THESIS

DEVELOPMENT OF AN ORGANICALLY CERTIFIABLE GROWTH MEDIUM FOR N-FIXING CYANOBACTERIA IN A RACEWAY BIOFERTILIZER PRODUCTION SYSTEM

Submitted by

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In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Fall 2014

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ABSTRACT

DEVELOPMENT OF AN ORGANICALLY CERTIFIABLE GROWTH MEDIUM FOR N-FIXING CYANOBACTERIA IN A RACEWAY BIOFERTILIZER PRODUCTION SYSTEM

The on-farm cultivation of N-fixing cyanobacteria in raceway ponds may provide an alternative N source in organic farming systems. The cultivation of cyanobacteria in an organic farming system requires an organically certifiable growth medium. Additionally, efficient cyanobacterial cultivation depends on production methods that reduce the severity of the three growth limiting factors present in outdoor raceway cultivation: inefficient solar irradiance, growth medium nutrient depletion, and day-night temperature fluctuations. The purpose of this work was two-fold, first to develop and test an organically certifiable growth medium, and secondly to test four specific production methods so as to optimize cyanobacterial growth and N-fixation. The four raceway production methods tested separately included: batch (B) versus semi-continuous (SC) operation mode, a culture depth of 20-cm versus 25-cm, bicarbonate supplementation in the growth medium, and four different cover plastics over raceways. All studies used a cyanobacterium cultured from a Fort Collins, CO lake, with 99% similarity to *Anabaena cylindrica*. Cyanobacterial growth was estimated by optical density (OD) and chlorophyll content and cyanobacterial N-fixation was estimated by net Total Kjeldahl Nitrogen (TKN).

In chapter 2, “Comparison of cyanobacterial growth and nitrogen fixation in a newly developed organically certifiable growth medium and Allen and Arnon growth medium”, a laboratory and raceway study were conducted. In the lab study, the nutrients of Allen and Arnon

(AA) that were not organically certifiable were replaced with organically certifiable nutrients to compose the organic medium (RB). The exponential growth rate was significantly higher in the RB medium compared to AA. Conversely the net TKN in the RB medium was 37% lower than that of AA. The lower N-fixation in the RB medium was attributed to the presence of N in the P source used for RB medium (bone meal). In the raceway study, there was no significant difference in growth between the two treatments despite lower concentrations of P, Co, Zn, and B in the RB medium. An overarching limiting factor evident in both treatments such as light limitation or C depletion could explain why there was no observed growth effect due to the low P, Co, Zn, and B concentrations of RB medium. The net TKN between the two treatments was not statistically different, which suggests similar N-fixation. The conclusion of similar N-fixation was questioned due to the contribution of dissolved N from bone meal. Together, the studies support that the RB medium supports growth similar to that of the AA medium in raceway cultivation. However, since N was present in the RB medium, it is possible that maximal N-fixation was not achieved. Recommendations to increase nutrient concentrations in RB medium are discussed in chapter 4, “Future recommendations”.

In chapter 3, “Biomass yield and nitrogen fixation of cyanobacteria in outdoor raceways under batch versus semi-continuous operation”, a SC treatment was operated under a 25% harvest regime every other day beginning on day 6. The B treatment was grown for 14 days, then 85% of the treatment was harvested and the remaining 15% was used as seed to begin a second B set. At the end of four weeks, biomass yield and total N fixed was calculated for the B and SC treatments. There was no difference in biomass yield or N yield between the two treatments. More than likely the SC was harvested when the culture density was above the optimal cell density range, resulting in a lower total biomass and N yield than what could have been achieved

within the optimal cell density range. Determination of the optimal cell density and a specific harvest regime that maintains the SC within the optimal cell density would result in a higher total SC biomass and N yield compared to that of B. Possible experiments to determine the optimal cell density are discussed in chapter 4, "Future recommendations".

In Appendix II, "Cyanobacterial growth and nitrogen fixation in response to depth, bicarbonate supply, and hoop house coverings in outdoor culture", three separate batch studies were conducted in 1.2-m (l) by 0.6-m (w) by 0.3-m (h) tanks. The first experiment compared the growth and N-fixation of batch cultures grown at two different depths (20-cm and 25-cm). Raceway depth did not have an effect on total growth or net N-fixation. The second experiment compared cyanobacterial growth and N-fixation in AA medium supplied with 0 mM (control), 0.2 mM (low treatment), and 2.0 mM (high treatment) of potassium bicarbonate ($KHCO_3$). There was no increase in growth or N-fixation due to addition of $KHCO_3$. It was concluded that inadequate $KHCO_3$ was added to significantly increase growth and that the addition of $NaHCO_3$ rather than $KHCO_3$ is necessary to assure adequate Na concentrations needed for maximal bicarbonate uptake. The third experiment compared the growth and N-fixation of cultures grown under different hoop house plastics (Thermax, Luminance, Dura-film Super 4, and 4 mil Husky construction plastic) and a no-cover control. None of the covers tested in the study increased the growth compared to the no-cover control. Zn slowly leached from the cultivation tanks, so that by the end of the third study, Zn toxicity clouded the interpretation of results.

ACKNOWLEDGEMENTS

First, to both Dr. Heather Storteboom and Dr. Jessica Davis, who have been the most amazing advisors and mentors. Heather, you have so patiently and wisely guided me through every skill needed in the research world and it is because of you that I can move confidently into the next big step at ECHO. Jessica, thank you for presenting me with the opportunity to do my master's degree and for instilling great trust and confidence into my research. You have a huge role in building my passion for soil and crop research and I thank you for that. To Dr. Graham Peers, thank you for being willing to instruct me in the use of instruments and for very clearly explaining specifics of cyanobacterial growth. To my amazing research team: Sophia Barstad, Natalie Yoder, Arina Sukor, Phasita Toonsira, Katie Buchan, Yun-Ya Yang, Hong Wang, Keegan Athey- you ladies rock! All of you taught me what true teamwork looks like and I will always cherish the summer of 2013 with you gals. To Dr. Ann Hess, thank you for all of your statistics consultation. Your passion for teaching statistics motivated me to really understand statistics. To Dr. Jim Self, thank you for teaching me laboratory analysis. To the construction mastermind, Swanie Swanson, who greatly helped build the infrastructure needed in this project. Thank you to Solix BioSystems Inc, for very graciously providing our team with tank models and pumps. To the funding source that allowed this project to be possible-Western Sustainable Agriculture Research Education. And I can't forget my mommy and daddy, the two who were always there, ready to instill encouragement into me when I was discouraged in my research. To my amazing friends, Becky Boo, Kelsita, Alissa, Emily, and many more who were always ready to also speak encouragement to me. Finally, to the One who strengthens me, fills me with peace, and is my source of joy in the ups and downs of research- my Lord Jesus.

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CHAPTER 1: INTRODUCTION

Nitrogen (N) is the most limiting nutrient for farmers throughout the world, and yet there are limited N fertilization options for organic farmers. N sources such as composted manure or fish emulsion are seldom produced on-farm and must be transported, incurring both time and expense. Other sources such as cover crops that are produced in rotation with cash crops can become weeds in following crops and use limited water resources in dry areas (Dabney 2001). Clearly, an N fertilizer that can be produced on-farm, independently of primary cropland could be beneficial in organic agricultural systems. The cultivation of cyanobacteria is one possibility for an on-farm N biofertilizer.

Cyanobacteria are phototrophic, N-fixing bacteria commonly found in soil and water that can be cultivated on-farm to provide an alternative N source for organic farmers. Cyanobacteria are either unicellular or filamentous. Within filamentous cyanobacteria, there are non-heterocystous filamentous forms or heterocystous filamentous forms. Heterocystous, filamentous cyanobacteria have specific N-fixing cells called heterocysts where the enzyme nitrogenase is located. Nitrogenase catalyzes the following N-fixation reaction (Kaplan et al. 1986):

$N_2 + 6H^+ + 6e^- + 12MgATP \rightarrow 2NH_3 + 12MgADP + 12P_i$

Intercellular NH_3 is then assimilated into organic N compounds such as amino acids. N-fixation is an energy intensive process supplied by photosynthetic energy in cyanobacteria.

Cyanobacteria have to date been used as a biofertilizer in rice paddies and as a soil inoculum in dryland crops. Cyanobacteria are inoculated in rice paddies to establish cyanobacterial populations in the water and thus increase biological N fixation. Rice paddy inoculation can result in 30 kg of fixed N per hectare in flooded rice fields each season and a 10

to 20% increase in rice grain yield (Malliga and Subramanian 2002). The increased soil N-fixation due to cyanobacterial soil inoculation in wheat resulted in significantly higher dry weight, total nitrogen, and pigment content of wheat compared to wheat grown on soil that was not inoculated (Abd-Alla et al. 1994). In addition to supplying N, cyanobacteria secrete plant growth hormones and build soil organic matter (Malliga and Subramanian 2002).

Use of cyanobacteria in rice paddies and in soil as an inoculum is meant to establish populations of cyanobacteria to increase biological N-fixation. Rather than increasing biological N-fixation within the soil or rice paddies, our research focuses on mass, concentrated cultivation of cyanobacteria in order to apply cyanobacteria as an N fertilizer. The decomposition of applied cyanobacteria in the field results in plant-available N. Cyanobacteria are grown in shallow, circulating, elliptical ponds called raceways. A growth medium supports cyanobacterial growth and N-fixation in the raceways. After a complete growth cycle (approximately every 2 weeks), the liquid culture of cyanobacteria can be applied to crops via fertigation.

There are clear advantages to a cyanobacterial biofertilizer compared to typical organic N fertilizers. Raceways can be constructed on non-cultivatable land, and the production of cyanobacteria can occur simultaneously with cash crop production. Thus land and time allotted to the main cash crop is maximized as an N source is simultaneously produced. Additionally, the ease of application via drip irrigation requires minimal equipment for spreading and incorporating the biofertilizer.

The production of cyanobacteria in raceways is one culture technique that is cheap, feasible for on-farm production, and is a high biomass production system. However, if high biomass production of cyanobacteria specifically in organic farming systems is to be achieved, two challenges must be overcome: (1) The application of cyanobacterial biofertilizer on certified

organic land requires a growth medium of organically certified nutrients since the growth medium is also applied to crops. There is no published organically certifiable growth medium for cyanobacteria. (2) Raceway production of cyanobacterial biomass is greatly reduced by three limiting factors: inefficient use of solar energy, nutrient limitation (e.g., C), and temperature (Richmond 1986).

The stratification of light irradiance in a raceway causes poor conditions for optimal photosynthesis. The cells at the surface of the raceway experience high light irradiation that can cause photoinhibition, while cells at the bottom of the raceway experience darkness due to light absorption by biomass at the surface (Ritchie and Larkum 2012). The zones of high light and low light reduce photosynthesis and in turn, biomass production. As photosynthesis occurs in raceways, C is depleted. Without the addition of C (e.g. CO_2 or HCO_3^-) to raceways, severe C limitations can develop (Richmond 1999). In turn, production is further reduced. In outdoor production, cultures are exposed to night and day temperatures. The optimum temperature range for cyanobacteria is between 30 to 35 °C (Fontes et al. 1987). Nighttime temperatures can lower the culture temperature below the optimum growing range.

The severity of limiting factors can be reduced by specific production methods. A culture maintained at a lighter cell density can increase light penetration. Constant maintenance of a lighter culture density is achieved by operation of a semi-continuous culture rather than a batch culture. A semi-continuous culture requires frequent biomass harvests and subsequent medium replenishment. A shallower depth can reduce the extent of the unilluminated strata where little net photosynthesis occurs in raceways (Ritchie and Larkum 2012). Also photoinhibition due to high light intensity can be decreased by operating raceways under a hoop house cover (Richmond et al. 1990). A hoop house cover can also maintain heat throughout the night and thus

reduce the negative growth effect due to low night temperatures. A C nutrient limitation can be avoided by the direct addition of C either in the form of CO_2 or HCO_3^-.

The overall objective of this work was to address these two challenges in cyanobacterial production by: (1) Developing an organically certifiable growth medium that can support growth and N-fixation comparable to a commonly-used non-organic cyanbacterial growth medium. (2) Reducing the severity of limiting factors by testing the growth and N-fixation in two culture depths, different concentrations of C supplementation, two operation modes, and covering raceways.

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CHAPTER 2: COMPARISON OF CYANOBACTERIAL GROWTH AND NITROGEN FIXATION IN A NEWLY DEVELOPED ORGANICALLY CERTIFIABLE GROWTH MEDIUM AND ALLEN AND ARNON MEDIUM

Preface

On-farm cultivation of the phototrophic, N-fixing microorganism, cyanobacteria, could provide organic farmers an alternative N source. Cyanobacteria are grown in shallow, circulating ponds called raceways and supplied with a growth medium. The application of a cyanobacterial biofertilizer on certified organic land requires a growth medium of organically certified ingredients. The goal of this work was to develop an organically certifiable growth medium that can support cyanobacterial growth and N-fixation in raceway cultivation comparable to that achieved in Allen and Arnon medium (AA). One lab study and one field study were conducted. Both studies used a cyanobacterium cultured from a Fort Collins CO lake, with 99% similarity to *Anabaena cylindrica*.

In the lab study, the nutrients of AA that were not organically certifiable were replaced with organically certifiable nutrients to compose the organic medium (RB). The cyanobacterial growth (estimated by optical density (OD)) and cyanobacterial N-fixation (estimated by net Total Kjeldahl Nitrogen (TKN)) in RB medium was compared to the AA medium in the lab study. The exponential growth rate was significantly higher in the RB medium. Conversely, the net TKN in the RB medium was 37% lower than that of the AA medium. The lower N-fixation in the RB medium was attributed to the presence of N in the P source used for RB medium (bone meal).

n the field study, there was no significant difference in growth between the two treatments despite lower concentrations of P, Co, Zn, and B in the RB medium. An overarching

limiting factor evident in both treatments such as light or C depletion could explain why there was no observed growth effect due to the low P, Co, Zn, and B concentrations in RB medium. There was no difference in net TKN between the two treatments, which suggests similar N-fixation. The conclusion of similar N-fixation was questioned due to the contribution of dissolved N from bone meal. Together, the studies suggest that the RB medium supports growth similar to that of the AA medium in raceway cultivation. However, since N was present in the RB medium, it is possible that maximal N-fixation was not achieved. Improvements for the RB medium, specifically to increase the Co and P concentrations, are discussed.

Introduction

The use of N-fixing cyanobacteria as a potential organic N biofertilizer requires mass cultivation of cyanobacteria in outdoor raceways. After a complete growth cycle (approximately every 2 weeks), the liquid culture of cyanobacteria is applied to crops via fertigation. Large-scale cultivation of N-fixing cyanobacteria requires a growth medium that specifically encourages high biomass production and N-fixation. Many N-free growth media have been developed for N-fixing cyanobacteria by first modeling the nutrient concentrations in the natural environment in which cyanobacteria thrive (Mandalam and Palsson 1998). Further development of media was by modification of an already existing growth medium (Allen 1952). Sufficiently high concentrations of nutrients are present in media in order to assure a non-growth limiting quantity (Lee and Shen 2004). However, very few media have been developed based on detailed study of actual nutrient requirements.

This study specifically compared cyanobacterial growth and N-fixation in two media, Allen and Arnon medium (AA) and an organically certifiable growth medium (RB) developed by the author. The application of cyanobacteria to organic crops requires a growth medium that

contains organically certified chemicals approved by the Organic Materials Review Institute (OMRI). OMRI reviews chemicals against the National Organic Standards to determine whether those chemicals should be allowed in certified organic production. Production of cyanobacteria thus far by this research team has used N-free Allen and Arnon medium (AA) (Allen and Arnon 1955). However, AA contains specific chemicals noted as "prohibited" on the OMRI Generic Materials List, which are therefore not allowed in certified organic production. Currently, there is no published organically certified, N-free growth medium for cyanobacterial cultivation. Thus, the purpose of this work was to develop an organically certifiable growth medium that can support cyanobacterial growth and N-fixation comparable to that of AA growth medium. To achieve this goal, the following objectives were identified: (1) Develop an organically certifiable medium (RB) based on the target concentrations of nutrients supplied in AA; (2) At lab-scale, compare the growth and N-fixation of cyanobacteria cultured in the developed RB medium to those grown in AA; (3) At field-scale, compare the growth and N-fixation of cyanobacteria cultured in the developed RB medium to those grown in AA.

