Waters Acquity UPLC coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer. Chromatographic separations were carried out on a Waters UPLC T3 C18 stationary phase (1 x 100 mm, 1.7 µM) column. Phytohormone compounds included in the assay were: trans-Zeatin, trans-Zeatin riboside, abscisic acid, diphaesic acid, giberellic acid-3, giberellic acid-4, indole-3-acetamide, indole carboxylic acid, indole-3-acetyl alanine, indole-3-acetic acid, indole-3-acyrylic acid, indole-3-acetylnitrile, indole-3-butyric acid, jasmonic acid, methyl jasmonate, salicylic acid, benzoic acid, methyl salicylate, S-adenosylmethionine, and 1-aminocyclopropane carboxylic acid.

Cyanotoxins

On August 23, 2016, one day after the end of the 14-d Experiment 3, 50mL of culture were drawn and filtered through 1.2 micron Whatman grade GF/C, 47mm diameter glass microfiber binder free filters (GE Healthcare Life Sciences, USA), cryopreserved and submitted to the Center for Coastal Studies Plankton Laboratory at Texas A&M University, Corpus Christi, for mass spectroscopy cyanotoxin analysis. Samples were extracted in ACN/water/formic acid (80/19.9/0.1) solution at 4 ºC for 20 h. The presence of toxin was determined using an Agilent 1200 HPLC equipped with an Agilent 6140 triple quadruple mass spectrometer. HPLC mobile phase A was 10% CAN, and mobile phase B was 100% ACN, both with 0.1% formic acid. The bioactive compound was loaded onto a Phenomenex C18 Luna, 3 µm, 150 x 3 mm column. The

flow rate was set to 0.3 mL/min and the initial solvent composition was 10% mobile phase B. This gradient was held for two min and then was raised to 100% mobile phase B over 45 min. The 100% mobile phase B was held for 5 min and then reduced to starting conditions. Column temperature was set at 35 ºC. The data were analyzed using MassHunter Workstation B.03.01 software. Limits of detection for microcystins was > 0.05 µg L^{-1}, for saxitoxin > 0.5 µg L^{-1}, and anatoxin-a > 0.06 µg L^{-1}.

Statistics

Statistical analysis was performed using SAS 9.4 University Edition (SAS Institute Inc., Cary, NC). Significance was defined as $\alpha = 0.05$ for all statistical analyses. Analysis of mean biomass, total N and phytohormone concentrations between the experiments was performed using a one-way ANOVA (proc mixed). Comparison of mean phytohormone concentrations between treatment combinations was performed using two-way ANOVA (proc mixed). Multiple linear regression, with top ten model selection based on Mallow's C(p) (proc reg) was used to identify relevant treatment and culture variables.

Results

Biomass and Total Nitrogen

When Experiments 2 and 3 were combined, no significant differences in 14-d biomass or N were found between the four treatment combinations using two-factor ANOVA. Mean 14-d biomass was 541 +/- 44 mg L^{-1}, and mean total N was 60.3 +/- 8.2 mg L^{-1}. One-way ANOVA (proc mixed) showed that neither 14-d mean biomass nor total N were significantly different between Experiment 2 and Experiment 3. Experiment 1 cultures (23cm depth) were all in decline

after day 10 of the batch. Experiment 1 achieved peak biomass at day 10 of 247 ± 41 mg L^{-1} and 10d total N of 25.3 ± 4.0 mg L^{-1}, less than 50% of the 14-d (peak) concentrations in Experiment 2 and Experiment 3 (13cm culture depth). Due to the depth difference and decline of the Anabaena sp. in all troughs, the results from Experiment 1 were not combined with Experiment 2 and Experiment 3 for statistical analysis. Phytohormone concentrations from day 14 of Experiment 1 are still relevant to one of the research goals, so are presented separately at the end of this section.

Endogenous Phytohormones Detected

Six phytohormones were detected during the assay. ABA, IAA, indole-3-carboxylic acid (ICA), and SA were detected above the limit of quantification. Indole acetamide (IAM) and trans Zeatin riboside (tZr) were detected above the limit of detection. One-way ANOVA (proc mixed) showed that mean concentrations of SA were significantly different between Experiment 2 and Experiment 3.

Abscisic Acid

The assay detected a mean ABA concentration of 0.819 +/- 0.366 ng mg^{-1}. ABA concentrations were significantly higher in cultures grown inside the high tunnel (Figure 13). However, ABA concentrations were only significantly different between the high density high tunnel and low density outside treatments (Table 9). Multiple linear regression with model selection resulted in a significant regression equation with the following predictor variables: high tunnel (location) treatment and 14-d biomass gain ($R^2 = 0.51$). Average peak dissolved oxygen

was removed from the original model selected (p=0.0745) (see Table 11 for model with coefficients).

Auxins

Three auxins were detected during the assay. The mean concentration of IAA was 1.13 +/0 0.286 ng mg^{-1}. The mean concentration of IAM, an IAA precursor, was 0.813 +/- 0.272 ng mg^{-1}. The mean concentration of ICA was 0.691 +/- 0.183.

IAA and IAM concentrations were significantly higher in cultures grown with full outdoor exposure compared to those within high tunnels (Figure 14a and b). Cultures inoculated at higher densities (HD) also showed higher concentrations of IAA and IAM than those inoculated at the low densities (LD). IAA and IAM concentrations were significantly higher in the high density outside (HDO) treatment (Table 9). Conversely, ICA concentrations were found to be greater in cultures grown within high tunnels with no difference between inoculation densities. ICA concentrations were not significantly different among treatment combinations (Figure 14c).

Significant regression equations were found to predict IAA, IAM, and ICA concentrations using treatment or other culture variables. The regression equation for IAA included the initial (inoculation) biomass ($R^2 = 0.50$) (Table 11). Location was included in the top model, but was removed ($p = 0.737$). The variability in IAM concentrations was best explained by the initial biomass and total solar irradiance, adjusted for high tunnel light transmission ($R^2 = 0.61$) (Table 11). Variability in ICA concentration was predicted by the location treatment, solar irradiance adjusted for high tunnel light transmission, 14-d biomass gain, and the experiment number ($R^2 = 0.56$) (Table 11).

Cytokinins

The only cytokinins included in the assay were trans Zeatin and (tZr). TZr reached detectable levels in most troughs, with a mean concentration of 0.0242 +/- 0.0184 ng mg^{-1}. No significant difference in concentration was found between locations or inoculation densities, nor between treatment combinations (Figure 15, Table 9). A significant regression equation was calculated to predict tZr concentrations using the variables solar irradiance, average temperature at 14:00, and trough coating color (($R^2 = 0.51$) (Table 11).

Salicylic Acid

A mean salicylic acid (SA) concentration of 4.81 +/- 2.37 was measured during the phytohormone assay. SA concentrations were significantly higher in Experiment 3 compared with Experiment 2 (Table 10). SA concentrations in the high density inoculation outside (HDO) were significantly lower than the low density high tunnel (LDHT) treatments in Experiment 2; otherwise there were no significant differences among treatments in Experiment 2 or Experiment 3 (Table 10, Figure 16). Multiple linear regression with model selection resulted in a significant regression equation that included the variables experiment number, inoculation biomass, and 14-d biomass gain ($R^2 = 0.78$) (Table 11). When solar irradiance was broken out between day 1 to 7 (Week 1) and day 8 to 14 (Week 2) of the experiment, where the growth appears to shift from exponential to stationary, the regression equation included the location (high tunnel), solar irradiance (Week 1), and solar irradiance (Week 2) ($R^2 = 0.80$) (Table 11).

Cyanotoxins

Cyanotoxin analysis performed on the twelve troughs in Experiment 3 showed no detectible concentrations of anatoxin-a, microcystin, or cylindrospermoposin.