Methods

Nutrient substitutions

The chemicals used in AA medium that are prohibited on the OMRI Generic Materials List were replaced with chemicals listed as allowed or allowed with restrictions to compose the RB medium (Table 1). The theoretical nutrient concentration in AA was used to determine the appropriate amount of each substitute chemical for the RB medium (Allen and Arnon n.d). There were multiple chemical substitutes available for Co, Fe, and Mn. The substitute with the highest water solubility was selected. The limited substitutes available for P included rock phosphate and bone meal. Both are P fertilizers intended for use in acidic environments due to their limited P

dissolution in basic pH's (Nelson and Janke 2007). Cyanobacteria grow best in a basic environment, which necessitates a basic growth medium. It was understood that P concentrations in RB medium (pH 7.13) would be lower than P concentrations of the AA medium due to the limited solubility of either organic P source in a basic medium. The P mineral in bone meal is more soluble than rock phosphate and therefore, bone meal was selected as the P source for RB medium (Nelson and Mikkelsen 2008).

Microorganism

A xenic culture of *Anabaena cylindrica* was used for this study. The heterocystous filamentous, N-fixing cyanobacteria used for inoculation of all experiments was cultured from sediment collected from Richard's Lake in Fort Collins, CO and maintained in AA medium. DNA was extracted as described by Morin et al. (2010). Sequence analysis of a 446 bp region of 16S DNA was amplified with the primers and conditions described in Nubel et al. (1997). The region was cloned and sequenced. Clones shared 99% similarity with *Anabaena cylindrica*.

Lab study

The lab experiment was set up as a randomized complete block design (RCBD) with four replicates and two treatments: RB medium and AA medium. The 0-10-0 Hi-Yield® Bone Meal was delivered in the RB medium by preparation of a stock solution. A stock solution of water and bone meal was prepared by dissolving 87.16 g L^{-1} of bone meal and shaking (150 rpm) the solution for 1 h. The stock solution was filtered to remove bone meal debris, and 0.75 L of the filtered 87.16 g L^{-1} bone meal stock was used in the RB medium. See the Appendix for calculations of the bone meal quantity (Calculation 1AI). A sample of the filtered 87.16 g L^{-1} bone meal stock was also analyzed for trace nutrients by inductively coupled plasma spectroscopy (ICP) at the Colorado State University (CSU) Soil, Water, and Plant Testing Lab.

Cyanobacteria were grown for fourteen days in 500 mL Erlenmeyer flasks. Flasks were agitated at 150 rpm on a G10 Gyrotary orbital shaker (New Brunswick Scientific Co., Inc., Edison, NJ, USA) under a 2500-lux grow light (Growl LED Inc., Wildomar, CA, USA) that operated with a 12-h/12-h light/dark cycle. To prevent carry over of cellular nutrient reserves from culture maintenance in AA medium, the cells used for the RB treatment were cultured for two weeks in the RB medium before use as the RB treatment inoculum (Lange 1974). A 1:10 culture to media ratio was used. Optical density (OD) and chlorophyll content were used as estimators of growth. Triplicate subsamples from each flask were analyzed to measure OD and chlorophyll content at 665 nm. OD was determined spectrophotometrically with a microplate reader (Model 680, Bio Rad Laboratories, Inc., Japan). OD was measured every other day for the first seven days and every day for the remaining seven days. For each experiment, the medium that cyanobacteria were cultured in was used as the blank. Chlorophyll was analyzed on days 0, 7, 10, and 14. Chlorophyll was extracted from samples as described by Charpy et al. (2010), and the chlorophyll content was determined using Eq. 1 (Montana State University n.d)

$$C = \frac{D}{(d * a)} \quad \text{where} \qquad (1)$$

C = chlorophyll *a* concentration (mM)
D = optical density of chlorophyll extract
d = inside path length of spectrophotometer (0.26-cm)
a = extinction coefficient for chlorophyll *a* (75.05 L/mmol-cm)

During the fourteen-day growth period, N-fixation was estimated by net Total Kjeldahl Nitrogen (TKN). TKN is an analysis of organic N and NH_4^+. One sample from each flask was analyzed for initial and final TKN. The initial and final nutrient concentrations of AA medium and RB medium were analyzed by ICP at the CSU Soil, Water, and Plant Testing Lab.

Field study

The experiment was conducted in July 2013 at the CSU Horticulture Research Center in Fort Collins, CO. Two 16-m by 6-m hoop houses (WEST, EAST) were used in the study. Within these hoop houses, there were six 5.6-m by 1.8-m by 0.3-m raceways prototype raceways, each with a surface area of 9.6 m^2 (4 in hoop house WEST and 2 in hoop house EAST). To provide mixing during the study, each raceway contained a 6-blade aluminum paddlewheel powered by a 60-hertz motor (Dayton Electric Mfg. Co., Niles, IL, USA). Paddlewheels ran continuously between 6AM and 8PM each day and were run for two 30-min segments during the night. It was difficult to obtain the same paddlewheel rotational speed for all raceways, and the average paddlewheel rotational speed for raceways with AA medium and RB medium were 38 ± 9-cm s^{-1} and 47 ± 14-cm s^{-1}, respectively. No supplemental CO_2 was added to the raceways. Due to the size limitations when scaling up to field conditions, 15 g L^{-1} bone meal was used in the RB medium in the field experiment. See the Appendix (Calculation 1AI) for details of how this concentration was determined. Bone meal was contained in 47-cm x 38-cm muslin bags that were placed in the raceways for the entire experiment. This inhibited dispersal of bone meal particles throughout the culture, which would cause turbidity. Both AA and RB treatment raceways were inoculated in a 1:23 dilution ratio with inoculum previously cultivated outdoors in the RB medium. Maximal culture depth was 0.25-m. Media was made with municipal water, and water loss from evaporation was replenished daily.

Cyanobacteria were grown in the prototype raceways in a replicated RCBD with three replicates and two treatments: RB medium and AA medium. Triplicate subsamples from each raceway were analyzed daily to measure OD and chlorophyll content. Samples were immediately stored at 4 °C and analyzed the same day. One subsample from each raceway was measured

every other day for TKN. TKN was determined using a HACH Simplified TKN instrument (TNT 880, Hach Company, Loveland, CO, USA). All samples were frozen (-20 °C) prior to analysis. Net TKN was calculated by $TKN_{day\ x}$–$TKN_{day\ 0}$. The initial and final media nutrient concentrations of each raceway were analyzed (P, Mg, Ca, Na, Mo, Zn, Cu, B, V, and Co) by ICP at the CSU Soil, Water, and Plant Testing Lab. The samples were centrifuged for 10 min (3500 rpm) to remove cyanobacterial biomass. The supernatant was then frozen (-20°C) until analysis. Cyanobacterial biomass was removed from nutrient samples before analysis. Duplicate subsamples from each raceway were microscopically analyzed to qualitatively monitor health and estimate heterocyst frequency on days 7, 10 and 14. Heterocyst frequency (number of heterocysts/total cell number) was determined according to Allen 1952; Hitch and Millbank 1975; Henskens et al. 2012. A Biological Microscope (model MT4310H, Meiji Techno Co., LTD., Japan) was used for microscopy. Water temperature, pH, and dissolved oxygen (DO) were measured daily between 11:30AM and 1PM with an Orion 5 Star portable meter (Thermo Fisher Scientific Inc., Beverly, MA, USA). Readings were taken from the east and west side of each raceway. Three times a week, samples were taken between 1:30PM and 2:30PM to analyze the efficiency of electron transfer through photosystem (PS) II (quantum yield of PS II) (Fv/Fm). This was measured with a dual pulse amplitude modulation (PAM) fluorometer (DUAL-PAM-100, Walz, USA). Samples were dark adapted (1 h) and then filtered on a Millipore 13-mm glass fiber prefilter prior to analysis.

N dissolution from bone meal

Despite the advertized 0-10-0 NPK analysis of Hi-Yield® Bone Meal, N was detected in the bone meal used in the RB medium. To estimate the maximum amount of N from bone meal that could be dissolved into the RB medium in the field study, N dissolution from two separate

Hi-Yield® Bone Meal bags was analyzed in duplicate over a two week period in a lab study. Lab-sized muslin bags similar to those used in the field were filled with 15 g L^{-1} bone meal (concentration used in the field study) and placed at the bottom of Mason jars. The RB medium (230 mL) was aliquoted into the Mason jars. The jars were capped and shaken (100 rpm) for two weeks on a G10 Gyrotary orbital shaker. The initial and final TKN, $NH_3 + NH_4^+$, and $NO_3^- + NO_2^-$ of the RB medium was measured in each Mason jar using a HACH Simplified TKN instrument.

Field bone meal reduction study

A laboratory experiment tested if a 57% reduction in the bone meal amount used in the field would decrease the P concentration of the RB medium. The two treatments were RB medium prepared with 15 g L^{-1} and 6.5 g L^{-1} of bone meal. To simulate field conditions, the bone meal was supplied within a lab-sized muslin bag and placed at the bottom of 500 mL Erlenmeyer flasks. Flasks were arranged in a RCBD with three replicates per treatment on a G10 Gyrotary orbital shaker (100rpm) under a 2500-lux grow light that operated with a 12-h/12-h light/dark cycle. A 1:9 inoculum to medium ratio was used, and inoculum cells were depleted of P before use by cultivation in P-free RB medium (no bone meal) for 24 h before use as inoculum. The initial P concentration of the RB medium, the chlorophyll content of cyanobacteria (day 0, 5, 7, 10, 12, 14, 16 and 17), and final cyanobacterial biomass of the two treatments were compared.

Statistical analysis

Data were analyzed using SAS 9.3 (SAS Institute Inc., Cary, NC). Alpha was set at 0.05 to determine significance for all tests. Replicate was not found to be significant in any experiment evidenced by $p>0.05$ for replicate effect in two-way ANOVA analyses. Thus, all two-way ANOVA analyses were run as one-way ANOVA in order to exclude replicate in the

model. Treatment difference in OD exponential and linear growth rates and nutrient concentrations were obtained from a one-way ANOVA (PROC MIXED). Treatment difference in chlorophyll and initial, final, and net TKN for the lab study were also obtained from a one-way ANOVA (PROC MIXED). Due to multiple day measurements for TKN, chlorophyll, in the field study, a repeated measures analysis was used (PROC MIXED). A repeated measures analysis was also used to analyze multiple readings for heterocyst frequency, Fv/Fm, DO and pH. An ANCOVA was used in the field study to test for a paddlewheel speed effect since paddlewheel speeds vary across and within treatments. The paddlewheel speeds were only measured on day 10 of the field study, thus day 10 values for TKN, chlorophyll, Fv/Fm, OD, DO, and heterocyst frequency were evaluated in an ANCOVA with paddlewheel speeds as the covariate.

Results and discussion

Lab study: cyanobacterial growth and N-fixation in RB medium versus AA medium

When OD data were *ln* transformed, two bacterial growth phases were evident: the exponential from day 0 to 7 and the linear growth phase from day 8 to 14 (data not shown). RB medium had a significantly higher exponential growth rate than AA (Table 2). There was no treatment difference in the linear growth rate or in the initial, mid-way, or final chlorophyll content. Conversely, the net TKN of AA was significantly higher compared to RB (Table 2). Assuming net TKN represents the N in a culture due to N-fixation, the difference in the net TKN between the RB medium and AA medium represents a reduction in RB N-fixation of 37% compared to the AA medium. The lower N-fixation in the RB medium was attributed to the presence of N in the P source used for RB medium (0-10-0 NPK Hi-Yield® Bone Meal). The significantly higher initial TKN of the RB medium (Table 2) suggested dissolution of N from

bone meal into the RB medium. It was verified that the higher initial TKN was not due to a difference in the TKN of the inoculum used for both the AA and RB treatments. Cyanobacteria reduce N-fixation as N (NH_4^+, NO_3^-, organic N sources such as amino acids) is available for uptake from the growth medium (Layzell et al. 1985; Liengen 1999). The activity of N-fixation in the RB treatment was verified by the positive difference in the final TKN of the RB treatment (average 28.79 mg L^{-1}) and initial TKN (average 11.27 mg L^{-1}). The difference can be attributed to N-fixation since bone meal was filtered out of the RB medium, eliminating further N dissolution from bone meal during the study. Thus, it appears that cyanobacteria fixed N, and the decline in N-fixation can be attributed to simultaneous assimilation of the N from bone meal.

The higher initial N concentration of RB medium may also explain the higher exponential growth rate for the RB treatment. There is a lower energy expenditure for assimilation of reduced N forms to that of N-fixation (Herrero et al. 2001). Therefore, cells spent less energy on N-fixation due to the supply of N, and more energy could be used for growth. This was supported by Layzell et al. (1985) who found that a 17% higher *Anabaena flos-aquae* growth rate was obtained when the culture fixed N while supplied with NO_3^- (5 mM) compared to a culture with no added NO_3^-. It is likely that as the N from bone meal was used and depleted, N-fixation by cyanobacteria increased, and the growth rate of RB declined to that of AA in the linear phase. From this experiment, it can be concluded that the RB medium can support cyanobacterial growth comparable to that of AA, but due to the N contribution from bone meal, RB did not support as much N-fixation as AA.

Lab study: nutrient concentrations in RB medium versus AA medium

The initial concentrations of RB medium were significantly lower than AA medium in B, Cu, Fe, P and Zn and higher in Ca, K, Mg, Mn, Na, S, and V. Bone meal contains other nutrients

in addition to P. The 87.16 g L^{-1} bone meal stock had detectable levels of Ca, K, Mg, Mn, Na, and V. The supply of these nutrients from bone meal could explain the higher concentration of these nutrients in RB. In the following field study, the RB nutrients that were of higher or lower concentration to that of AA are discussed. Reasons for the higher and lower concentrations of nutrients, the function of those nutrients in the cell, and the effect due to higher or lower concentrations are discussed.

Field study: effect on measurements due to varying paddlewheel speeds

For all the measurements considered, only DO was affected by the varying paddlewheel speeds among raceways as determined by ANCOVA ($p<0.05$). Therefore, only the analysis of DO included paddlewheel speed as a covariate in the repeated measures analysis.

Field study: cyanobacterial growth in RB medium versus AA medium

The growth and N-fixation of cyanobacteria cultivated in outdoor raceways with the RB medium and the AA medium were compared. When OD data were *ln* transformed, two bacterial growth phases were evident: the exponential and linear growth phases (Fig. 1). Growth rates for each phase were calculated, and the RB exponential growth rate was compared to the AA exponential growth rate. The same was done for the linear growth rate. There were no statistical differences between the treatment growth rates of the exponential or linear phases suggesting that the RB treatment supported cyanobacterial growth similar to that of AA. When the chlorophyll content was analyzed for each measured day, the RB treatment was significantly higher than AA on day 4 and every day from 6 to 14 (Fig. 1). A possible explanation for higher chlorophyll content in the RB medium is discussed in the following section.

Field study: cyanobacterial N-fixation in RB medium versus AA medium

Only on days 6 and 8 was the net TKN of the RB treatment significantly higher than that of AA ; all other days were not significantly different (p<0.05) (Fig. 2). This would at first suggest similar N-fixation between the two treatments, which was surprising given that N-fixation was suppressed in the lab study. In the lab study, a reduction in N-fixation was supported by two results: one, the significantly higher initial TKN of the RB medium which suggested N dissolution from bone meal, and two, a significantly lower final net TKN in the RB medium to that of AA. Neither of these results was observed in the field study. However, the low initial TKN of the RB medium in the field study can be explained by the manner bone meal was delivered and should not be cited to suggest that dissolution of N from bone meal did not occur during the study. Bone meal was delivered in large muslin bags that encouraged slow dissolution of N from bone meal over time. At time 0, N dissolution from bone meal was minimal and did not increase the initial TKN of the RB medium.

Dissolution of N from bone meal, and furthermore cyanoabacterial utilization of that N in the field study can be verified by the results of the N Dissolution from Bone Meal lab study and the higher chlorophyll content of the RB in the field study. The average amount of final TKN in the RB medium resultant from the release of N from bone meal contained in muslin bags was 5.21 mg L^{-1} (Table 3). The results of this lab study suggest that N was probably added to the RB medium from bone meal in the field study.

Chlorophyll content is affected by nutrient deficiencies. One general response to deficiencies in N, P, S, Si, Mg, Fe, K, and Mo is a decrease in the content of photosynthetic pigments such as chlorophyll *a* content per cell and an increase in carbohydrate content (Kaplan et al. 1986). Thus, any of these specified nutrients that are lower in the AA medium compared to

the RB medium and at deficient concentrations could account for the lower chlorophyll content per cell in the AA medium. Of these specified nutrients, only S, Si, and Mg are at lower concentrations in the AA medium compared to the RB medium (Table 1A). S, Si, and Mg would have to be at deficient concentrations in AA medium to result in lower chlorophyll content. Both Mg and S concentrations were probably not at deficient concentrations in AA medium because both were above the theoretical concentration of AA (Table 1A). Si is a micronutrient (Kaplan et al 1986) and is required in concentrations similar to other micronutrients (Fe, B, Mn, Mo, Zn, Cu, V, and Co). The measured AA concentration of Si in the field study (69.08 μM) was above all theoretical concentrations of micronutrients in AA, which suggests it was not at deficient concentrations in the AA medium. Additionally, Si was not provided by stock solutions in the AA.

The significantly higher chlorophyll content in RB and lower chlorophyll content in AA may confirm a slight N_2 deficiency in the AA treatment that was not evidenced in the RB treatment due to the presence and uptake of combined N from the bone meal. N deficiency can occur in raceway production when low turbulence limits N_2 gas dissolution into the culture (Fontes et al. 1987). According to Richmond and Grobbelaar (1986), the average stirring rate of RB (47-cm s^{-1}) and AA (38-cm s^{-1}) treatments were slow stirring speeds. In their study, which used raceways about 5 times smaller in surface area and a culture depth 10-cm shallower than our depth, a stirring rate of 58 cm s^{-1} was considered slow due to the reduction in the output rate at high population densities compared to faster paddlewheel rotational speeds. If both AA and RB raceways were limited in N_2 due to the slow turbulence, addition of N by bone meal and cyanbacterial uptake of provided N could result in less N limitation and in turn a higher chlorophyll content compared to AA. Sanz et al. (1995) found that the chlorophyll content of

Anabaena variabilis increased when supplied 0.1 mM NH_4^+ or 0.1 mM NO_3^- compared to cultures solely relying on N_2.

The gradual release of N from bone meal over two weeks may have contributed to the net TKN in RB from day 2 to day 14. Therefore, despite similar net TKN in the RB medium and the AA medium throughout the study, not all the net TKN in RB should be assumed to be solely from N-fixation, but also from N dissolution of bone meal. If RB N-fixation was equal to that of AA in the field, a significantly higher final (day 14) net TKN of RB would be expected due to the additional 5.20 mg L^{-1} contribution of N dissolved from bone meal over two weeks that was predicted from N Dissolution from Bone Meal lab study. The final net TKN of RB was not significantly higher than AA, and therefore, a reduction in RB N-fixation in the field study can be inferred.

The exact % reduction in N-fixation cannot be quantified as done in the lab study; however, the hypothetical maximal % reduction can be calculated. Assuming the average 5.20 mg L^{-1} TKN dissolved from muslin-bag contained bone meal over two weeks in the N Dissolution from Bone Meal lab study simulated the field study, the subtraction of 5.20 mg L^{-1} TKN from the final net TKN (12.25 mg L^{-1}) of RB medium in the field study represents the amount of TKN from N-fixation (7.04 mg L^{-1}). Compared to the final net TKN of AA (10.99 mg L^{-1}), a maximal 36% reduction in N-fixation is hypothesized in the RB treatment, similar to that found in the lab study. This is presented as maximal % reduction because the N Dissolution from Bone Meal lab study may have overestimated the amount of N dissolution from bone meal in the field study. A proportional amount of bone meal to medium in the field study was used in the N Dissolution from Bone Meal lab study; however, the surface area of bone meal to water was different across studies. The large quantity of bone meal compared to the size of the muslin bags

resulted in bone meal being highly compacted into the muslin bags used in the field study. The smaller scale of the lab study, allowed bone meal to not be compacted into muslin bags, and thus, there was a higher bone meal to medium surface area. Water weakens the protein-mineral bond of bone meal and encourages N release from bone meal (Endt and Ortner 1984). An increase in the bone meal surface area to water encourages dissolution of the protein-mineral, increasing N dissolution from the bone meal in the N Dissolution from Bone Meal study compared to the field study.

Literature review of the effect of combined N additions on N-fixation

There have been studies conducted to determine the reduction in *Anabaena* sp. N-fixation when N is added to the growth medium (Layzell et al. 1985; Bone 1971; Liengen 1999; Kaplan et al. 1986; Sanz et al. 1995; Rhee and Lederman 1983; Mishra 1997). In these studies, the form of N (organic N, NH_4^+, or NO_3^-) added was specified because the form of N affects the degree of N-fixation reduction. All forms (organic, NH_4^+ and NO_3^-) can be assimilated by cyanobacteria. However, cyanobacteria preferentially use NH_4^+ compared to NO_3^- and organic N because NH_4^+ is the most reduced N form needed for assimilation into cellular organic N (Kaplan et al. 1986). Therefore, NH_4^+ also reduces N-fixation to a greater degree than NO_3^- and organic N (Liengen 1999).

It is difficult to use these studies as a comparison to our study for further estimation of the % reduction in N-fixation that occurred in the field study. All published studies determine the effect on N-fixation due to a single form of N added into the medium. However, N dissolution from bone meal contributes all three forms of N into the RB medium at once (Table 3). Additionally, the majority of studies use much higher concentrations of combined N than those in our study.

Field study: heterocyst frequency

There was no significant difference in heterocyst frequency between the two treatments on day 7, 10 or day 14 (Fig. 1AI). Heterocysts only differentiate when there is insufficient N (NH_4, NO_3 and organic N) in the medium to fully support growth (Smith et al. 1987). The N released from bone meal was not enough to fully support growth, as it takes 20 to 40 mM NO_3^- to fully support *Anabaena cylindrica* population at 6 to 8 gm dry wt L^{-1}(Allen and Arnon 1955). It is probable that cyanobacteria of the RB treatment relied on both N-fixation and uptake of N from bone meal due to insufficient concentrations of N from bone meal to fully support growth. Layzell et al (1985) have found that an uptake of combined N, even if the culture continues to fix N, is coupled by a decrease in heterocyst frequency due to the large energy expenditure to maintain heterocysts. However, the heterocyst frequency is affected by other factors in addition to the N concentration in the medium, which may explain similar heterocyst frequency despite the suspected reduction in N-fixation of the RB treatment. One factor that affects heterocyst frequency is the extracellular Ca^{2+} concentration (Smith et al. 1987). The RB medium had a significantly higher Ca^{2+} (0.38 mM) concentration than the AA medium (0.15 mM). Extracellular Ca^{2+} regulates both heterocyst frequency and nitrogenase activity. Smith et al. (1987) found that an increase in extracellular Ca^{2+} concentration increases heterocyst frequency and decreases nitrogenase activity in *Nostoc* sp. The higher Ca^{2+} concentration in the RB medium could explain why the possible reduction in N-fixation of the RB medium was not coupled with a reduction in heterocyst frequency.

Field study: nutrient concentrations in RB medium versus AA medium

The RB medium had significantly higher initial concentrations of Ca and Mg and significantly lower initial concentrations of P, Co, Zn, and B compared to AA (Table 1A). In the

lab study, the contribution of K, Mn, and V in addition to Ca and Mg increased the concentration of these nutrients to higher concentrations than that of AA. However, less bone meal per liter of RB medium was used in the field study compared to the lab study, which may explain the lower K, Mn, and V concentrations in the RB medium. The lower P concentration can be explained by limited P dissolution from bone meal in a basic pH medium. The lower Co concentration was due to the water insolubility of $CoCO_3$. The lower Zn and B concentrations in both field and lab study were surprising, as the chemicals and concentrations used in the RB medium were the same as the AA chemicals. It is possible that the Zn and B concentrations were lowered by a precipitation reaction with another chemical in the RB medium.

There was no concern of growth inhibitory concentrations with the higher Ca (15 mg L^{-1}) and Mg (10.84 mg L^{-1}) concentrations in the RB medium. Both Ca and Mg are macronutrients in cyanobacteria and are needed in large quantities (Kaplan et al 1986). One lab study reported that 20 mg L^{-1} Ca produced optimum growth of *Anabaena cylindrica* (Allen and Arnon 1955). Ca is required in cell wall formation (Kaplan et al. 1986). Mg is required for the uptake of phosphate, is a component of chlorophyll, and the activation of ATP requires a bond with Mg (Healey 1973).

P, Co, Zn, B are needed for specific cellular functions, and therefore adequate concentrations of each nutrient are important. P is also a macronutrient and a component of the energy rich ATP molecules that are needed for energy transfer. B is a key component in the maintenance of heterocysts (Bonilla et al. 1990). Co is a component of the vitamin B_{12}, which is a cofactor for the B_{12}-dependent methionine synthase (Kaplan et al. 1986). Zn is an essential component of photosynthetic electron transport enzymes and carboxysomal carbonic anhydrase (the catalyst for dehydration of cytoplasmic bicarbonate) (Cavet et al. 2003; Tang et al. 2013).

There was no statistical difference in growth between AA and RB treatments, despite significantly lower P, Co, Zn, and B concentrations in the RB medium. Two explanations for this are highlighted: One, the P, Co, Zn, and B nutrient concentrations were sufficient to support similar growth to that of AA because the lower nutrient concentrations were still within the necessary nutritional requirement for high biomass production. Two, the lower nutrient concentrations were not sufficient to support high biomass production similar to AA, but lower RB growth was not observed either due to an overarching limiting factor evident in both treatments that equally capped growth, or the presence of N in the RB medium reduced the need for these nutrients in N-fixation, and thus, lower concentrations were acceptable.

Nutrient concentration range for high biomass growth

The required concentration for each nutrient in a growth medium for high biomass production depends on many external factors such as light, temperature, pH, growth rate, and population density (Kaplan et al. 1986). Due to the difficulty in identifying a specific P, Co, Zn and B requirement for *Anabaena cylindrica*, these nutrient concentrations in the RB medium were compared to the P, Co, Zn and B concentrations in three media specifically made for high biomass production: AA, BG-11_0 and D medium (Table 4) (Castenholz 1988).The RB concentrations of P, Co, and Zn were lower than the concentration range formed by the three media, and B was within the high biomass media range. This suggests that P, Co, and Zn may not be at sufficient concentrations to support high biomass like that of AA and other media made for high biomass growth. Rather, similar growth between AA and RB treatments was achieved due to an overarching limiting factor or the effect of N in the RB medium. Of particular importance, P and Co were at or below nutrient concentrations of Chu-10 (modified) medium, a medium that is less nutrient concentrated and specifically made for low biomass growth and

simple cell maintenance (Castenholz 1988). Obviously, increasing the concentration of P Co, and Zn nutrients should be a focus and is discussed in Chapter 4 "Future recommendations".

Field study: photosynthetic activity, stress, and limiting factors

P, Co, and Zn concentrations in the RB medium may have been below sufficient levels to support high biomass production comparable to that of AA. The similar growth between treatments despite the lower RB concentrations could be explained by an overarching limiting factor evident in both treatments. The major limiting factors in phototrophic microalgae growth include temperature, light, and CO_2 (Richmond 1986). Because photosynthesis is affected by all three limiting factors, photosynthetic activity can be used to identify the presence of a limiting factor. Fv/Fm and the DO are measures of photosynthetic activity. Photosynthesis is the first physiological function to respond to stress, thus a rapid decline in either measurement indicates that the culture is stressed (Richmond 1999). A rapid decline in DO or Fv/Fm in one treatment, while the other treatment remains level, would suggest one culture was more stressed than the other. There was no treatment difference in Fv/Fm or in DO (Fig. 3). A typical Fv/Fm value for for cyanobacteria is 0.5, although this is affected by the species, the irradiance, the chlorophyll content, and the phycobiliprotein content (Campbell et al. 1998; Harel et al. 2004). For the majority of the days, both RB and AA Fv/Fm values were below 0.5, suggesting reduced efficiency of photosystem II due to some stress. AA and RB treatments were similarly stressed despite below optimal nutrient concentrations (P, Co, and Zn) in the RB medium. This could be due to an overarching limiting factor such as temperature, light or CO_2 supply that masks the effect of low nutrient concentrations in RB medium (Richmond 1999).

The temperature range of the culture was not growth limiting (21.6 to 37.5 °C for the RB treatment and 21.8 to 36.05 °C for the AA treatment). The optimum temperature range for

Anabaena sp. growth is 30 to 40 °C (Guerrero et al. 1988). However, Fontes et al. (1987) reported that *Anabanea varabilis* maintained at a constant 30 °C resulted in the same productivity of *Anabanea varabilis* cultured outdoors that experienced uncontrolled temperature fluctuations from 20 to 35 °C.

Light and CO_2 supply are more likely to be limiting factors. The average irradiance within a raceway is either in excess (during a dilute culture) or deficient (a dense culture), marking light as a constant overriding limiting factor in raceway production (Vonshak et al. 1982). Cyanobacteria are sensitive to high light intensity, and the photosystem II is damaged (photoinhibition) by high light irradiance (Richmond 1999). A decline in Fv/Fm indicates exposure to excess radiation usually due to a dilute culture concentration (Richmond 1999). The Fv/Fm declined from day 1 to day 3 in both treatments (Fig. 3), suggesting the initial culture population was dilute enough that both treatments were stressed due to excessive light. As cell density increases in batch cultures cultivated in raceways, the average irradiance within the raceway becomes limited due to mutual shading (Kromkamp et al. 2009). Light deficient growth can explain the change in growth rate from exponential to linear phase growth (Sinetova et al. 2012). In a photobioreactor system, the increase in pigmentation (chlorophyll content) during the linear phase was most likely due a response to light deficiency (Sinetova et al. 2012), which was also seen in this experiment. The greatest increase in chlorophyll content over time (slope) occurred from day 7 to 10, as the cultures were in transition from the exponential to the linear phase (Fig.1).

Carbon composes 50% of the dry matter of microalgae cells and thus represents a crucial nutrient (Kaplan et al. 1986). A severe C limitation rapidly develops when C is not added to an actively growing culture, resulting in reduced growth and productivity (Richmond 1999). Culture

pH rises continuously as a result of C depletion through photosynthesis (Richmond 1999). C-limitation can be identified by a pH that exceeds 9.0 (Giordano et al. 2005). Both treatments exceeded pH 9.0 on day 4 and remained above 9.5 for the remainder of the study, suggesting CO_2-limited growth (Fig. 4). As cyanobacteria uptake CO_2 in a non-buffered system, the pH increases and reduces the CO_2:HCO_3^- ratio. Between a pH of 9.0 and 9.5, cyanobacteria become dependent on the energy expensive uptake of HCO_3^- due to limited CO_2 availability. Above a pH of 9.5, most of the carbon is in the form of CO_3^{2-} and unavailable for cyanobacteria (Giordano et al. 2005). The presence of both light and CO_2 limitations in this experiment can cause an interaction between light and CO_2 (Young and King 1980). Essentially, the effect of light limitation on the growth rate was dependent on the intensity of CO_2 limitation. In turn, both CO_2 and light could be overriding limiting factors that capped growth in both treatments.