Experiment 1 Phytohormone Concentrations

Despite the Anabaena sp. decline in all Experiment 1 troughs after day 10, the same six phytohormones were detected. Mean ABA concentrations in Experiment 1 were not significantly different from the other experiments (Figure 17). IAA, IAM, ICA, and tZr concentrations were significantly different between Experiment 1 and Experiment 3, but were not significantly different between Experiment 1 and Experiment 2. SA concentrations were significantly different among all three experiments (Figure 18). One trough in Experiment 1 had SA concentrations of 46.0 ng mg^{-1} biomass. The Bonferroni-adjusted outlier test failed to identify the trough as an outlier. Even so, analysis without this trough showed that Experiment 1 SA concentrations were still significantly higher than those in Experiment 2 and Experiment 3.

Discussion

Influence of Field Production Parameters on Phytohormone Concentrations

Abscisic Acid

Growing Anabaena sp. inside the high tunnel had a significant positive effect on ABA concentrations while 14-d biomass gain had a negative influence on ABA concentrations (Figure 1, Table 11). Pouneva (2006) and Stirk et al. (2014) saw increased ABA concentrations in microalgal cultures in response to energy stress. Although solar irradiance was not selected by the model, high tunnel light transmission was measured at 84% of outdoor levels, suggesting

potential energy limitation. The negative correlation of ABA concentrations with biomass gain, a likely response to increased energy availability, also leads to the likelihood of energy stress.

Oxidative or salt stress, reported to trigger ABA production, do not appear to be likely sources of ABA in Anabaena sp. cultures based on the results of this study. DO was not selected as a significant variable in the multiple regression. The highest DO measured during any experiment was 13.8 mg L^{-1}, below the 20 mg L^{-1} that might be expected to cause oxidative damage (Vonshak, 1997). Freshwater Anabaena species vary in their tolerance to salt (Rai et al., 2014). Studies on microalgal stress generally use 100 mM or greater NaCl concentrations when investigating the effects of salt stress (Yoshida et al., 2004; Srivastava et al., 2010), though Rai et al. (2014) saw effects on Anabaena L31 with 62 mM NaCl. These salt concentrations translate to an electrical conductivity (EC) range of 5.67 to 13.7 milliSiemens/cm (mS/cm). AA liquid growth medium, in comparison, results in 3-4 mM salt concentrations. Laboratory cultures of Anabaena sp. in twice the AA concentration had an EC of 740 µS/cm at inoculation (0.178 OD_{750nm}) and a high of 1070 µS/cm in 28 day old cultures. Up to 10% of the culture volume in the field was lost daily to evaporation. 18% evaporative loss in laboratory cultures shifted EC from 1010 to 1230 µS/cm. Salt concentrations in on-farm cultivation may not reach the levels that would stress less salt-tolerant Anabaena sp. strains. These results show that a likely avenue for manipulating ABA concentrations under field conditions is through energy stress. Physical shading of ponds or allowing Anabaena sp. cultures to grow beyond optimal cell density (maximal productivity) to increase self-shading could increase ABA concentrations.

Auxins - Indole3- Acetic Acid and Indole-3 Acetamide

The treatment variable, inoculation density (inoculation biomass), had a positive influence on IAA concentrations, accounting for 50% of the variability between among treatment combinations. IAM, a precursor to IAA, was strongly correlated to IAA concentrations (Pearson Correlation Coefficient 0.91801, $p < 0.001$). Inoculation biomass and solar irradiance, adjusted for high tunnel transmission, showed a positive influence on IAM concentrations.

The positive effect of Anabaena sp. inoculation biomass on IAA and IAM concentrations suggests that IAA concentrations were established to some degree by initial environmental conditions. Under full outdoor light exposure, a less dense inoculation biomass experiences more photoinhibition, diverting metabolic resources to repair of photosystem II (Richmond, 2004; Vonshak, et al., 2014). Photoinhibition would discourage cell growth. This is supported by Stirk et al. (2013) who saw higher IAA concentrations in the faster growing of 24 microalgae strains. Stirk et al. (2014) also found relatively static IAA concentrations over 48 hours of Chlorella minutissima growth. They also reported higher concentrations of IAA in C. minutissima cultures grown under light:dark or supplemented with glucose and grown under complete dark conditions when compared to completely dark conditions. Assuming day 1 or 2 of batch production are not cloudy, daily growth rates decline over the course of the batch culture. Decreasing rates of cell expansion and division would limit the need for higher IAA concentrations, tying IAA levels tightly to light (energy) availability. Prasanna et al. (2010) tracked IAA in two Anabaena strains over 21 days and found that IAA concentrations leveled off by day 14 under 55 $\mu mol\ m^{-2}\ s^{-1}$ light intensity. Increasing culture density would be expected to reduce light availability over time, especially 14 days into a growth cycle.

IAM concentrations in the present study were also tied to inoculation biomass, but were driven primarily by light, suggesting a dynamic response to energy availability. IAM production would replenish IAA lost to storage or catabolism (Cohen and Bandurski, 1982; Seidel et al., 2006; Spaepen and Vanderleyden, 2011).

The cultures grown outside the high tunnel exhibited a more olive-green color than the blue-green cultures within the high tunnels, even at end of batch densities. Photoinhibition has been shown to change the carotenoid concentrations in cyanobacteria (Mulders et al., 2014; Nichols 1973; Schagerl and Müller, 2006; Sedoud, 2014; Takaichi, 2011; Wolk, 1973) Greater photoinhibition may be occurring outside compared to inside the high tunnel. One source of this photoinhibition would be UV-A (Singh et al., 2013). Cyanobacteria produce scytonemins as a protective measure in response to UV-A (Karsten, 2008). Indole-3 pyruvic acid (IPyA), a precursor to scytonemins was not present at detectable concentrations in the culture. However, the presence of IAM in the culture indicates that tryptophan, a precursor to both IAM and IPyA was being synthesized. UV-A related stress at the end of the batch appears to be insufficient to raise IPyA to detectable levels in outdoor production cultures

In on-farm Anabaena sp. production, utilizing an inoculation rate towards the upper end of the optimal cell density may establish high IAA baselines, while maximizing light availability would keep IAM production high. This strategy is effectively the same as maximizing productivity of the culture for biomass and N concentration.

Auxins - Indole-3 Carboxylic Acid

The presence or role of ICA in bacteria is unclear. In Arabidopsis, ICA production and transport to cell walls was a response to a necrotic fungal pathogen (Gamir et al., 2012). Forcat et

al. (2010) found that ICA can act as a substitute for callose, building up on plant cell walls to restrict bacterial multiplication. ICA has been detected in algae, and callose has been found in some filamentous green algae (Schiewer, 1967; Herburger and Holzinger, 2015). No filamentous green algae were identified during microscopic observation of culture samples in the present study. However, Ochromonas sp. and a small single-celled algae were frequently observed in all trough cultures. This may be a more likely source of ICA than cyanobacteria.

Cytokinins

Tzr, but not trans Zeatin, was detected during the assay. TZr concentrations were negatively correlated to solar irradiance, average temperature at 14:00 and the metal coating of the trough interiors (underneath the plastic lining). White coating had no effect compared to the other two coatings, and red trough coatings positively influenced tZr concentrations (Red Coating $p = 0.721$). Cytokinins appear to be related to increases in transcriptional activity, cell division, and growth in algae and cyanobacteria (Burkiewicz, 1987; Selivankina et al., 2006; Stirk et al., 2013; Lu et al., 2014). Higher rates of cell division would be expected with greater light availability, reducing accumulated tZr levels (Stirk et al., 2014). Stirk et al. (2013) detected up to 20 cytokinins across 24 algae strains. In the algae C. minutissima, Stirk et al. (2014) detected up to 15 cytokinins over a 2 day period, with tZr among the bottom third by concentration, and several orders of magnitude lower than cis trans zeatin concentrations. Hussain et al. (2010) found five cytokinins in five cyanobacteria stains, including three in an Anabaena strain (“CK1”). TZr may not be representative of active cytokinin concentrations. A wider cytokinin assay may identify additional cytokinins in greater concentrations, allowing for improved correlation to field production conditions.