N in the RB medium may have reduced the P, Co, Zn, and B requirement

As explained earlier, it is probable that cyanobacteria in the RB treatment simultaneously fixed N and assimilated N provided by bone meal, which implies a reduction in N-fixation to that of AA. The reduction in N-fixation could have reduced the requirement for P and B since these two nutrients are required for N-fixation. B is a key component in heterocyst structure, and without B, the heterocyst envelope is prone to O_2 diffusion (Bonilla et al. 1990). Nitrogenase activity in N-fixing cyanobacteria is related to the availability of phosphate in the cultures (Kaplan et al. 1986). ATP is required for nitrogenase activity (Stewart and Alexander 1971).

In a chemostat culture under steady state growth conditions provided with a continuous P concentration (0.05 mM P), the P cellular content and PO_4 assimilation rate of *Anabaena flos-aquae* were compared in cultures provided with NO_3^- (5mM), NH_4^+ (5mM), or solely N_2 (Layzell et al. 1985). The cellular P content and PO_4 assimilation rates were higher in the culture relying

completely on N_2 fixation compared to cultures provided with NO_3^- and NH_4^+, which may support that P requirements in RB were lower. However, in another chemostat culture with a continuous P concentration (0.002 mM), there was no difference between the P requirement of cells provided with 0.25 mM NO_3^- and cells with no combined N in the medium (Rhee and Lederman 1983).

Iron concentrations

An adequate supply of bio-available Fe is very important in cyanobacterial growth media (Gibson and Smith 1982). Fe is required for photosynthetic electron transport proteins such as ferredoxin and cytochromes; the reduction of organic compounds; DNA, RNA, and chlorophyll synthesis; and is a component of the iron-molybdenum cofactor that catalyzes the nitrogenase enzyme (Sun et al. 2005). The Fe concentration for both AA and RB initial and final media samples was below the detection limit (<0.018 μM of Fe). The field Fe concentrations of RB and AA were below the lab study concentrations (0.6 μM for RB and 2.0 μM for AA) despite the same theoretical Fe concentration from lab to field for each medium. The field concentrations in both RB and AA were below the minimal non-limiting requirement for prokaryotic cells (0.4 μM to 4 μM) (Weinberg 1989). A chelating agent could be used to increase soluble Fe concentrations in the field RB medium, as detailed in Chapter 4 "Future recommendations".

In the AA medium, the use of ethylenediamine tetraacetic acid (EDTA) chelates Fe to assure Fe remains soluble. The majority of growth media use a chelating agent such as EDTA, nitrilotriacetic acid, trisodium hydroxyethylene diaminetriacetate, or citric acid. Available dissolved Fe in oxygenated water is oxidized and forms Fe^{3+} oxides. The solubility of these oxides is very low, and cyanobacteria can hardly take up this Fe form (Hori et al. 2003). In order to avoid Fe deficiency, the use of a chelating agent is very important to assure solubility by

preventing formation of Fe oxides (Weinberg 1989; Kosakowska 2007). EDTA is not allowed as an organically certifiable source, and no chelating agent was used in the RB medium. Potential chelating agents that are allowed in the RB medium include: citric acid, humic acids that are not fortified with a prohibited substance, tartaric acid, synthetic lignin sulfonate, and nonsynthetic amino acids. The possibility of using one of these chelating agents is discussed in Chapter 4 "Future recommendations".

Conclusion

The purpose of this work was to develop an organically certifiable growth medium that would support cyanobacterial growth and N-fixation comparable to that of AA medium in outdoor, raceway cultivation. The development of RB medium in the lab study resulted in an organically certifiable growth medium that supported similar cyanobacterial growth comparable to that of AA medium. However, due to the N contribution from bone meal (the organic P source), N-fixation was reduced by approximately 37% in the RB medium. The growth and N-fixation in the RB medium was compared to AA medium in outdoor raceway production. RB medium supported similar growth to AA despite significantly lower P, Co, Zn, and B concentrations compared to AA. N-fixation in the RB medium was most likely reduced compared to the AA medium due to the N dissolution from bone meal. Ultimately, as a biofertilizer, the RB treatment still provides the same amount of N as AA medium. However, not all N in the RB treatment is derived from N-fixation; some is provided by bone meal. To improve the RB medium, P, Zn, Co, and Fe concentrations should be improved by various means suggested in Chapter 4 "Future recommendations".

TABLES

Table 1. The OMRI-certifiable chemicals used in the organic medium (RB) that replaced non-organic chemicals in the Allen and Arnon medium (AA).

Nutrient	AA Source	RB Organic Source
P	K_2HPO_4	Hi-Yield® Bone Meal
K	K_2HPO_4	KCl
Ca	$CaCl_2\ 2H_2O$	Hi-Yield® Bone Meal
Fe	FeNaEDTA	$FeSO_4$
Mn	$MnCl_2\ 4H_2O$	$MnSO_4$
V	NH_4VO_3	----------
Co	$CoCl_2\ 6H_2O$	$CoCO_3$

Table 2. Growth and N-fixation parameters of a xenic culture of *Anabaena cylindrica* grown in RB and AA media in lab study. The exponential growth rate was calculated by the increase in the ln transformed OD over first 7 d of the study. N-fixation was estimated by net Total Kjeldahl Nitrogen (TKN in mg L^{-1}). Distinct letters indicate significant difference (p<0.05) as determined by one-way ANOVA. (n=4, mean ± standard deviation).

Treatment	**Avg. exponential growth rate**	**Avg. TKN**		
		Initial	**Final**	**Net**
AA	0.212 ± 0.023 b	4.20 ± 1.84 b	31.91 ± 1.39 a	27.71 ± 0.66 a
RB	0.268 ± 0.026 a	11.27 ± 2.41 a	28.79 ± 4.44 a	17.53 ± 5.09 b

Table 3. The N concentration and N form in RB medium (no cyanobacteria) after two weeks of dissolution of bone meal contained in muslin bags in the N Dissolution from Bone Meal lab study. Two different bags of Hi-Yield® Bone Meal were used (Bag 1 and Bag 2). Results are averages of duplicate samples per bone meal source ± standard deviation.

TKN Analysis (mg L^{-1})			
Bone Meal Source	Initial	Final	Final Avg.
Bag 1	0.00	7.03 ± 1.88	5.21 ± 2.38
Bag 2	0.00	3.40 ± 0.47	
N Form Analysis (mg L^{-1})			
Bone Meal Source	Final NH_4^+	Final NO_3^-	Final organic N
Bag 1	3.77 ± 0.57	0.70 ± 0.11	3.26 ± 1.32
Bag 2	0.61 ± 0.56	0.47 ± 0.08	2.78 ± 0.09

Table 4. The P, Co, B, and Zn theoretical concentrations of three high biomass media (BG-11_0, D, and AA) (Castenholz 1988). The initial P, Co, B and Zn concentration of the RB medium in the field study were compared to the nutrient range formed by the three high biomass media.

Medium	P (mM)	Co (µM)	B (µM)	Zn (µM)
BG-11_0	0.17	0.20	44.80	0.75
D	0.78	0.10	4.04	0.87
AA	0.76	0.04	11.66	0.19
Nutrient Range	(0.17 to 0.78)	(0.04 to 0.20)	(4.04 to 44.80)	(0.19 to 0.87)
RB	0.04	0.02	9.40	0.02
AA	0.48	0.05	11.19	0.18
RB medium within nutrient range				
	No	No	Yes	No

FIGURES

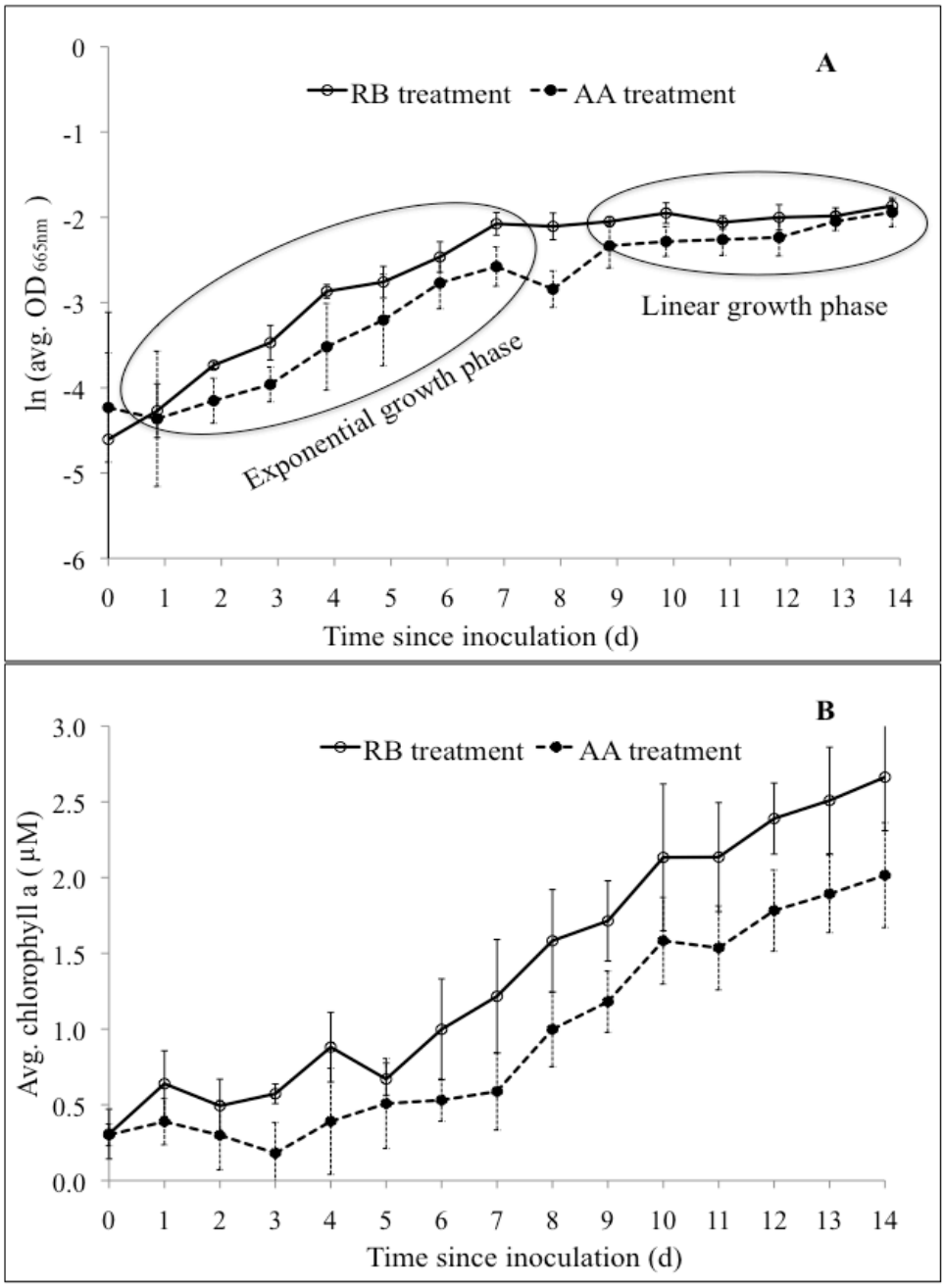

Figure 1. The natural log transformed optical density (OD) (A) and chlorophyll *a* content (B) of a xenic culture of *Anabaena cylindrica* cultivated in outdoor raceways in two media: an organically certifiable medium (RB) and Allen and Arnon (AA). Error bars represent standard deviation (n=3). The exponential and linear growth rate was calculated as the slope (A). There was no significant difference in treatment growth rates (A), and on days 4 and 6 to 14, RB chlorophyll content was significantly higher compared to AA as determined from repeated measures analysis (p<0.05).

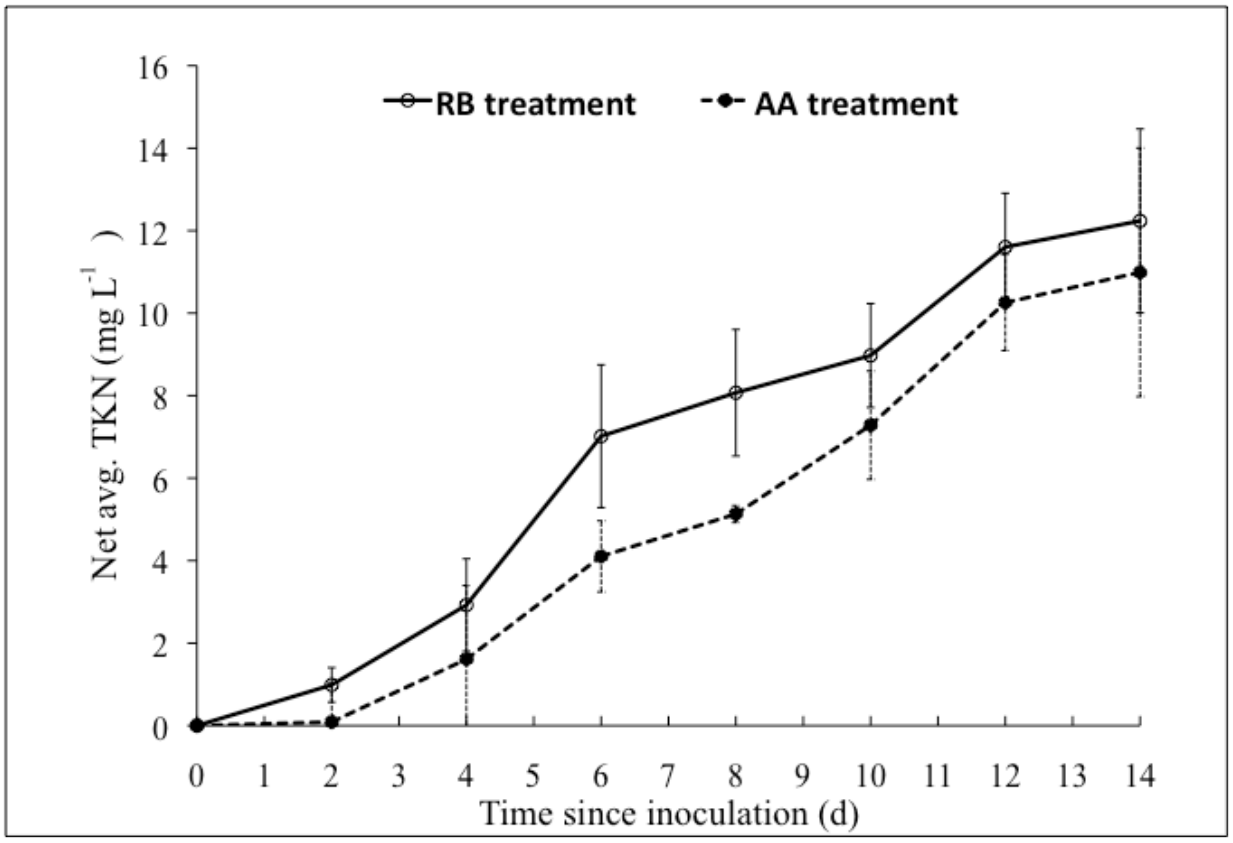

Figure 2. The Total Kjeldahl Nitrogen (TKN) culture of a xenic culture of *Anabaena cylindrica* cultivated in outdoor raceways in two media: an organically certifiable medium (RB) and Allen and Arnon (AA). Error bars represent standard deviation (n=3). On days 6 and 8, RB was significantly higher than AA in TKN as determined by repeated measures analysis (p<0.05).

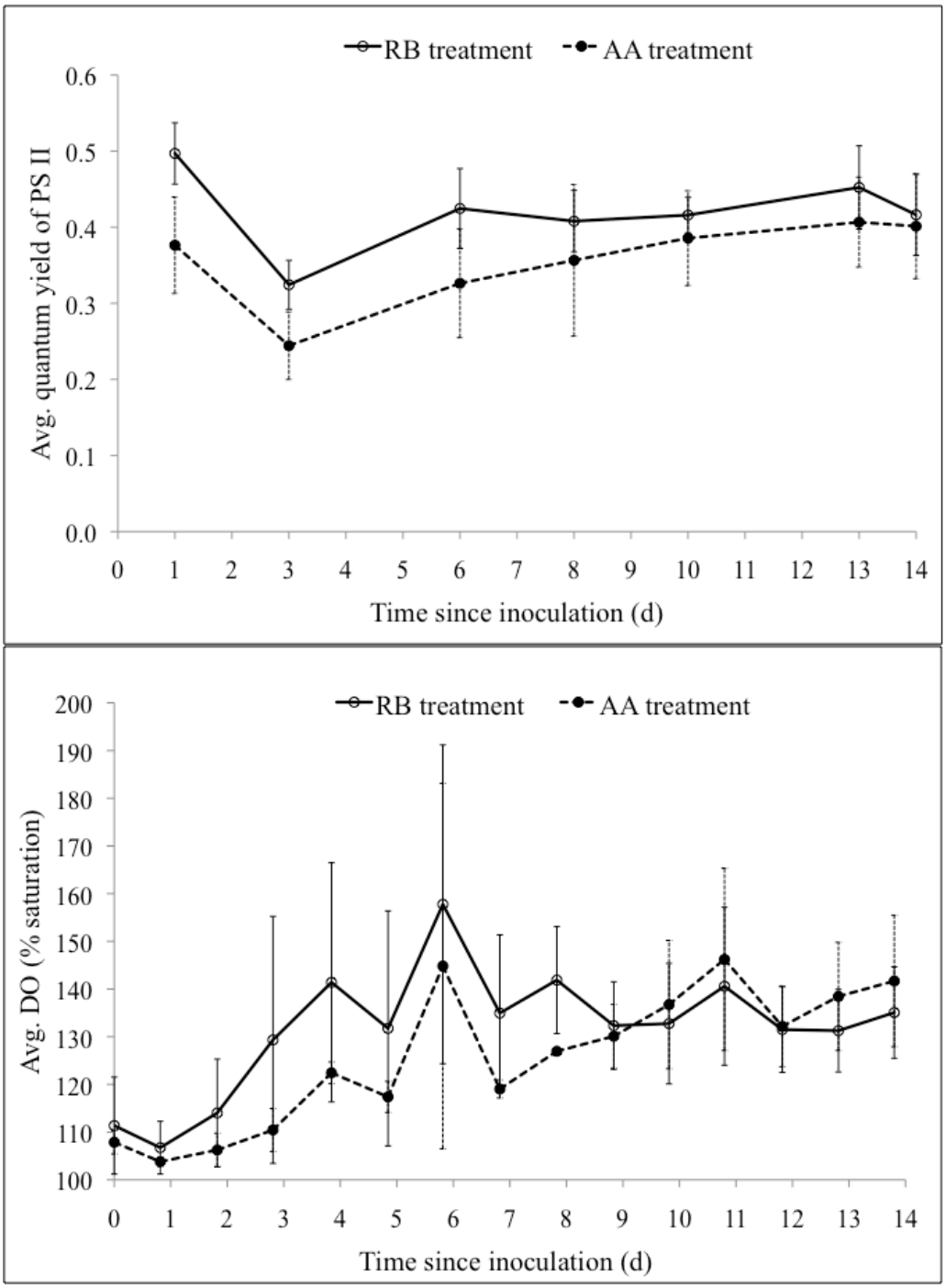

Figure 3. The quantum yield of photosystem II (PS II) (Fv/Fm) (A) and dissolved oxygen (DO) concentration (B) of a xenic culture of *Anabaena cylindrica* cultivated in outdoor raceways in RB and AA media measured between 12PM to 2PM. Error bars represent standard deviation (n=3). Only on Day 1 was RB significantly higher in Fv/Fm compared to AA, and there were no treatment differences for DO, as determined by repeated measures analysis (p<0.05).

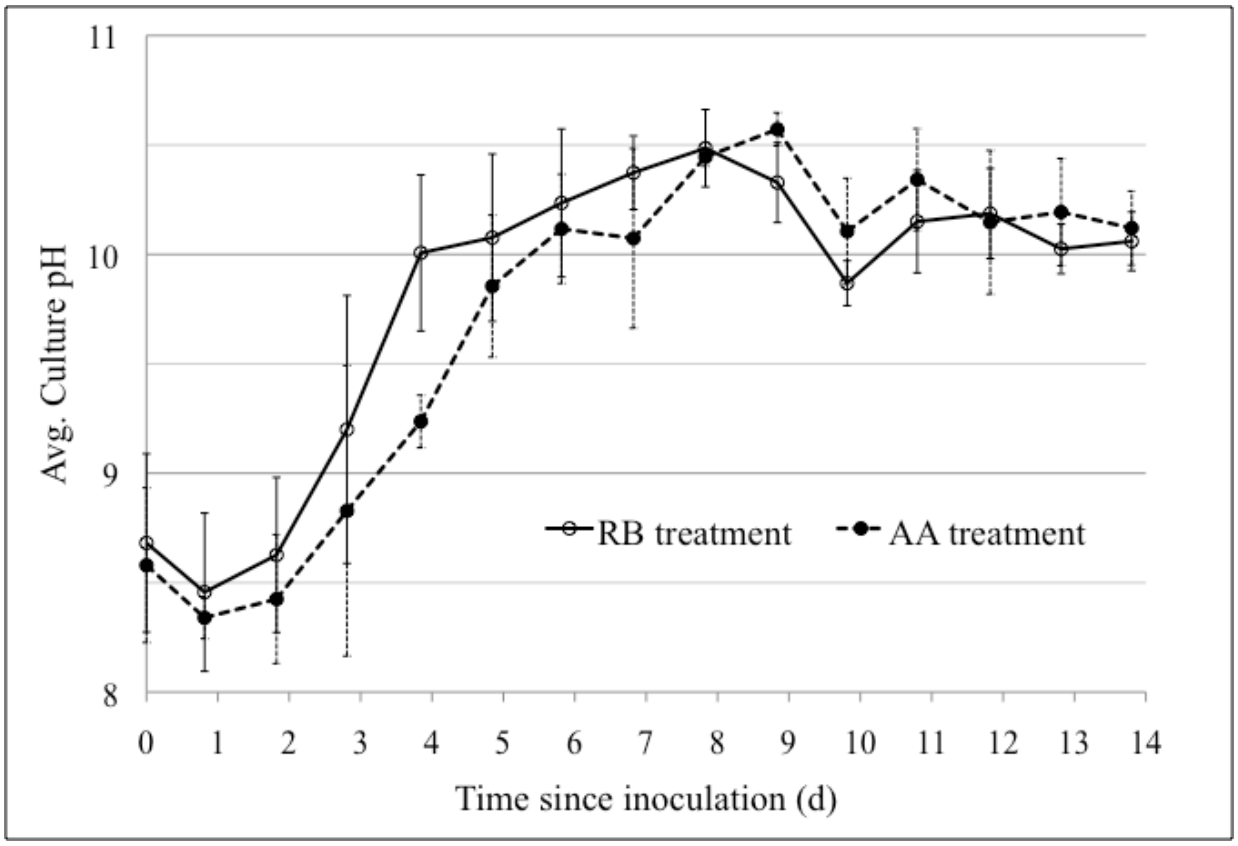

Figure 4. The pH of a xenic culture of *Anabaena cylindrica* cultivated in outdoor raceways in RB and AA media measured between 12PM to 1PM. Error bars represent standard deviation (n=3). RB was significantly higher than AA on day 4, as determined by a repeated measures analysis (p<0.05).

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CHAPTER 3: BIOMASS YIELD AND NITROGEN FIXATION OF CYANOBACTERIA IN OUTDOOR RACEWAYS UNDER BATCH VERSUS SEMI-CONTINUOUS OPERATION

Preface

N-fixing cyanobacteria can be cultivated in raceways to provide an organic, on-farm N fertilizer. Increasing biomass and N yield in raceway cultivation of cyanobacteria is critical to success. Two operational modes in raceways were tested for biomass and nitrogen (N) yield in this four-week study: semi-continuous (SC) and batch (B). After six days of growth, 25% of the SC treatment was harvested and filled to volume with fresh RB medium every other day. After 14 days of growth, 85% of the B treatment was harvested and the remaining 15% was used as seed to begin a second B set. Biomass yield was estimated by optical density (OD) and chlorophyll content, and N-fixation was estimated by Total Kjeldahl Nitrogen (TKN). At the end of four weeks, biomass yield and total N fixed was calculated for the B and SC treatments. There was no difference in biomass yield or N yield between the two treatments. More than likely the SC was harvested when the culture density was above the optimal cell density range, resulting in a lower total biomass and N yield than what could be achieved within the optimal cell density range. Determination of the optimal cell density and a specific harvest regime that maintains the SC within the optimal cell density would result in a higher total SC biomass and N yield compared to that of B.

Introduction

The on-farm cultivation of phototrophic, N-fixing cyanobacteria offers an alternative N fertilizer source. Cyanobacteria are grown in shallow, circulating ponds called raceways and

supplied with a growth medium. The cyanobacteria are pumped into drip irrigation to fertigate crops. Raceway cultivation can be operated as a batch (B), continuous, or semi-continuous (SC) culture. There are no nutrient medium additions added after cyanobacterial inoculation in a B culture, whereas continuous and SC cultures require frequent medium input and biomass harvest.

Thus far in our research, a B culture has been used. Two weeks after inoculating a raceway, 85% of the culture is harvested and the remaining 15% is used as seed to begin another two-week growth cycle. In outdoor raceways, microalgae is often produced as continuous or SC cultures rather than B cultures because they are marked by higher biomass production (Sassano et al. 2007). In a continuous culture, the continual medium input and biomass output maintains constant cell density and growth rate in the culture, whereas cell density continues to increase as growth rate declines in a B culture. A constant cell density in the continuous culture allows for a relatively constant growing environment, known as a steady state, where parameters such as light penetration, pH, dissolved oxygen (DO), and nutrient concentrations remain constant (Avila-Leon et al. 2012).

In contrast, the ever-changing cell density in the B culture causes an ever-changing growing environment where nutrients are depleted as cell density increases (Rhee 1980; Liere and Walsby 1982). As cell density increases, the majority of the sunlight is absorbed within the surface layer and cells below the surface layer do not receive enough light for maximal photosynthesis (Rhee 1980; Liere and Walsby 1982) This ever-changing growth environment in the B culture imposes growth limitations on the organism. The transition from exponential growth phase to linear growth phase is often due to limited light, and the transition from linear growth phase to stationary growth phase is often due to nutrient depletion and/or accumulation of excretory products in B cultures (Rhee 1980; Richmond 1999). In a continuous culture, growth

limitations such as light, depleted nutrients, and build up of excretory products are less severe, allowing the culture to be maintained in the exponential growth phase indefinitely. Over a production period, indefinite exponential growth produces more biomass than a B culture that goes through all growth phases (Tempest 1970).

To achieve maximal productivity in a continuous culture, optimum cell density should be maintained during exponential growth. The optimum cell density is the range of cell density that yields the highest productivity due to the most efficient use of resources such as light and nutrients (Richmond 1986). When a culture is below the optimum cell density, productivity is negatively affected by photo-inhibition, and above the optimum cell density, productivity is negatively affected by limited light penetration (photolimitation) (Grobbelaar 2007; Morais et al. 2009).

A continuous culture requires specific infrastructure for a continual outflow and inflow of biomass and medium. For our purposes, a SC culture is a more feasible approach that is designed to achieve similar end goals of a continuous culture. In a SC culture, rather than a constant inflow and outflow of medium and biomass, a portion of the culture is harvested and replenished with new medium at frequent time intervals (Rhee 1980). In this study, a SC method of production was tested. The goal of this study was to maximize biomass yield and total N fixed in cyanobacterial raceway cultivation through testing B and SC culture production systems. The objective of this study was to compare biomass yield and N production in a B culture versus SC culture.

Methods

Microorganism

A xenic culture of *Anabaena cylindrica* was used for this study. The heterocystous N-fixing cyanobacteria used for this study was cultured from sediment collected from Richard's Lake in Fort Collins, CO in October 2009. DNA was extracted from the enriched cyanobacterial culture as described by Morin et al. (2010). Sequence analysis of a 446 bp region of 16S DNA was amplified with the primers and conditions described in Nubel et al. (1997). The region was cloned and sequenced. Clones shared 99% similarity with *Anabaena cylindrica.*

Field site and raceway operation

The experiment was conducted during August 2013 at the Colorado State University (CSU) Horticulture Research Center in Fort Collins, CO. Two 16-m by 6-m hoop houses (WEST, EAST) were used in the study. Within these hoop houses, there were six 5.6-m by 1.8-m by 0.3-m raceways (four in hoop house WEST and two in hoop house EAST). To provide mixing and aeration during the study, each raceway contained a six-blade aluminum paddlewheel powered by a 60-Hz motor (Dayton Electric Mfg. Co., Niles, IL, USA). Paddlewheels were operated from 6AM to 8PM each day and for two 30-min segments during the night. Paddlewheels operated between 25 to 36 rpm due to difficulty in obtaining the same paddlewheel speed for each raceway. No supplemental CO_2 was added to the raceways. Raceways were inoculated in a 1:21 dilution ratio of culture to RB medium. Municipal water was used as the water source for RB medium. The maximum culture depth was 0.25-m. Evaporative water loss was replenished daily before measurements were made.

Experimental setup and measurements

Cyanobacteria were grown in the raceways in a replicated randomized block design with three replicates and two treatments: B culture and SC culture. Daily optical density (OD) and chlorophyll content were used as estimators of biomass. OD was determined spectrophotometrically with a microplate reader at 665nm wavelength (Model 680, Bio Rad Laboratories, Inc., Japan) and chlorophyll was extracted from samples as described by Charpy et al. (2010). The following algorithm (Eq. 1) was applied to the spectrophotomic absorbance readings to calculate the chlorophyll content (mM) (Montana State University n.d):

$$C = \frac{D}{(d * a)} \quad \text{where} \tag{1}$$

C = chlorophyll *a* concentration (mM)
D = optical density of chlorophyll extract
d = inside path length of spectrophotometer (0.26-cm)
a = extinction coefficient for chlorophyll *a* (75.05 L/mmol-cm)

Samples were taken at 10AM and immediately stored at 4°C and analyzed within five hours. On harvest days, the OD and chlorophyll samples for the SC treatment were taken before (10AM) and after harvest. N-fixation was estimated by Total Kjeldahl Nitrogen (TKN). A TKN subsample from each raceway was taken daily at 10AM (prior to harvest on harvest days). All TKN samples were frozen (-20°C) prior to analysis and analyzed with a HACH Simplified TKN instrument (TNT 880, Hach Company, Loveland, CO, USA). To qualitatively monitor culture health, microscopic analyses was performed on days 7, 16, 25, and 30 using a Biological Microscope (model MT4310H, Meiji Techno Co., LTD., Japan). Readings were taken from the east and west ends of each raceway. The efficiency of electron transfer through photosystem (PS) II (quantum yield of PS II) (Fv/Fm) was measured three times a week using a dual pulse

amplitude modulation (PAM) fluorometer. Samples were taken between 1:30PM and 2:30PM, dark adapted (1h) and then filtered on a Millipore 13-mm glass fiber filter prior to analysis.

Treatments

B cultures and SC cultures were operated simultaneously. After 6 days of growth, when the cultures had reached late exponential phase, 25% of the biomass from the SC raceways were harvested at 11AM and then filled to volume with fresh RB medium. This was continued every other day for the duration of the four-week experiment. After 14 days of growth, 85% of the batch culture was harvested. On day 15, a second set of B was started by using the remaining 15% of the first set as seed, and raceways were filled to volume with fresh medium. The second B culture set grew for 14 days.