Salicylic Acid

SA concentrations were significantly higher in Experiment 3 compared to Experiment 2. Neither treatment variable was selected by the model. The significant regression equation included Experiment 2, inoculation biomass and 14-d biomass, all exhibiting a negative effect on SA concentrations. Increased SA concentrations in lower inoculation biomass suggests SA is playing the signaling role for increasing antioxidant production. Greater photoinhibition or light acclimation would be expected in a relatively lower culture density, resulting in higher concentrations of ROS (Vonshak et al., 2014). This in turn would activate SA production (Muhlenbock et al., 2008; Chang et al., 2009). There was no significant difference in mean Anabaena sp. biomass concentrations between treatments at day 14, but the model showed that increased biomass gain depressed SA concentrations. As batch cultures increased in density and became more light acclimated, or limited, oxidative stress would be less likely. In an effort to better understand this process, the solar irradiance variable was split into two periods, solar irradiance, Week 1, included solar irradiance for days 1 to 7. Solar irradiance, Week 2 corresponded to days 8 to 14. While it was difficult to determine from the data in Experiment 2 and Experiment 3, the division of the 14-d batch period by week has corresponded to exponential and stationary growth in previous outdoor cultivation of Anabaena sp. (Barminski, 2014). Multiple regression with model selection using the split solar irradiance variables included only light related variables in the top model- the location treatment, solar irradiance Week 1, and solar irradiance Week 2 ($R^2 = 0.80$, see Table 11). Week 1 irradiance positively influenced SA concentrations, as would be expected in a less dense culture acclimating to light. Week 2 irradiance and the high tunnel location negatively influenced SA levels. The increasingly dense culture during the last half of the batch was increasingly light limited, so the buildup of ROS

could be expected to decrease in severity each day. Light availability would still drive biomass gain, but additional SA production is not needed to deal with ROS, and overall concentrations were diluted. If SA concentration increases are desired for application of Anabaena sp. based N fertilizer, this study indicates that SA could be maximized in Anabaena sp. cultures by maximizing light availability early in the batch period, including inoculation at the lower end of the density range.

Experiment 1 Phytohormone Concentrations

Despite four to five days of slowly declining biomass prior to sampling, day 14 Experiment 1 phytohormone concentrations were similar to those found in Experiments 2 and 3. ABA was not significantly different among any of the experiments, and SA was significantly higher than Experiments 2 and 3 (Figure 17 and Figure 18). It is not clear whether the decline was biological in nature or due to light or other limitation. The decline was gradual, rather than a "crash" or complete decline occurring in one or two days, and was not accompanied by increased predation by other protozoa, such as rotifers. Anabaena sp. filaments or cells did not appear to decline in health (breakage into individual cells, cell lysing, etc.), nor was an obvious shift to algae observed in the culture. If inoculation biomass is a significant determinant in IAA concentrations, as seen in Experiments 2 and 3 (Table 11), lower inoculation biomass in Experiment 1 to offset the 23cm depth could explain why IAA concentrations were lower. Increased light path (trough depth) resulting in greater light or energy limitation in Experiment 1 could also explain why ABA concentrations were not significantly different despite lower day 14 culture densities, and why IAM concentrations were lower than Experiments 2 and 3.

Effective Concentrations of Phytohormone Applications

Table 12 compares endogenous Anabaena sp. phytohormone concentrations detected in the study with phytohormone concentrations found to have plant beneficial effects. Plant beneficial effects are shown in Table 13. Endogenous SA concentrations reported in this study are at or near those reported to have beneficial effects on abiotic stresses such as cold, heat, and drought (Senaratna et al., 2000; Stevens et al., 2006; Sukor, 2016). ABA concentrations are within a few orders of magnitude of concentrations reported to enhance drought tolerance and chlorophyll and carotenoid production (Astacio and van Iersel, 2011; Barickman, 2014; Barickman et al., 2014). Hussain et al. (2010) detected exogenous levels of IAA and cytokinins in Anabaena sp. cultures that were equal to endogenous levels. Hartung (2010) noted that ABA produced in response to stress is generally released from the cell. Measuring the exogenous levels of phytohormones in Anabaena sp. on farm cultures and widening the assay to include more phytohormone compounds, such as cytokinins, could reveal much higher actual phytohormone concentrations.

Multiple regression of productivity with treatments and other culture variables tracked throughout the batch period (data not shown) did not establish an optimal cell density under outdoor production, suggesting that inoculation densities were already too high within the troughs or that something other than light was limiting growth (Richmond, 2004). Trough heights (30cm) were more than twice the depth of the culture (13cm). With a trough width of 52cm, direct light reaching the surface of the culture was delayed by an hour post sunrise and pre sunset each day. The sides of the troughs partially shaded the culture surface for all but 4 hours during the day. Where light availability influences phytohormone concentrations, resolving excess shading could improve both batch biomass yields and phytohormone concentrations.

Several avenues are immediately available, then, to identify or improve phytohormone production to a concentrations which are more likely to provide plant growth promoting benefits.

Cyanotoxins

Cyanotoxins are of concern when growing cyanobacteria for application to food crops. CSU has used ELISA tests to monitor cyanotoxins in on-farm Anabaena sp. production cultures. The cyanotoxin mass spectroscopy analysis performed on the twelve troughs from Experiment 3 showed no detectible concentrations of anatoxin-a, microcystin, or cylindrospermoposin. Anabaena sp. continues to be a strong candidate as a biofertilizer.

Effect of Xenic Cultures and Production Conditions on Phytohormone Concentrations

In a xenic culture, some portion of the phytohormones are invariably produced by the associated bacterial and protozoan community. Regular microscopic examination identified the presence of ciliates, amoebae, and photosynthetic protozoa similar to Ochromonas sp. as well as the occasional single-cell algae. Considering the detection of ICA, usually associated with algae or plant cell walls, isolation of Anabaena sp. as well as the phototrophic protozoa and algae could clarify the source of ICA. SA, while detected in some bacteria, has never been reported in cyanobacteria cultures. SA should be assayed from axenic Anabaena sp. cultures to confirm that concentrations detected in our on-farm cultures could be generated by Anabaena sp.

Bacterial populations visible during microscopy were dense in some samples, but many appeared to successfully pass through the 1.5 µm pore size used during filtration prior to cryopreservation of Anabaena sp. for phytohormone analysis. The primary bacteria visible under field production cultures resembled those seen in cultures grown under laboratory conditions at

up to 90% higher culture densities, suggesting that they do not negatively impact culture growth. Identifying the bacteria, their contribution to biomass, and their phytohormone profile could increase and improve phytohormone concentration estimates and our understanding of culture dynamics.

Submersible flow pumps used for mixing are energy efficient and provide sufficient mixing action. However, either the high flow rates in the troughs, measured at up to three times the recommended 0.30 m s^{-1} for algae cultures, or the chopping action of the impellers (Becker, 1994) kept Anabaena sp. filaments consistently shorter than those observed under bubbling agitation in the lab or paddlewheel mixing in larger outdoor raceway ponds. It is not clear whether consistent mechanical breakage may affect phytohormone concentrations. Improving on-farm production conditions for the Anabaena sp. culture should clarify or strengthen the models of the conditions that drive phytohormone concentrations.

Many phytohormones play a role in modulating each other. For example, ABA is known to have an antagonistic effect on CK and SA levels (Pál et al., 2011; Lu et al., 2014). With additional data on other community contributions, and increased biomass concentrations, we may be able to predict concentrations of multiple phytohormones using a single model.