Yield analysis

The biomass yield of the SC and B treatments was analyzed indirectly with both chlorophyll and OD. The TKN was used to estimate the N production from N-fixation. For both biomass and TKN, the yield of each treatment was calculated as follows. Total yield was calculated as time (t_1)=0 and t_2=30. Additionally, productivity from (t_1=0 and t_2=14) and (t_1=15 and t_2=30) was calculated for each treatment.

Average yield was determined using Eq. 2.

$$Y_{(t_2 - t_1)} = \frac{\left(\sum_{t_1}^{t_2} V_t * C_t \right)}{(t_2 - t_1)} \text{ where} \qquad (2)$$

Y = yield (µmol chlorophyll d^{-1} , mg TKN d^{-1}, or OD d^{-1})
t_1= Beginning day of production (day 0 or day 15)
t_2=Final day of production (day 30 or day 14)
V_t = volume of culture harvested at time t (L)
C_t = Concentration of culture harvested at time t (mmol chlorophyll L^{-1}, mg TKN L^{-1}, or OD L^{-1})

SC Growth Rates Analysis

The growth rate between consecutive harvests in the SC treatment was calculated (Eq. 3). Thus, for the thirteen harvest events over the total study, twelve growth rates were calculated.

$$\mu = \frac{\ln(OD)_{t_x} - \ln(OD)_{t_{(x-2)}}}{n} \text{ where} \quad (3)$$

u = the SC growth rate between two consecutive harvests (OD d^{-1})
t_x= time immediately before harvest on day x
$t_{(x-2)}$=time immediately after harvest two days prior to day x
n=time between t_x and $t_{(x-2)}$

Statistical analysis

Data were analyzed using SAS 9.3 (SAS Institute Inc., Cary, NC). Significance was defined as $\alpha = 0.05$ for all statistical analyses. Replicate was not found to be significant in the experiment evidenced by $p>0.05$ for replicate effect in two-way ANOVA analyses. Thus, all two-way ANOVA analyses were run as one-way ANOVA in order to exclude replicate in the model. Differences between treatments for biomass yield and N production were obtained from a one-way ANOVA (PROC MIXED). Analysis of treatment productivity from t_1=15 and t_2=30 excluded SC replicate 3 due to a culture crash during this time period. A repeated measures analysis was used to analyze DO and Fv/Fm data (PROC MIXED).

Results and discussion

Biomass and TKN yield

There was no treatment difference in the total (four-week) biomass (µmol chlorophyll d^{-1} and OD d^{-1}) and N yield (TKN d^{-1}) (Table 5). From t_1=0 to t_2=14, B was significantly higher in biomass and N yield than SC. From t_1=15 to t_2=30, SC was significantly higher in biomass, specifically chlorophyll (µmol chlorophyll d^{-1}), and N yield compared to B. The biomass and N yield within the B treatment declined from set 1 (t_1=0 to t_2=14) to set 2 (t_1=15 to t_2=30) (Table

5). The SC treatment increased in biomass and N yield from set 1 (t_1=0 to t_2=14) to set 2 (t_1=15 to t_2=30) (Table 5).

One explanation for similar total (four-week) biomass and N yield between treatments is that the cell density of SC was maintained outside of the optimal cell density range during the harvest regime. The optimum cell density is the range in cell density that yields the highest productivity due to the most efficient use of resources such as light and nutrients (Richmond 1986). Maintenance within the optimum cell density would result in a total (four week) biomass and N yield greater than that of B (Richmond 1986; Sassano et al. 2007), while maintenance outside of the optimum cell density lowers the total biomass and N yield. Determination of whether a SC was maintained within, above, or below the optimum cell density is usually achieved by composing productivity curves. Productivity curves are composed by determining the productivity of several different cell densities maintained by specific harvest regimes (Figure 4AI). Our experiment did not test several cell densities in the SC operation. However, cell densities below the optimum cell density are prone to photo-inhibition, which is evidenced by a declining Fv/Fm (Grobbelaar 2007). A declining trend in the Fv/Fm of the SC treatment was not observed once harvests began on day 6 and onward (Fig. 5). It is likely that the cell density was above rather than below the optimum cell density.

A cell density above the optimum cell density is hypothesized because harvests began in the late exponential growth phase, and optical densities eventually reached densities that corresponded to those of B in the linear growth phase (Fig. 6). The first harvest event occurred on the last day of the exponential growth phase, day 6. An insufficient quantity of biomass harvested per harvest event led to a gradual upward trend in the OD and TKN (Fig. 6). Eventually, the SC treatment transitioned from optical densities that correspond to late

exponential growth phase to the linear growth phase. At these high cell densities, mutual shading decreases the available light, and photolimitation can occur (Morais et al. 2009). A decline in the OD and TKN then occurred towards the end of the study. The average SC growth rate from consecutive harvests during the study would be expected to decline over time with a transition from the exponential to linear growth phase. It is difficult to distinguish a clear declining trend in the SC growth rates with increasing time (Table 6). Ideally in a SC culture, maintenance of an indefinite exponential growth phase results in higher biomass and N yield to that of B, which transitions from exponential to linear and stationary growth phases (Tempest 1970).

A decline in B from set 1 to set 2 can be explained by possible buildup of contaminants and the additional time required to transition out of lag phase in the second B set. In a B culture, there is minimal removal of contaminants via frequent harvests, allowing the population of contaminants to gradually increase (Richmond et al. 1990). Additionally, the contaminating bacterial population rises as a culture transitions to the stationary phase (Brown et al. 1993). This buildup of contaminants over the two-week duration is then carried over into the seed of the following B treatment. A heavily contaminated seed requires a longer time to transition out of the lag phase.

An increase in the biomass and N yield in the SC from set 1 (t_1=0 to t_2=14) to set 2 (t_1=15 to t_2=30) can be explained by the increasing SC cell density and more harvest events from (t_1=15 to t_2=30) contributed to a higher calculated yield. Hypothetically, in a SC culture maintained at a fairly constant cell density, the total biomass and N yield should not decline over time like that of a B culture. Reichert et al. (2005) have shown that in a lab study, a *Spirulina* SC culture maintained productivity for 7 weeks, after which productivity began to decline. Despite eventual

decline in SC yield, of importance is the longer time period for a SC treatment to decline in yield compared to a B treatment.

In SC production systems, the harvest regime is based on maintaining the identified upper and lower biomass concentrations that correspond to the optimum cell density. Harvest occurs every time the culture hits the maximum optimum cell density and is ideally harvested down to the minimum optimum cell density (Richmond 1986). A harvest regime based on the minimal and maximum optimum cell density maintains the cells in the exponential growth phase (Lierre and Walsby 1982). Knowledge of the upper and lower optimum cell concentration will aid in the maintenance of an approximate steady state for an outdoor raceway, allowing for cells to remain in the exponential growth phase. A possible experiment for determination of the optimal cell density is discussed in Chapter 4 “Future recommendations”.

Conclusions

No difference in the total (4-week) biomass yield and N-fixation between the B and SC in this study can be explained by maintenance of a cell density above the optimum cell density in the SC culture. Harvest events earlier in the exponential growth phase corresponding to the optimal cell density may have resulted in a total SC biomass and N yield greater than that of B. Determination of the optimal cell density must be experimentally determined, as described in the Chapter 4 “Future recommendations”.

TABLES

Table 5. Between treatment comparison of semi-continuous (SC) and batch (B) biomass yield (µmol chlorophyll d^{-1} and OD d^{-1}) and N production (mg TKN d^{-1}). Distinct lower case letters within each time period indicate significant difference between treatments as determined by one-way ANOVA (p<0.05). Within treatment differences of biomass yield and N production from t_{0d} to t_{14d} versus t_{15d} to t_{30d} are denoted with distinct capital letters as determined by one-way ANOVA (p<0.05).

Time period	Treatment	µmol chlorophyll d^{-1}	OD d^{-1}	mg TKN d^{-1}
t_{0d} to t_{30d}	SC	223.7 ± 88.0 a	15.9 ± 6.2 a	1529.1 ± 411.9 a
	B	252.1 ± 38.2 a	18.7 ± 3.0 a	1711.0 ± 75.1 a
t_{0d} to t_{14d}	SC	202.4 ± 22.1 b, B	14.7 ± 0.8 b, B	1401.2 ± 163.0 b, B
	B	332.6 ± 76.4 a, A	20.1 ± 3.2 a, A	2149.1 ± 256.8 a, A
t_{15d} to t_{30d}	SC	367.0 ± 79.5 a, A	24.6 ± 4.9 a, A	2303.8 ± 148.9 a, A
	B	193.7 ± 14.4 b, B	18.7 ± 3.0 a, A	1416.1 ± 89.6 b, B

Table 6. The growth rate between consecutive harvests in the SC treatment as calculated from Eq. 2. Values are averaged over the three replicates in the SC treatment. SC replicate 3 crashed and was not included in the averaged growth rate from t_{12d} to t_{10d} and thereafter. The growth rate from t_{30d} to t_{28d} was not calculated due to a missing value.

Time (n)	SC growth rate (μ)
t_{8d}-t_{6d}	0.243
t_{10d}-t_{8d}	0.052
t_{12d}-t_{10d}	0.178
t_{14d}-t_{12d}	0.145
t_{16d}-t_{14d}	0.152
t_{18d}-t_{16d}	0.138
t_{20d}-t_{18d}	0.166
t_{22d}-t_{20d}	0.197
t_{24d}-t_{22d}	0.077
t_{26d}-t_{24d}	-0.580
t_{28d}-t_{26d}	0.278

FIGURES

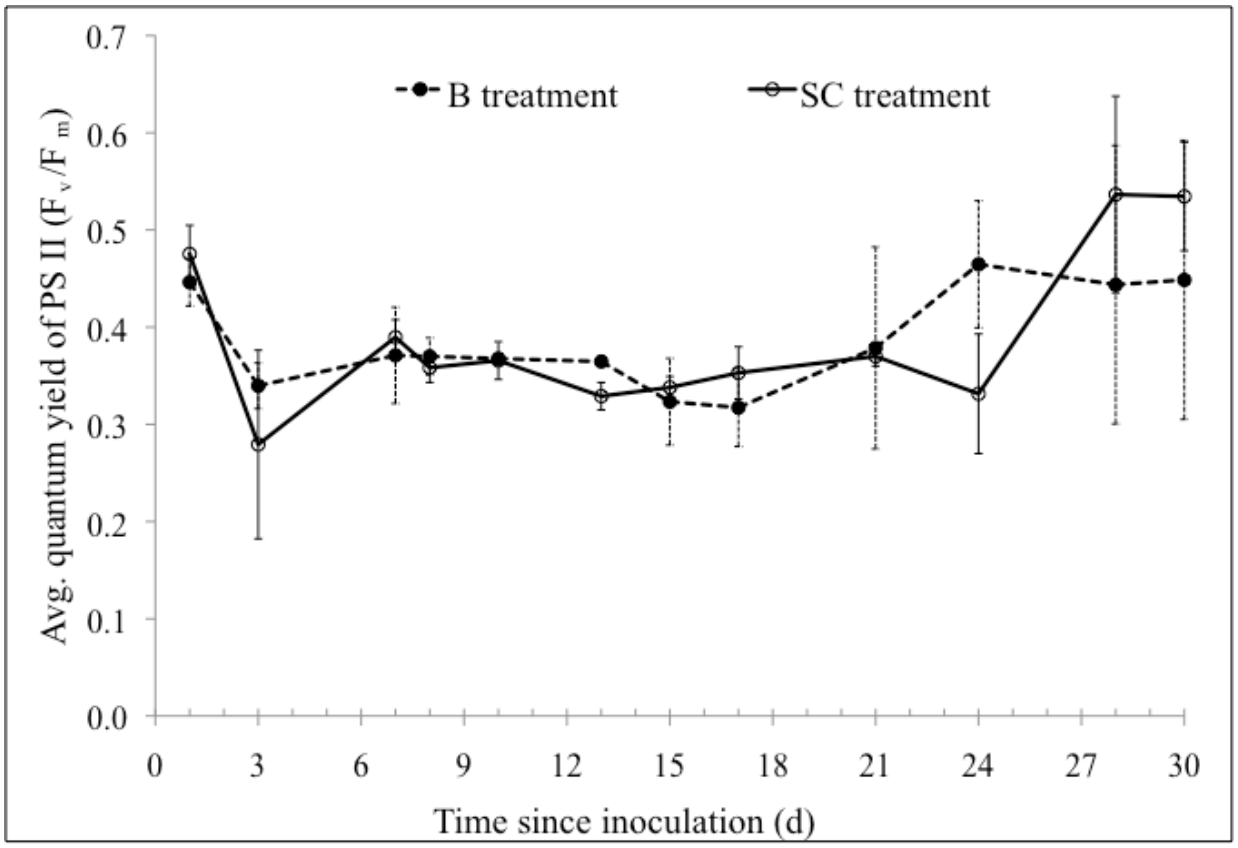

Figure 5. The quantum yield of photosystem II (PS II) (Fv/Fm) of a xenic culture of *Anabaena cylindrica* cultivated in outdoor raceways under batch (B) and semi-continuous (SC) operation. Standard deviation is represented by error bars (n=3). The third replicate of SC crashed on day 13 and was not evaluated from then on. B was significantly higher than SC only on day 24, as determined from repeated measures analysis (p<0.05).

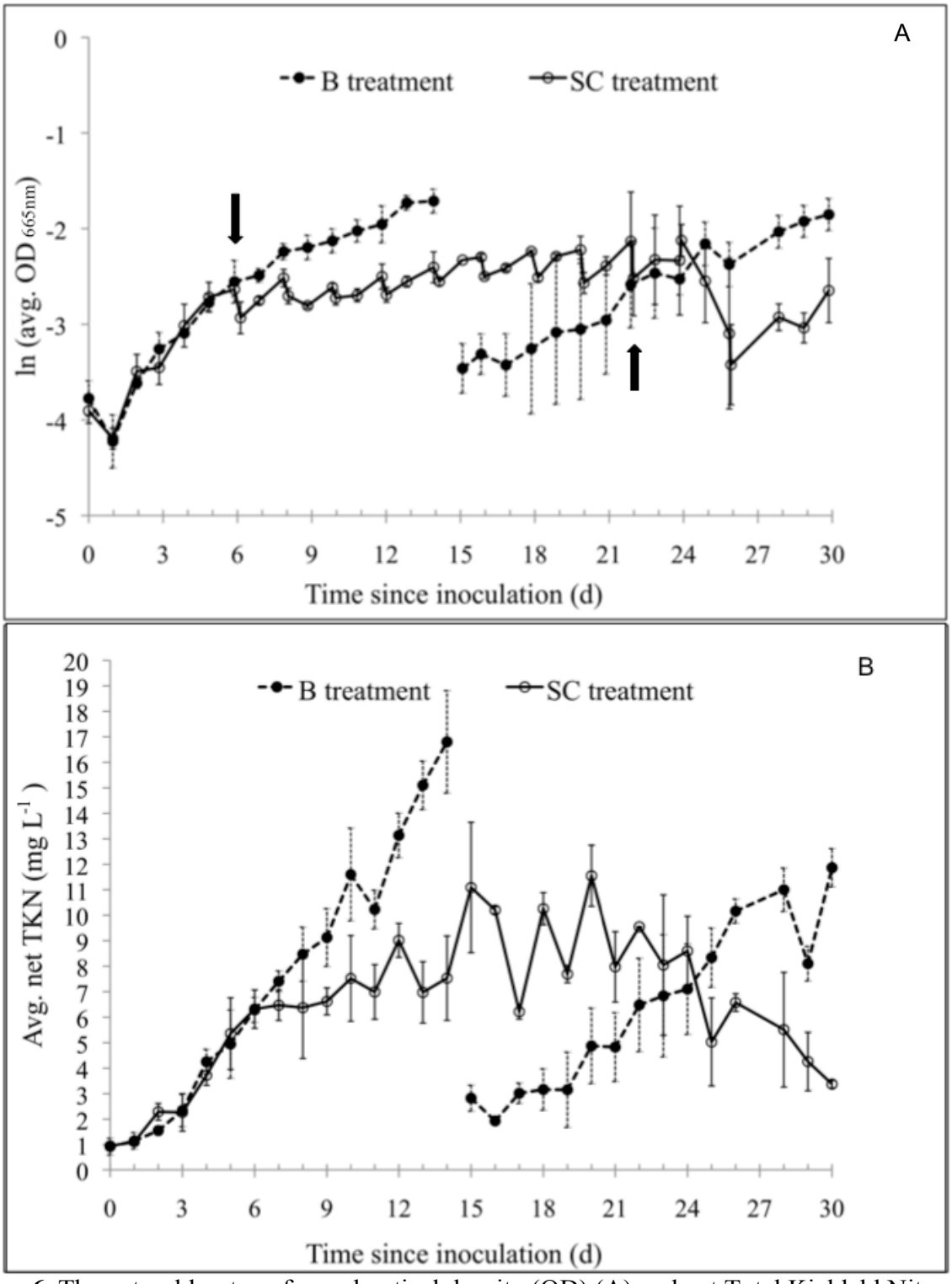

Figure 6. The natural log transformed optical density (OD) (A) and net Total Kjeldahl Nitrogen (TKN) (B) of a xenic culture of *Anabaena cylindrica* cultivated in outdoor raceways under two operational modes: semi-continuous (SC) and batch (B). Arrows indicate last day of exponential growth phase in B culture. The standard deviation is represented by error bars (n=3). The third replicate of SC crashed on day 13 and was not included from thereafter.