Conclusions

This study shows that open raceway Anabaena sp. based cultures may be a reliable source of a number of phytohormones that can improve plant health and crop yields. It is possible to significantly influence ABA, IAA, IAM, and SA phytohormone concentrations in an Anabaena sp. culture by manipulating light availability during batch production. ABA enhanced cultures cold be used to improve the tolerance of indoor starts to drying out after transplanting; SA could

be increased to relieve cold stress in early season soils; ABA, auxins, and SA concentrations could improve plant response to drought and phytopathogens. Auxin and cytokinin concentrations could be increased when growing conditions are ideal simply to speed up cell division and plant growth. The combination of phytohormone versatility within the Anabaena sp. culture under outdoor production and the flexibility provided by frequent (e.g., 7-d to 14-d) batch production results in a N fertilizer that can be regularly adjusted to address changing crop needs and stressors throughout the season.

Tables

Table 8. Inoculation optical density treatments, Experiments 1, 2 and 3. Target optical densities at 750nm expected to capture 91% and 84% peak photosynthetically active radiation (PAR) at a depth of 23cm or 13cm on day 1 of the experiment batch period.

Inoculation Date	Peak PAR ($\mu mol\ m^{-2}\ s^{-1}$)	Culture Depth (cm)	Target Optical Density (750nm)	Target PAR Captured ($\mu mol\ m^{-2}\ s^{-1}$)	Estimated % PAR Captured
May 27 (Experiment 1) High Density	2012	23	0.102	1839	91
May 27 (Experiment 1) Low Density	2012	23	0.070	1683	84
July 20 (Experiment 2) High Density	1969	13	0.164	1792	91
July 20 (Experiment 2) Low Density	1969	13	0.115	1654	84
August 9 (Experiment 3) High Density	1906	13	0.141	1734	91
August 9 (Experiment 3) Low Density	1906	13	0.100	1602	84

Table 9. Endogenous phytohormone concentrations in Anabaena sp. on day 14 of batch culture by treatment. High density (HD) and low density (LD) treatments are inoculation densities estimated to capture 91% and 84%, respectively, of maximum expected PAR on the inoculation date. High tunnel (HT) troughs are located under polyethylene covered high tunnel. Outside (O) troughs are located south of the high tunnel, under full exposure to outdoor light intensity. Concentrations are reported in nanograms phytohormone per milligram Anabaena sp. biomass, dry weight. Mean concentrations with different letters are significantly different ($p < 0.05$).

Concentrations in ng mg^{-1}, dw		Abscisic Acid	Auxins			Cytokinins
Treat	n	Abscisic Acid	Indole-3-Acetic Acid	Indole Acetamide	Indole Carboxylic Acid	Trans Zeatin riboside
Mean, ng mg^{-1}	21	0.817 ± 0.366	1.13 ± 0.286	0.813 ± 0.272	0.691 ± 0.183	0.025 ± 0.018
HDHT	4	1.17 ± 0.524 a	1.04 ± 0.138 b	0.749 ± 0.191 b	0.823 ± 0.228	0.028 ± 0.004
HDO	7	0.716 ± 0.244 ab	1.47 ± 0.212 a	1.092 ± 0.190 a	0.669 ± 0.153	0.020 ± 0.004
LDHT	5	0.967 ± 0.285 ab	0.913 ± 0.146 b	0.563 ± 0.095 b	0.767 ± 0.249	0.037 ± 0.035
LDO	5	0.558 ± 0.247 b	0.965 ± 0.145 b	0.741 ± 0.241 b	0.563 ± 0.058	0.015 ± 0.009

Table 10. Endogenous salicylic acid concentrations in Anabaena sp. culture on day 14 of batch by experiment and treatment. High density (HD) and low density (LD) treatments are inoculation densities estimated to capture 91% and 84%, respectively, of maximum expected PAR on the inoculation date. High tunnel (HT) troughs are located under polyethylene covered high tunnel. Outside (O) troughs are located south of the high tunnel, under full exposure to outdoor light intensity. Concentrations are reported in nanograms phytohormone per milligram Anabaena sp. biomass, dry weight. Concentrations with different letters are significantly different ($p < 0.05$).

Concentrations in ng mg-1, dw		Salicylic Acid				
Treatment	n	Combined	n	Experiment 2	n	Experiment 3
Mean, ng mg^{-1}	21	4.80 ± 2.39	9	2.70 ± 1.37 b	12	6.40 ± 1.57 a
HDHT	4	4.54 ± 1.14	1	3.86 ± 0.0 ab	3	4.70 ± 1.27
HDO	7	3.71 ± 2.48	4	1.82 ± 0.289 b	3	6.23 ± 1.31
LDHT	5	6.15 ± 1.92	2	4.59 ± 1.49 a	3	7.19 ± 1.49
LDO	5	5.23 ± 3.10	2	1.97 ± 0.043 ab	3	7.41 ± 1.23

Table 11. Multiple linear regression with model selection to explain phytohormone concentrations using treatments variables and culture conditions. Top model selected based on Mallow's C(p) (proc reg). Variables included in top model selected with $p > 0.05$ were removed prior to calculation of coefficients.

Phytohormone	Multiple regression model (variables included at $p < 0.05$)	R^2	F value, p-value of model	Notes
Abscisic acid (ABA)	1.8379 + 0.461 * High Tunnel Location (=1) - 0.00307 * 14-d Biomass Gain (mg L^{-1})	0.5072	F(2,18) = 9.26 p =0.0017	DO removed p= 0.0745
Indole-3 acetic acid (IAA)	0.003558 + 0.00775 * Initial Biomass (mg L^{-1})	0.5014	F(1,19) = 19.11 p =0.0003	Location removed, p = 0.0737
Indole-3 acetamide (IAM)	-0.92625 + 0.00591 * Initial Biomass (mg L^{-1}) + 0.0002077 * Solar Irradiance (kWm^{-2})	0.6072	F(2,18) = 13.91 p =0.0002	
Indole carboxylic acid (ICA)	13.9791 -1.613 * High Tunnel (Location) - 0.00184 * 14-d Biomass gain (mg L^{-1}) - 0.00256 * Solar Irradiance (kWm^{-2}) - 2.4 * Experiment #2 (=1)	0.5595	F(4,16) = 5.08 p =0.0078	
Trans Zeatin riboside (tZr)	1.104 - 0.00004 * Solar Irradiance (kWm^{-2}) - 0.0294 * Average Peak Temperature (^oC) - 0.0267 * Trough Coating(Metal) (=1) + 0.0122 * Trough Coating(Red) (=1)	0.5701	F(4,16) = 5.30 p =0.0065	Coating (red) p = 0.0721)
Salicylic acid (SA)	18.294 - 3.5 * Experiment 2 (=1) - 0.0437 * Initial Biomass ((mg L^{-1}) - 0.0142 * 14-d Biomass Gain (mg L^{-1})	0.7793	F(3,17) = 20.01 p < 0.0001	
SA – with Solar Irradiance split out between Week 1 and Week 2	127.700669 - 18.8 * High Tunnel (Location) + 0.8830*Solar Irradiance days 1to7 (kWm^{-2}) - 0.9818*Solar Irradiance days 8to14 (kWm^{-2})	0.7953	F(3,17) = 22.01 p < 0.001	
Treatment Variables	Location: Within polyethylene High Tunnel or Outside of high tunnel. Initial Biomass (Inoculation Density): Initial Biomass, mg L^{-1} – actual density of each trough, improved accuracy of high and low inoculation densities designed to capture 91% and 84%, respectively, of maximum PAR on inoculation date.			
Growth Conditions Variables	Solar Irradiance (kWm^{-2}): 14-d average of cumulative hourly solar irradiance measurements from Colorado Agricultural Meteorological network, ARDEC station, adjusted for high tunnel light transmission (kWm^{-2}). DO: 14-d average dissolved O_2 at 14:00, % saturation. Average Peak Temperature: 14-d average culture temperature at 14:00, ^oC. 14-d Biomass Gain: 14-d net gain in biomass, mg L^{-1}, dry weight. Coating: Trough interior color, underneath clear plastic. Experiment: Experiment Number.			

Table 12. Comparison of Anabaena sp. on-farm phytohormone concentrations with phytohormone concentrations shown to provide plant growth promoting benefit. Concentrations from literature cited converted to nM. Phytohormones applied via root drench unless otherwise indicated. Sukor and Shariatmadari sourced their respective phytohormones from cyanobacteria, Zhang and Ervin from seaweed extracts.

Phytohormone	Concentration (nM)	Level of Beneficial Effect (nM)	Crop	Citation
ABA	4.42×10^2	2.37×10^5 (> concentration had adverse effect); 1.89×10^3 (foliar)	Tomato	Astacio and van Iersel, 2011; Barickman, 2014; Barickman et al., 2014
IAA	6.09×10^2	1.94×10^4	Mentha piperita L.	Shariatmadari, et al, 2015
IAM	4.39×10^2	Unknown		
ICA	3.73×10^2	Unknown		
SA	2.59×10^3	1.44	Lettuce Sweet Corn	Sukor, 2016
SA	2.59×10^3	1×10^4 to 5×10^5 (>1×10^6, adverse effect)	Tomato; Tomato, beans	Stevens et al., 2006; Senaratna et al., 2000
Trans Zeatin riboside (tZr)	13	1×10^4 (foliar)	Creeping Bentgrass	Zhang and Ervin, 2008; Chang et al., 2013
Trans Zeatin (TZ)	13 (tZr)	7.1×10^4 (TZ)	Tomato	Basra and Lovatt, 2016

Table 13. Reported plant beneficial effects of bacteria-sourced phytohormone concentrations applied to horticultural crops or turf grass.

Phytohormone	Reported Beneficial Effect	Crop	Citation
ABA	Reduced transpirational water loss; Increased leaf and fruit chlorophylls and carotenoids.	Tomato	Astacio and van Iersel, 2011; Barickman et al., 2014a,b
IAA	Increased plant biomass, concentrations of essential oils.	Mentha piperita L.	Shariatmadari, et al, 2015
SA	Increased water use efficiency	Lettuce, Sweet Corn	Sukor, 2016
SA	Salinity Tolerance; Enhanced tolerance to heat, chilling and drought stresses	Tomato; Tomato, beans	Stevens et al., 2006; Senaratna et al., 2000
Trans Zeatin riboside (tZr)	Heat, drought tolerance	Creeping Bentgrass	Zhang and Ervin, 2008; Chang et al., 2013
Trans Zeatin (TZ)	Improved plant growth, yield and fruit quality.	Tomato	Basra and Lovatt, 2016

Figures

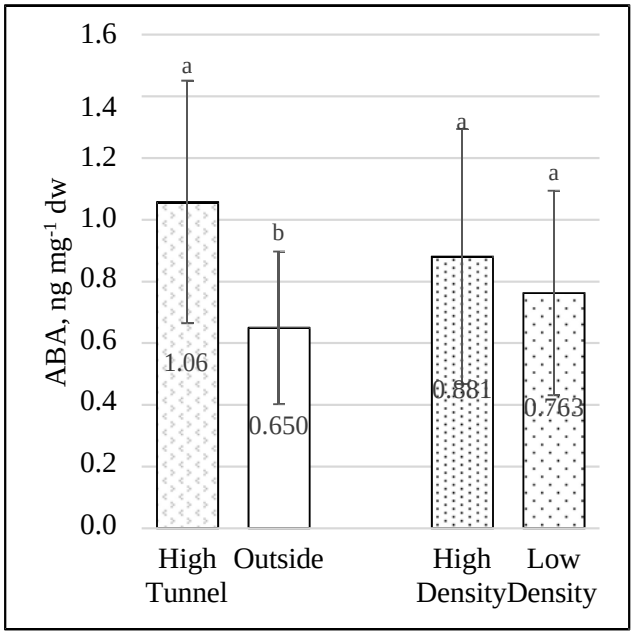

Figure 13. Endogenous abscisic acid (ABA) concentrations day 14 of batch culture by location and by inoculation density. High density and low density treatments are inoculation densities estimated to capture 91% and 84%, respectively, of maximum expected PAR on the inoculation date. Different letters are significantly different.

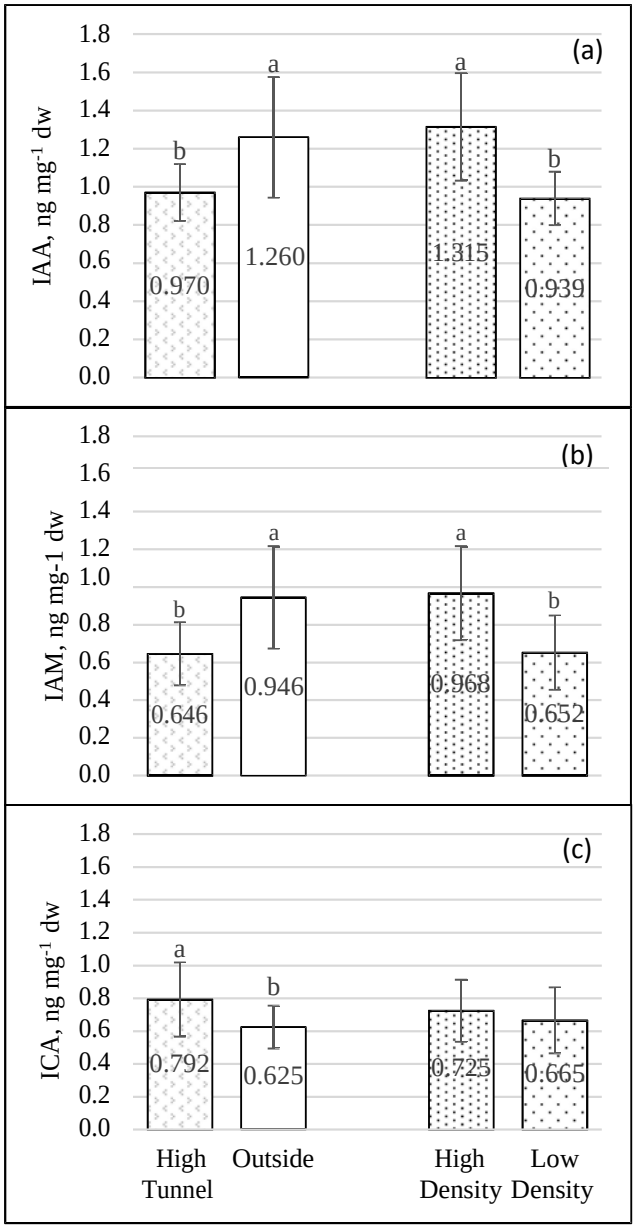

Figure 14. Endogenous indole-3 acetic acid (a), indole acetamide (b) and indole carboxylic acid (c) concentrations, day 14 of batch culture, by location and inoculation density. Troughs located in a polyethylene covered High Tunnel or Outside of high tunnel. High and Low Density are Anabaena densities that capture 91% and 84% peak PAR, respectively on day 1. Different letters are significantly different ($p < 0.05$).

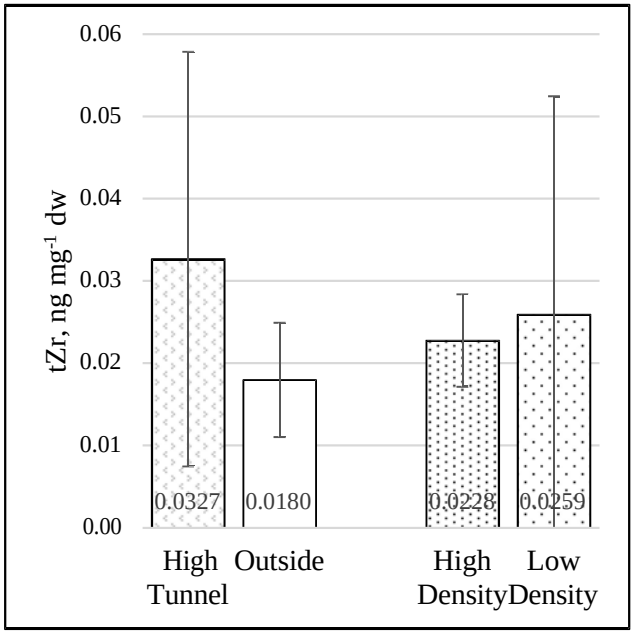

Figure 15. Endogenous trans Zeatin riboside concentrations, day 14 of batch, by location and inoculation density. Troughs located in a polyethylene covered High Tunnel or Outside of high tunnel. High and Low Density are Anabaena densities that capture 91% and 84% peak PAR, respectively on day 1. Different letters are significantly different ($p < 0.05$).