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CHAPTER 4: FUTURE RECOMMENDATIONS

Increasing biomass and N-fixation of cyanobacteria in raceway production, specifically in the RB medium, requires a specific chronology of improvements. First, possible growth-limiting nutrient concentrations in the RB medium must be corrected. Secondly, environmental conditions such as low nighttime temperatures can be improved by use of a cover with high thermicity (Thermax). Next, operational parameters, specifically increasing the turbulence, should be of focus. Increased turbulence enhances exchange rates of nutrients between the cultured cells and the growth medium. Thus, to be most effective, the nutritional concentrations of the medium should be improved prior to optimizing turbulence. Additionally, turbulence increases diffusion of N and C into the culture (Grobbelaar 2004). When turbulence is increased, the C nutritional requirement not satisfied by the increased turbulence could then be met by C supplementation via CO_2 sparging. Finally, the optimum cell density should be determined to reduce the effects of photoinhibition and photolimitation.

Improving the nutrient concentrations in RB medium

Thus far, sufficient RB nutrient concentrations for high biomass yield have been evaluated on the basis of the following criteria: (1) comparing initial RB concentrations to measured AA concentrations and other high biomass media and (2) comparing the growth and N-fixation of cyanobacteria grown in RB media to that of AA. It was hypothesized that RB was below adequate concentrations of P, Zn, Co, Fe, and possibly B, despite no difference in cyanobacterial growth in RB and AA. To verify whether RB medium causes nutrient deficiency in the field, three measurements can be analyzed:

(1) The nutrient concentrations of RB medium throughout the 14 d growth period. This allows for the development of medium nutrient depletion to be tracked throughout the experiment. Kim et al. (2010) reported a P medium deficiency due to initial BG-11 P concentration decreasing to <0.01 mg L^{-1} on day 4 and remaining at <0.01 mg L^{-1} throughout the experiment. For our purposes, every other day nutrient measurements of RB medium filtered of cyanobacterial biomass would be sufficient. **(2)** The nutrient percent uptake efficiency throughout the 14 d growth period. The nutrient percent uptake efficiency (Eq. 1) is a comparison of the amount of nutrient incorporated into cyanobacterial biomass to the nutrient concentration of the medium. It can be used to determine whether the medium concentration for a particular nutrient is sufficient for cellular demand (Kim et al. 2010).

$$\% \text{ uptake} = \frac{(B)(C)}{M} * 100 \quad \text{where} \tag{1}$$

%uptake = % nutrient (e.g. P, Co, or other nutrients) uptake efficiency
B= biomass concentration of culture (mg L^{-1})
C= standard elemental (e.g. P, Co, or other nutrients) composition of *Anabaena* (% by weight)
M= nutrient (e.g. P, Co or other nutrients) concentration of medium (mg L^{-1})

An uptake efficiency greater than 100% for a particular nutrient indicates a nutrient deficiency and the activation of nutrient conserving mechanism (Kim et al. 2010). A lower uptake efficiency indicates no nutrient deficiency (Kim et al. 2010). The standard elemental composition of *Anabaena* is a reported value from literature that is calculated with *Anabaena* cells grown in nutrient sufficient medium, allowing for a calculated uptake efficiency based on nutrient-sufficient cell composition (Kim et al. 2010). However, there are few reported values for the elemental composition of *Anabaena* genus specifically relying on N-fixation. Instead, *Anabaena* cells cultivated in the lab with nutrient sufficient AA medium could be elementally analyzed to obtain the elemental composition values. The elemental composition changes at

different stages of growth; therefore it would be important to analyze the elemental composition during the lag, exponential, and linear growth phases (Mandalam and Palsson 1998). The uptake efficiency can be easily determined every other day in parallel with the every other day RB medium sample suggested in (1). A comparison of the uptake efficiency for each nutrient in the medium will also aid in determining which nutrient is the most limiting to growth. The nutrient with the highest uptake efficiency compared to the other nutrient uptake efficiencies is likely to be the nutrient most limiting growth in the medium (Mandalam and Palsson 1998).

The lower Co concentration was due to the water insolubility of cobaltous carbonate. Other OMRI-certifiable sources for cobalt include cobalt acetate, cobalt oxide, and cobalt sulfate. Cobalt oxide is also insoluble in water. Cobalt acetate and cobalt sulfate are soluble in water and should be tested for replacement of cobaltous carbonate.

The Fe, P, and Zn concentrations in the RB medium were low. A chelating agent in the RB medium could increase the biological availability of Fe, P (indirectly through the chelation of Ca), and Zn concentrations. The chelating agents that are organically-certified for use in RB include organic acids such as: humic and fulvic acids (not fortified with prohibited substances), nonsynthetic amino acids, nonsynthetic lactic acid, nonsynthetic tartaric acid, and nonsynthetic citric acid (JH Biotech 2014; OMRI 2014). Synthetic lignin sulfonate is also allowed as a chelating agent. A chelating agent should be selected using the following criteria:

(1) Chelating agent must be commercially available.

(2) Chelating agent must not inhibit cyanobacterial growth.

(3) An amino acid as a chelating agent cannot supply bio-available N. All amino acids contain N; however, not all amino acids are utilized by cyanobacteria for growth and have an

effect on N-fixation (Kaplan et al. 1986). The only amino acids that *Anabaena* are able to utilize for growth include: glutamate, arginine and ornithine.

(4) Chelating agent must have a high affinity for Ca. Bone meal is a calcium-phosphate apatite mineral and dissolves as described in Eq. 2 (Nelson and Janke 2007).

$$(Ca_{9.5}\ (HPO_4)_{0.5}(PO4)_{5.5}(OH)_{1.5} + 13H^+ \leftrightarrow 9.5\ Ca^{2+} + 6H_2PO_4^- + 1.5H_2O \quad (2)$$

From Eq. (2), the two possibilities to increase bio-available $H_2PO_4^-$ concentrations are: (1) decreasing medium pH; and (2) limiting Ca reactions with $H_2PO_4^-$ (Nelson and Mikkelsen 2008). Since cyanobacteria grow best at neutral to basic pH, limiting the Ca reactions with $H_2PO_4^-$ is more plausible. The chelation of Ca can inhibit reactions with $H_2PO_4^-$, and in turn, increase bio-available $H_2PO_4^-$ concentrations (Nelson and Janke 2007).

(5) Chelating agent must have moderate affinity for Fe and Zn. A specific chelating agent may form a strong bond with Fe or Zn due to its exceptionally high affinity for Fe or Zn. Too strong of a bond with Fe or Zn could cause Fe or Zn to be unavailable for cyanobacterial uptake (JH Biotech 2014).

Amino and humic acids are not viable chelating agents for cyanobacteria

Most all commercially available amino acids are synthetic (USDA 2007). Additionally, amino acids in solution can decompose into bio-available N such as ammonium, and specific amino acids such as glycine are inhibitory of cyanobacterial growth due to feedback inhibition (USDA 2007; Eisenhut et al. 2007). Humic acids have been shown to be growth-inhibiting for cyanobacteria. The addition of 1.0 mg L^{-1} humic acid to CT medium caused complete cell desiccation of *Anabaena circinalis* within 1 h (Sun et al. 2005). The cause of this toxic effect is not completely understood. Sun et al. hypothesized that the UV irradiation of humic acid in the medium produced peroxides that then reacted with Fe to produce oxidizing free radicals that damaged cell membranes (2005).

Citric acid, lactic acid, tartaric acid, lignin sulfonate, fulvic acid are viable chelating agents

Nonsynthetic citric acid, lactic acid, fulvic acid and tartaric acid are commercially available. The allowed synthetic form of lignin sulfonate is also commercially available. The only manner of growth inhibition with these chelating agents would be through increased solubility and bio-available concentrations of heavy metals such as Cu, Pb, Zn, Cd, Cr, Ni due to chelation (Wuana et al. 2010; Sinhal et al. 2010). However, the additions of these metals in the medium from stock solutions, bone meal, or municipal water should be relatively low; therefore, increased chelation of these metals should not present a problem. The affinity of citric, tartaric, lactic, fulvic and lignin sulfonate for Ca, Fe, and Zn varies and thus requires additional study, as detailed below.

Citric, tartaric, lactic, fulvic and lignin sulfonate should be tested in RB medium. The amount of chelating agent to add is a critical question, as higher concentrations of chelating agents will increase the number of chelated ions (Ekholm et al. 2000). Due to the increasing effect of chelating action with higher concentrations, it would be beneficial to include two factors in this study: concentration and chelating agent. Of the chelating agents in question, citric acid has been used in some growth media, usually in conjunction with EDTA. Chu's #10 medium is one of the few media that use citric acid without EDTA (Purdue University and Wichita State University 1995). The citric acid concentration in Chu's #10 medium (3.5 mg L^{-1}) would be an appropriate estimate for each chelating agent. Concentrations above and below 3.5 mg L^{-1} should also be tested for each chelating agent. Additionally, it is important to add the chelating agent to the medium before nutrient additions, in order to avoid immediate precipitation reactions (Australian National Algae Culture Collection 2014).

A chelator's affinity for Fe, Ca, and Zn should be evaluated by measuring the initial, mid-way, and final concentrations of the media during cyanobacterial cultivation. The phosphate concentration should also be measured to evaluate whether chelation of Ca increased available phosphate. It would be important to measure the initial, mid-way, and final cyanobacterial biomass composition for each of these ions (Fe, Zn, and P) to ensure that chelation improved bioavailability and uptake of these nutrients. The concentration of the heavy metals in the medium and cyanobacterial biomass composition should be monitored throughout the experiment to monitor whether growth-inhibiting concentrations of heavy metals are present and being assimilated.

Improving the efficiency of bone meal as a P source

Bone meal is the most expensive ingredient in the RB medium due to the large quantity (80 lb raceway $^{-1}$) needed to provide P. Even with this large quantity, the P concentration of RB medium is very low. Bone meal can be more cost effective by either reducing the quantity of bone meal used or by increasing P dissolution from bone meal.

The Bone Meal Reduction study tested the effect on initial P concentration and cyanobacterial growth in RB medium with a 57% reduction in bone meal (6.5 g L^{-1}) to that of the current field amount (15 g L^{-1}). The initial PO_4 concentration of 15 g L^{-1} bone meal was 206 mg L^{-1} PO_4 while that of 6.5 g L^{-1} bone meal was 210 mg L^{-1} PO_4. The two treatments were also no different in growth, verified by chlorophyll content and final biomass. It is possible that over a 50% reduction in bone meal in the field could be achieved while obtaining the same P concentration and growth as the current 15 g L^{-1} bone meal used in the RB medium. This would increase the cost effectiveness of bone meal. The high pH of the medium (low availability of H^+ ions) is the limiting factor in P dissolution from bone meal (Eq. 2). Therefore, a reduction in

bone meal will still result in an equal amount of $H_2PO_4^-$ because of limited H^+ ions needed for $H_2PO_4^-$ dissolution.

Phosphate rock (the other OMRI certified P source) and bone meal are both apatite minerals, thus their dissolution is dependent on Ca concentrations and pH (Nelson and Janke 2007). Phosphate rock has been noted to reach maximal solubility 4 to 8 weeks after application to soils with pH greater than 5.5 (Nelson and Janke 2007). Due to the similarity of the apatite mineral in rock phosphate and bone meal, maximum solubility of bone meal may also be 4 to 8 weeks after application to soils. The difference between soil and water dissolution should be considered; however, it may be possible that bone meal could be used up to 8 to 9 weeks in muslin bags before necessary replacement with new bone meal. Less frequent replacement of bone meal would further increase the cost effectiveness of bone meal.

Water weakens the protein-mineral bond of bone meal and encourages P release from bone meal (Endt and Ortner 1984). Increasing the bone meal to water surface area encourages dissolution of the protein-mineral, increasing P dissolution. The major limitation to bone meal to water surface area in the raceways is the compaction of 20 lbs of bone meal into one muslin bag. The majority of the bone meal was not in constant contact with water, which could decrease the P dissolution. One possibility to increase the surface area of bone meal is to use an increased amount of smaller sized muslin bags. However, increased N dissolution could also occur from increased surface area. Surface area would be maximally increased if bone meal was not contained and was allowed to free float in the medium. However, there is concern that the turbidity of bone meal would lower light penetration and increase protozoa contamination.

Increasing the P concentration of RB medium may aid in the dry harvesting process. Self-flocculation of cyanobacteria is affected by macronutrient concentrations (P, K, Mg, Ca). Lower

macronutrient concentrations lower self-flocculation (Silva and Silva 2007). It is possible that with increased P concentrations in the RB medium, there will be an increase in self-flocculation, which could enhance biomass settling needed for harvests.

The use of an organically certified P source that is N-free should be considered. Phosphate rock, the alternative to bone meal is a mined source and the major phosphate mineral is fluoroapatite. Fluoropapatite is less soluble than the main phosphate mineral in bone meal, hydroxyapatite (Nelson and Mikkelsen 2008). Assuming there is no N in rock phosphate, the reduced P concentration in exchange for no N would not be worthwhile. The addition of N to the RB medium is relatively small and in the end, application of cyanobacteria still provides the same amount of N to that of AA. Further reduction in P by using rock phosphate when P concentration is already significantly low could cause a further reduction in growth.

Finally, all current lab cultures are maintained in AA medium. There has been noted difficulty in transitioning cells from AA to RB while simultaneously scaling up the size of the culture for intended field use. A lab culture in RB medium should be maintained year-round, so as to have a culture that is already acclimated to the RB medium when it is time culture scale up for field studies.