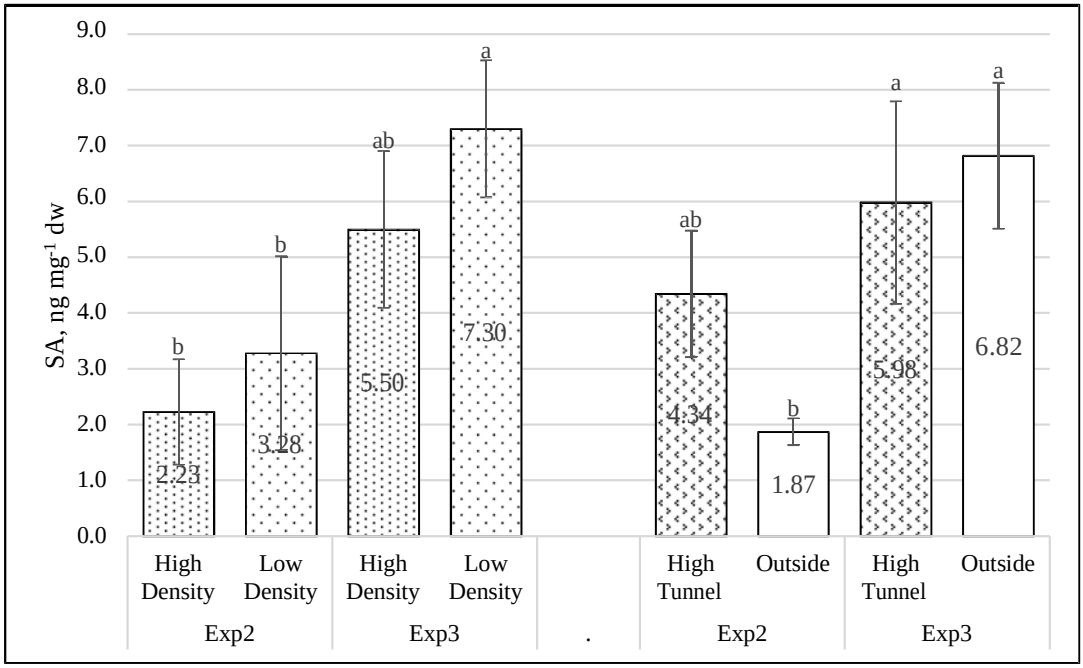

Figure 16. Salicylic acid concentrations, day 14 of batch, by location and inoculation density treatments, broken out by Experiment 2 and Experiment 3. Troughs located in a polyethylene covered High Tunnel or Outside of high tunnel. High and Low Density are Anabaena densities that capture 91% and 84% peak PAR, respectively, on day 1. Different letters are significantly different ($p < 0.05$).

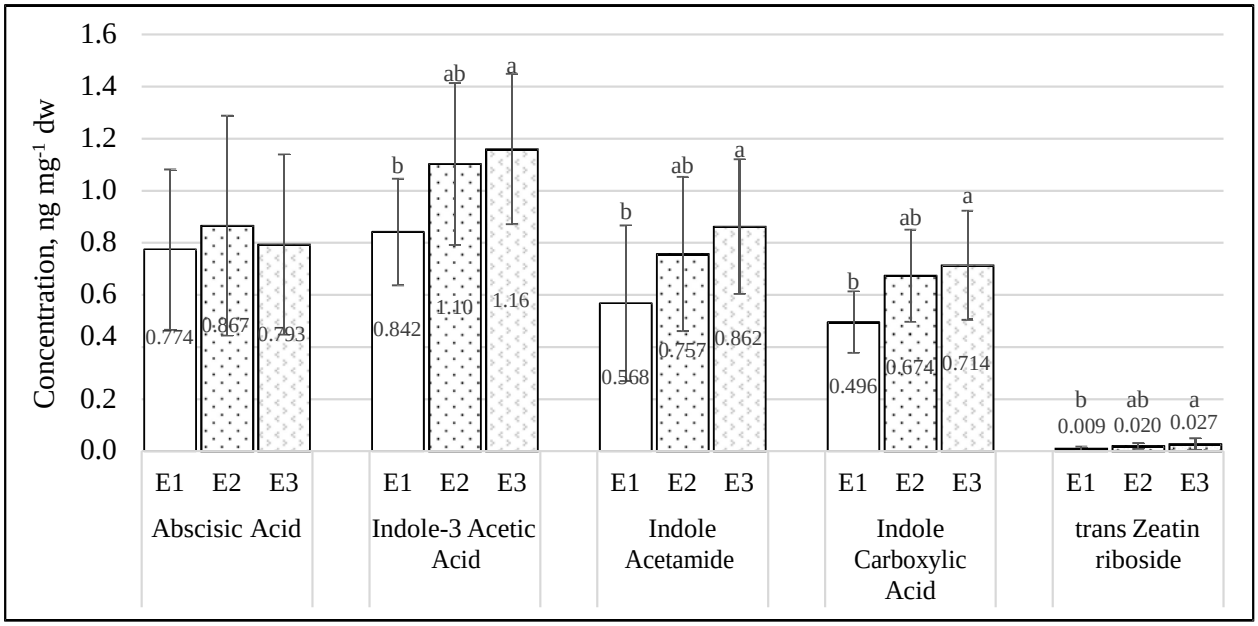

Figure 17. Endogenous phytohormone mean concentrations of Anabaena sp. culture, day 14 of batch by Experiment (E). Different letters are significantly different concentrations between experiments ($p < 0.05$). E1, n =12; E2, n =9; E3 n =12.

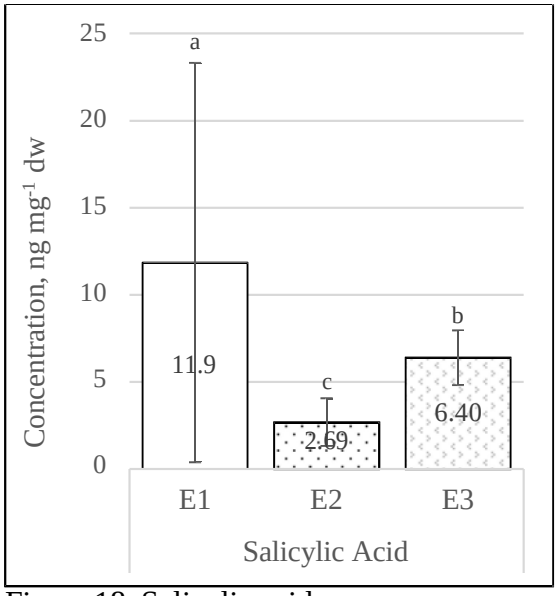

Figure 18. Salicylic acid mean endogenous concentrations, day 14 of batch by Experiment (E). E1, n =12; E2, n =9; E3 n =12. No troughs excluded from E1. Different letters are significantly different concentrations between experiments ($p < 0.05$).

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APPENDIX I: CO_2 SUPPLEMENTATION AT 9.5 PH OF AN ANABAENA SP. CULTURE GROWN IN OPEN RACEWAYS UNDER HIGH TUNNELS.

Introduction

As noted in Chapter 1, CO_2 supplementation of cyanobacterial cultures is required to maintain sufficient stocks of dissolved inorganic carbon (DIC) during mass production, particularly during peak periods of photosynthesis. Maintaining pH 9.0 or lower in cyanobacterial cultures ensures that most DIC is available as HCO_3^-, which cyanobacteria can utilize through the carbon concentrating mechanisms (CCM) (Giordano et al., 2005). Above 9.0, carbonate (CO_3^{2-}) is the primary form of DIC, and cannot be utilized for photosynthesis (Borowitzka and Moheimani, 2013). Below pH 8.0 could result in more volatile forms of CO_2, increasing the opportunity for atmospheric loss and decreasing the efficiency and cost effectiveness of supplementation (Putt et al., 2011). Limiting pH maximum to between 8.0 and 9.5 should be sufficient to enhance CO_2 availability and on-farm biomass and total nitrogen (N) yields of on-farm Anabaena sp. Supplemental CO_2 when pH reaches 9.5 is common in the production of Arthrospira platensis (Grobbelaar, 2012). The pH of Anabaena sp. cultures not supplemented with CO_2 regularly exceed 10.0 when exposed to sunlight. Supplementing CO_2 at a pH 9.5 threshold will provide additional DIC during the middle of the day when photosynthetic rates are highest while minimizing input costs of on-farm Anabaena sp. production. CO_2 supplementation at pH 9.5 was tested in production scale open raceways. The hypothesis was that supplementing CO_2 to prevent pH from exceeding 9.5 in open raceways under high tunnels will result in significantly higher 14-d biomass and N concentrations compared with control raceways.

Methods

Experimental Design

CO_2 supplementation of open raceways to 9.5 pH of open raceway batch cultures was conducted during July of 2014. Six 1.87m by 3.58m open raceways were installed on bare soil covered with weed block fabric in 6.1m x 15.2m east-west oriented high tunnels covered with 6 mil UV resistant polyethylene plastic (AT Films, Edmonton, AB Canada). Raceways were lined with 6 mil clear plastic. A central divider was installed to create a continuous channel within each raceway. Four of the raceways were located in the westernmost high tunnel at the research farm and two in the east high tunnel. In an effort to minimize potential differences in light and temperature between high tunnels and between north and south positions within each hoop house, the experiment was set up using a Latin square blocking structure. Each north/south pair of raceways received one control and one treatment. In the west hoop house east/west (row) raceway pairs received control and one treatment. In the west hoop house east/west (row) raceway pairs received one control and one treatment. The CO_2 treatment and control treatment were each applied to three raceways.

Raceways were filled to approximately 2,200 L, equivalent to 25 cm average depth from the bottom of the raceway. Paddlewheels powered by electric motors maintained a flow of at least 30 cm s^{-1}. Rosalyn Barminski (RB) growth medium (Barminski et al., 2014) was added to the raceway and allowed to mix prior to inoculation. Raceways were inoculated with Anabaena sp. the evening prior to day 1 at a mean optical density (750nm) (OD_{750nm}) of 0.011 +/- 0.003.

CO_2 Supplementation

Three raceways were fitted with a PINPOINT pH Controller (American Marine, Inc.) to continuously monitor pH. A brass AC110V solenoid (Duda Energy, Alabama, U.S. Model 2W-200-20N, ¾" fitting size) was attached to the pressure regulators on a 20lb compressed CO_2 tank and plugged into the pH controller. CO_2 was bubbled into the raceway using 1.5" x 0.5" Sweetwater ceramic diffusers attached to ¼" ID x 3/8" OD ATP Vinyl-Flex clear plastic tubing. The terminal 20 cm of tubing was inserted into a drilled two-liter bottle cap. The caps were then screwed into two liter bottles with 15 cm x 15 cm panels removed from one side (Figure 19). This allowed the bottle to remain partially submerged and was intended to increase residence time of the CO_2 with the surface of the water (Vasquez and Heussler, 1985). The pH controller for each raceway was set to open the solenoid when the pH reached 9.6 and bubble CO_2 until the pH reached 9.5.

Health, biomass and N concentrations of the production cultures were monitored between 14:00 and 15:00 as described in Chapter 1. 15 mL samples were collected at the same three data points within each raceway for measurement of optical density and microscopy. Every three to four days, 50mL of additional culture was sampled from the three data points in each raceway for measurement of total N and Anabaena sp. biomass.

Results

One of the control raceway cultures shifted from Anabaena sp. to one with algae resembling Scendesmus on day 2 of the batch production. The culture began shifting back to Anabaena sp. beginning day 13, but was excluded from statistical analysis. Mean 14-d biomass of the CO_2 supplemented raceways was 256 +/- 17 mg L^{-1} compared with 218 +/- 6 mg L^{-1} for the control.

14-d N concentrations were 31.2 +/- 3.1 for the CO_2 supplemented cultures and 28.0 +/- 1.2 mg L^{-1} for the control cultures. Neither difference was significant.

Repeated measures analysis of productivity showed that the CO_2 supplemented cultures had significantly higher productivity on days 8 and 11. Multiple regression with model selection to predict productivity using the treatment variable, CO_2 supplementation, the previous day's OD_{750nm}, the pH and temperature measured at sampling time, and average hourly solar irradiance identified solar irradiance as the only significant variable ($R^2 = 0.34$) for days 8 and 11 (see Table 14 for regression equations). Multiple regression with model selection to predict productivity using the same variables for the full 14-d batch identified culture density at the start of the 24hr daily productivity period, and pH and temperature measured at sampling time ($R^2 = 0.30$). Finally multiple regression to predict productivity of CO_2 raceways only identified OD_{750nm} at the start of the 24hr period, solar irradiance, and pH at sampling as significant variables ($R^2= 0.41$)

Discussion

CO_2 supplementation at pH 9.5 did not significantly increase biomass and total N concentrations compared with control raceways. The results fail to support the hypothesis that open raceways located inside high tunnels would achieve higher biomass and N concentrations if supplemented with CO_2 when pH exceeded 9.5.

The regression equations (Table 14) suggest that pH may still be a significant variable when CO_2 is supplemented at culture pH 9.5. Lowering the maximum pH to 9.0 may result in significant biomass and N gains as the availability of CO_2 increases in forms that cyanobacteria can take up (Badger and Price, 2003; Giordano et al., 2005; Burnap et al., 2013). However, it is

not clear whether such a difference would be sufficient to justify extra costs. Light availability, both light intensity and the density of the culture, were identified as significant variables influencing daily productivity. The greenhouse plastic currently used on the high tunnels is rated at 91% light transmission. Moving production out of high tunnels may improve light availability. However, removing production from high tunnels is not without risks. The high tunnel could play an important role in reducing photoinhibition, buffering low and high diurnal and seasonal temperatures, and reducing evaporation and contamination rates. Daily productivity never exceeded 30 mg L^{-1}, less than half that achievable under laboratory conditions in 14 days. Despite the risks, the increases to light intensity gained by fully exposing cultures to sunlight, coupled with CO_2 supplementation at pH 9.0, may be necessary and worthwhile in achieving significant and practical increases in batch concentrations of Anabaena sp. biomass and N.

Tables

Table 14. Multiple regression with model selection based on Mallow's C(p). Variables in selected model with $p > 0.05$ were removed prior to calculation of coefficients and model significance. CO_2 treatment raceways were supplemented with CO_2 when pH exceeded 9.5. Control raceways received no supplemental CO_2. Solar irradiance for 24hr hour productivity period was average of hourly measurements for Colorado Agricultural Meteorological network station "ARDEC".

Model	n	R^2	F-value	p-value (Model)	Notes
Productivity, all raceways, Days 8 and 11 = 1.875570 + 0.053043* Solar Irradiance (kWm^{-2})	5	0.34	F(1,18) = 9.09	0.0075	pH (p=0.0577) removed from initial model
Productivity, CO_2 and Control Raceways Days 1 to 14 = 18.725929 + 57.491754 * OD_{750nm}, start of 24hr period - 6.107157 * pH at sampling time + 1.686679 * Temperature (^oC)	70	0.30	F(3,66) = 9.46	< 0.0001	Solar Irradiance (p=.0509) removed from the initial model.
Productivity, CO_2 Raceways only Days 1 to 14 = -0.413176 + 75.126810 * OD_{750nm}, start of 24hr period - 1.300201 * pH at sampling time + 0.060203 * Solar Irradiance (kWm^{-2})	42	0.41	F(3,38) = 8.93	0.0001	

Figure 19. Raceway and CO_2 supplementation setup for on-farm high tunnel production of Anabaena sp.

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APPENDIX II: RECOMMENDATIONS.

Introduction

Despite increases in 14-d Anabaena sp. biomass and N concentrations, troughs located outdoors have failed to achieve higher biomass and N concentrations than troughs located within the high tunnel over 14-d batch periods. Therefore, additional trough-scale studies may provide additional clarity on production parameters, and further improve batch biomass and N concentrations. Determining whether there is a significant relationship between Fv/Fm and productivity, and identifying (or strengthening) any significant variables driving productivity should improve batch production methods. Once production parameters are more fully understood, and ideally, light availability is established as the primary limiting factor in production, experiments should be conducted at the raceway scale (at least 9.26m^2, 13cm to 25cm depth). These experiments would use CO_2 supplementation at pH 9.0 and the most productive inoculation rates identified in this study (capturing 84% PAR estimated on the inoculation date) to test methods for improving light availability. Growing cultures outside of high tunnels and installation of inexpensive delta foils, which have been shown to improve mixing and increase algal biomass concentrations by more than 25% are promising avenues for increasing biomass production further (Laws et al., 1983, Vaughan, 2013). When biomass and N concentrations have been maximized, additional studies should be initiated to identify the full range and concentrations of phytohormones present in Anabaena sp. production cultures.

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Productivity

Even when accounting for day-to-day variability in light intensity, no regression equation that predicted productivity included both light intensity and culture density as significant variables. This suggests that light is not the limiting factor (Fontes et al. 1987; Richmond, 2004). Higher biomass and N concentrations could be attainable if other limitations within the cultures are resolved. Only one treatment on one day across the two experiments showed significantly higher productivity. On Day 12 of Experiment 3 in 2016, low density high tunnel treatment had significantly higher biomass gain that the two outdoor treatments. This underscores the inability to establish a multiple regression equation to predict productivity based on the data collected.

Collecting additional data on culture conditions throughout the day could strengthen modeling efforts and identify significant production variables. For example, a pre-sunrise (daily low) and mid or late afternoon (peak) culture temperature would capture the full range of temperature stress the culture may experience. While temperature is difficult to control in outdoors open ponds, small changes in production practices could help buffer low or high temperatures by a few degrees if temperature is a significant variable. More frequent measurements of pH, such as every 30 to 60 min when pH exceeds 9.0 (e.g. 10:30 to 16:00 daily) would ensure that CO_2 supplementation is occurring evenly across all cultures and could also act as quality control on pH monitoring and CO_2 bubbling equipment.

The failure of the cultures grown outdoors to exceed 14d biomass and N concentrations of cultures grown in high tunnels suggests that light or energy limitation is not currently the only factor limiting production. The 13cm depth cultures exhibited a more olive green color at the end of the batch compared to cultures grown under high tunnels. This suggests, as noted in Chapter 1, that photoinhibition may be occurring. This was unexpected at higher densities, but may have

been a result of the reduced culture depths compared with previous experiments and field production. Furthermore, the 30cm trough sides shaded the surface of the 13cm deep culture to some degree before 11:00 and after 14:00 each day. It is possible that the reduction in surface area exposed to light (e.g., 30cm of 52cm width of trough surface was shaded until 08:00) when early morning and late afternoon light intensities were more ideal for photochemically efficient production (less photoinhibition), reducing biomass gains. Furthermore, when light reached the entire culture surface between 11:00 and 14:00, it was also the most intense, increasing the potential for photoinhibition and photodamage. Using a vessel with a height closer to the culture depth may allow for more biomass gain throughout the day and a more complete light acclimation as light intensities increase. It would also provide more accurate data on Fv/Fm early in the batch period.

Identifying and resolving, where possible, abiotic, non-light related production limitations of an Anabaena sp. culture should allow for development of a more reliable productivity curve, which will in turn aid in the identification of practical and cost effect batch period lengths and batch biomass and N concentration expectations.

Fv/Fm and Productivity

Identifying, and where possible, resolving culture-limiting variables so that light intensity or culture density are the primary factors in on-farm productivity would maximize the value of additional studies on Fv/Fm. Analyzing the relationship between Fv/Fm and productivity early in the batch period could help determine whether significant differences in Fv/Fm reported between inoculation densities are also practically significant. Additional Fv/Fm measurements, later in the batch period could (e.g., days 7 and 10) could confirm whether the observed differences in

culture colors during the 2016 summer trough scale experiments are indeed due to photoinhibition, and whether that photoinhibition translates to significant differences in productivity, as Vonshak et al., (2014) reported with A. platensis.

Mixing

Mixing will be an important factor in raceway production (Grobbelaar, 2012). The horizontal mixing provided by paddlewheels generally results in stratification of culture layers. Improving vertical mixing within the culture should improve mass transfer of nutrients, including CO_2, to the cyanobacteria, thereby increasing photosynthetic efficiency (Grobbelaar, 1994). Laws, et al. (1983), for example, saw a 200% increase in growth using foils to improve mixing in open raceways. The Utah State University (USU) raceway hydraulics group designed delta wings that improve vertical mixing (vs. the horizontal mixing provided by the paddlewheel) (Voleti, 2012; Lance, 2012; Vaughan, 2013). The USU group determined that delta wings at a 40-degree angle, spanning the width of the raceway channel, and placed every 1.65m could achieve sustained vortices throughout the raceway. When these delta wings were added to USU model raceways in a greenhouse, the biomass of Chlorella vulgaris (a green algae) increased by 27.1% compared with controls (Vaughan, 2013). Using the methodology described in Appendix I and Vaughan (2013), two 30.5cm (leg length) equilateral delta wings, made from 1mm thick aluminum sheeting were placed side by side for a total of five two-delta arrays were placed 5.4 ft apart along the channels of three raceways, except where the paddlewheel was attached. Three raceways served as controls. The experiment, conducted in August and September, 2014. There were no significant difference in biomass and N concentrations by 18d (Wenz, et al., 2015). However, cultures supplemented with CO_2, and grown under full exposure to sunlight may

benefit from improved mixing and achieve biomass gains similar to those reported by Vaughan (2013).

Phytohormones

If batch biomass and N concentrations are increased significantly beyond those in this study, a new and more complete phytohormone assay may be valuable, since phytohormone concentrations likely increase exponentially rather than linearly with biomass (Stirk, et al., 2013). At a minimum, an assay including a wider range of brassinosteroid, cytokinin, and giberrellin compounds should be conducted to confirm whether or not they reach detectable concentrations in an Anabaena sp. based culture. Measurement of exogenous concentrations in the media is likely to reveal greater concentrations of at least some phytohormones (Hartung et al., 2010; Hussain et al., 2010). Isolation to get a pure Anabaena sp. culture could help identify whether SA and ICA, which have not been reported as cyanobacterial metabolites prior to this study, are actually produced by Anabaena sp. From a practical standpoint, it would be challenging to maintain a xenic Anabaena sp. culture during outdoor production, so it may also be valuable to identify major constituents of the Anabaena sp. inoculum, including the protozoa resembling Ochromonas sp., one or two small algae frequently encountered (more so during culture decline), and bacteria that may contribute significant biomass to the culture. In addition to their identification, a study of the phytohormone production of these Anabaena sp. culture community members, individually and cooperatively could add to our understanding of Anabaena sp. growth and phytohormone production dynamics.

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