Determining optimum cell density

Harvests in SC cultures should maintain the culture within the optimum cell density range in order to maximize productivity (Richmond et al. 2003). When a culture reaches the maximum within the optimum cell density range, biomass is harvested down to the minimum within the optimum cell density range. This maximizes the amount of harvested biomass in each harvest (Richmond 1986). Only when the optimum cell density range is known can harvesting purposefully maintain the culture in this range. An estimate of the optimum cell density range for

Anabaena genus is 2.0 mg chlorophyll *a* L^{-1} to 3.5 mg chlorophyll *a* L^{-1} (Fontes et al. 1987; Guerrero et al. 1988). However, the optimum cell density changes depending on culture depth, turbulence, and light irradiance (Fontes et al. 1987) and, therefore, an accurate range is obtained by experimentation. Fontes et al. (1987) tested six different cell densities in SC cultures in order to conclude the optimum cell density range of *Anabaena variabilis* in 0.25 m^2 tanks. Every afternoon, the cultures were harvested down to the cell density for each treatment (2.0, 2.5, 3.0, 3.5, 4.0 and 4.5 mg chlorophyll *a* L^{-1}). The productivity of each treatment was calculated as the increase in biomass between two consecutive harvests (24 h). This resulted in a curve of cell density versus productivity (Fig. 2AI). From this curve, a constant, maximal productivity occurred with cell densities from 2.0 mg chlorophyll *a* L^{-1} to 3.5 mg chlorophyll *a* L^{-1}. The use of 0.5 mg chlorophyll *a* L^{-1} incremented treatments allowed for a range in the optimum cell density to be identified. Guerrero et al. (1988) conducted a similar experiment; however cell densities selected for treatments were 2, 4, 6, 8 mg chlorphyll a L^{-1}. As seen from the resultant productivity curve (Fig. 3AI), only 2 mg chlorophyll *a* L^{-1} resulted in maximal productivity and a range including 2.5, 3.0 and 3.5 mg chlorophyll *a* L^{-1} could not be concluded.

An experimental design for our research would designate the biomass concentration on day 2, day 3, day 4, and day 5 as the treatment cell densities. All treatment raceways would simultaneously begin as a batch culture. On day 2, the biomass concentration of three raceways would be recorded, 24 h later the culture would be harvested down to the biomass concentration of day 2, cultivated for 24 h, and again harvested to the recorded biomass concentration of day 2. On day 3, the OD of three different raceways would be recorded, cultivated for 24 h, harvested down to the biomass concentration of day 3, etc. The same would be done for three raceways corresponding to the biomass concentration for day 4 and three raceways corresponding to the

biomass concentration for day 5. The flaws in this design include the twenty raceways needed for this experiment, and harvesting twenty raceways at once is unrealistic.

An alternative to the suggested experiment is the use of productivity models. In general, these models require two input variables: the temperature and the surface irradiance range that a culture experiences (Grobbelaar et al. 1990). It is assumed there are no nutrient limitations. The productivity for a specific biomass concentration can be calculated. Determination of the productivity for multiple biomass concentrations creates a productivity versus cell density curve, and optimum cell density can be clearly distinguished from this curve (Fig. 4AI) (Grobbelaar et al. 1990).

Finally, the time of harvest can increase productivity in a SC culture. Nocturnal biomass loss due to respiration can account for 5 to 21% biomass loss in open raceways (Richmond et al. 1990). Harvests in the evening rather than morning reduces nocturnal losses to respiration. In commercial *Spirulina* production, late afternoon/evening harvests were accompanied by a 15-20% increase in net productivity to that of the previous year when harvesting was done in the morning (Richmond et al. 1990).

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APPENDIX I

Table 1A. The initially measured nutrient conentrations in Allen and Arnon (AA) medium and organically certifiable medium (RB) in the lab and field study compared to the theoretical AA concentrations. Distinct capital letters indicate significant difference in lab study and distinct lower case letters indicate significance difference in field study (p<0.05) as determined by one-way ANOVA. The concentration of Cl was not analyzed. No theoretical concentration for Si was calculated because Si was not listed on the AA recipe (Allen and Arnon n.d).

	Theoretical	Measured Lab				Measured Field			
	AA	AA		RB		AA		RB	
Nutrient	(M)	(M)				(M)			
K	1.52E-03	1.467E-03	B	1.735E-03	A	1.231E-03	a	1.218E-03	a
Cl	1.34E-03	---		---		---		---	
Na	1.08E-03	1.305E-03	B	1.439E-03	A	1.592E-03	a	1.529E-03	a
P	7.62E-04	5.993E-04	A	4.011E-04	B	4.839E-04	a	3.701E-05	b
Mg	2.56E-04	3.669E-04	A	1.235E-03	B	3.262E-04	b	4.460E-04	a
S	2.60E-04	4.414E-04	B	1.022E-03	A	3.560E-04	a	3.815E-04	a
Ca	1.29E-04	3.066E-04	B	1.341E-03	A	1.479E-04	b	3.823E-04	a
Si	---	1.386E-04	B	1.934E-04	A	6.908E-05	b	8.143E-05	a
Fe	1.81E-05	1.996E-06	A	5.721E-07	B	1.791E-08	*a	1.791E-08	*a
B	1.17E-05	2.375E-05	A	1.056E-05	B	1.119E-05	a	9.400E-06	b
Mn	2.29E-06	6.689E-08	B	2.685E-07	A	1.335E-07	a	2.002E-07	a
Mo	1.27E-06	1.516E-07	A	2.092E-07	A	2.745E-07	a	2.641E-07	a
Zn	1.93E-07	2.830E-07	A	3.059E-09	B	1.835E-07	a	2.039E-08	b
Cu	7.96E-08	3.366E-06	A	5.209E-07	B	4.721E-08	a	1.574E-08	*a
V	4.95E-08	3.445E-07	B	1.361E-06	A	1.374E-07	a	1.178E-07	a
Co	4.24E-08	6.321E-08	A	3.691E-08	A	4.525E-08	a	1.700E-08	*b

* Signifies concentrations were below detection limit (LoD) of 0.001 mg L^{-1}. The LoD was converted into M for each applicable nutrient.

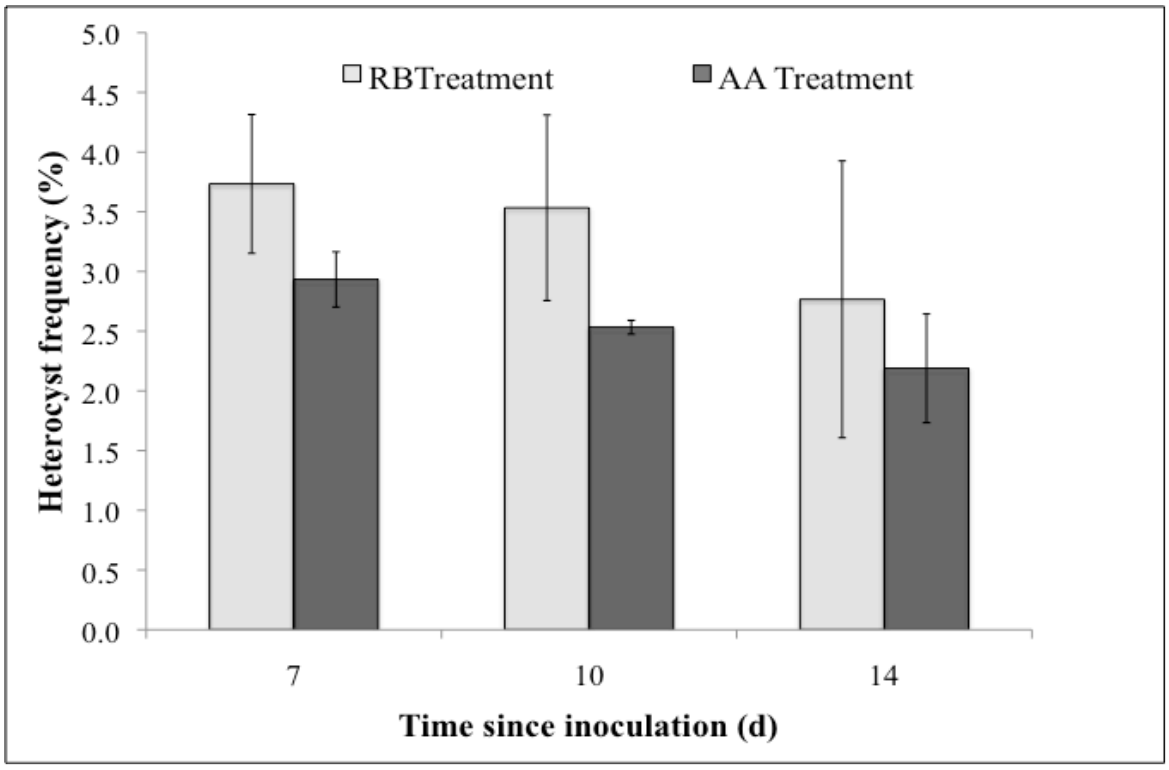

Figure 1AI. The heterocyst frequency (%) of filamentous cyanobacterial cells cultivated in outdoor raceways in RB and AA media. Error bars represent standard deviation (*n*=3). No significant difference was found between RB and AA on days 7, 10, and 14 as determined by repeated measures analysis.

Calculation 1AI. The replacement of Hi-Yield® 0-10-0 Bone Meal for K_2HPO_4 as a phosphorus (P) source in the RB medium required the following calculations. First, the theoretical P concentration of the 42.8 g L^{-1} K_2HPO_4 stock solution used in the AA medium was determined (7.61 g L^{-1}). The Hi-Yield® 0-10-0 Bone Meal has a 4.364% P concentration. The amount of bone meal to compose a stock solution that was equal in P concentration to that of the K_2HPO_4 stock solution was calculated with the following equation.

$$\frac{7.61}{0.04364} = 174.37 \text{ g } L^{-1}$$

However, only 87.18 g L^{-1} bone meal was used in order to increase the water to bone meal ratio for increased solvent dissolution.

To compose the lab version of the RB medium, 750 ml L^{-1} of 87.16 g L^{-1} bone meal stock solution (pH 6.93) was used. Thus, the bone meal concentration of the RB medium was 65.37 g L^{-1}. The addition of 750 ml of the bone meal solution was determined with the following equation.

$$\frac{36.1}{x} = 27.2 \text{ where}$$

36.1= the measured P concentration (mg L^{-1}) of the bone meal solution pH adjusted to 9.01
27.2 = the measured P concentration (mg L^{-1}) of AA medium
x= the volume (L) of bone meal solution (0.75 L)

Field Concentration

The transition from lab to field version of the RB medium required reductions in the amount of bone meal. Additionally, a stock solution of bone meal for the field version was no longer used. Instead, due to the low solubility of bone meal at neutral pH, we considered that bone meal could be supplied in a muslin bag to allow for the bone meal to continue dissolving

into the RB medium as it was utilized by cyanobacteria. To figure adequate reductions of bone meal, in a lab study, the organic medium (RB) was prepared with 4 dosages of bone meal (65.37(the original lab version concentration), 15.5, 6.537, and 1.09 g L^{-1}). The bone meal was supplied within a 4-layer muslin bag placed at the bottom of the flasks. These treatments were then compared on the basis of cyanobacterial growth and N-fixation. A bone meal concentration of 15.5 g L^{-1} was adequate for cyanobacterial growth and N-fixation. However, due to difficulties in scale-up of the muslin bags, only 15.16 g L^{-1} bone meal was supplied in the field.

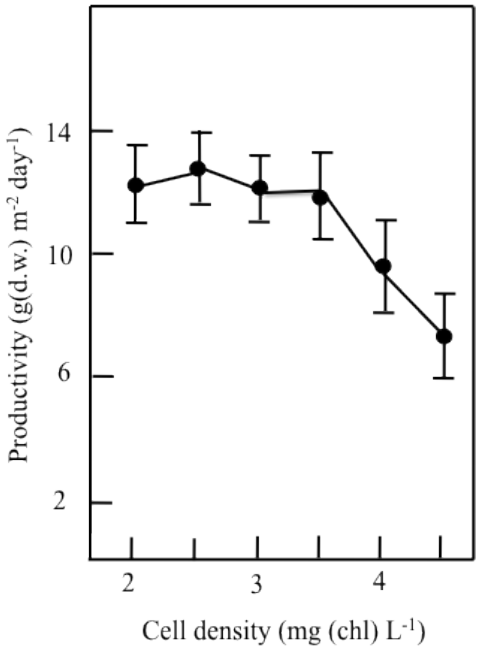

Figure 2AI. The productivity versus cell density curve for *Anabaena variabilis* cultivated in 0.25 m^2 outdoor tanks under SC harvests that maintained different cell densities (figure was redrawn from Fontes et al. 1987). Productivity was determined as the increase in biomass between two consecutive harvests (24 h).

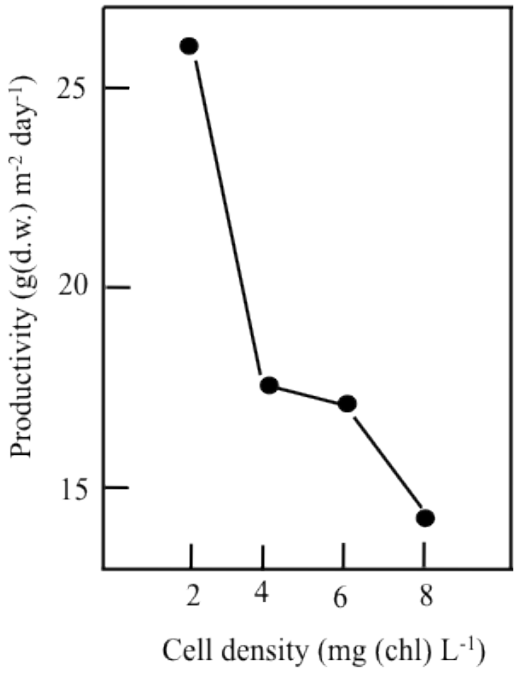

Figure 3AI. A productivity versus cell density for *Anabaena* ATCC 33047 cultivated in SC mini raceways (1-m^2 and 10-cm depth) (figure was redrawn from Guerrero et al. 1988). Each cell density was maintained by 24 h harvests and the experiment was operated for four days during the summer.

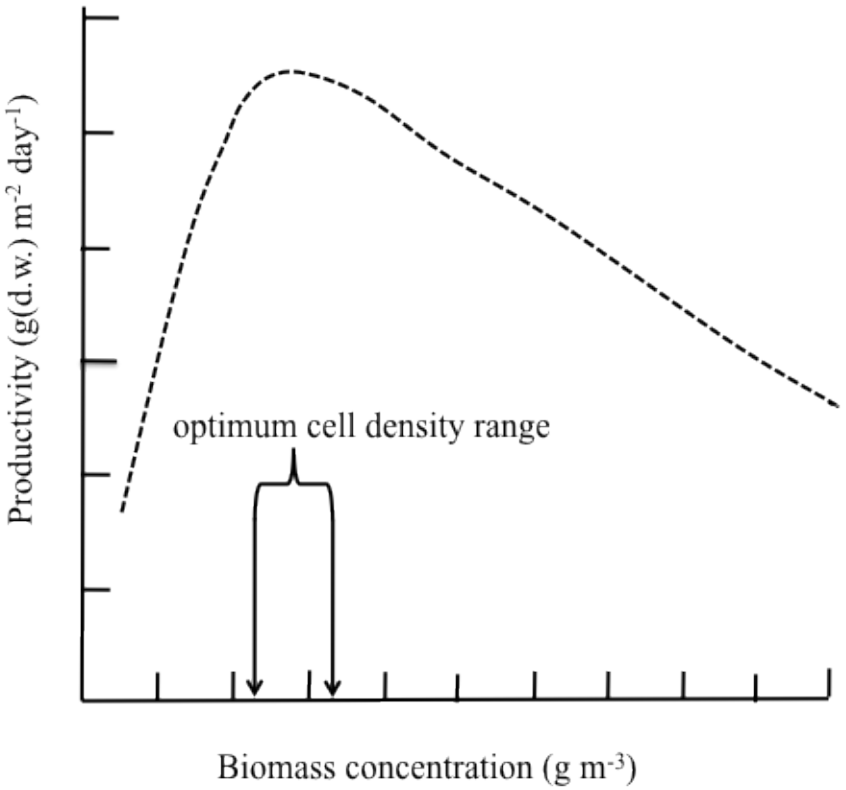

Figure 4AI. Productivity curve resultant from a Grobbelaar et al. model (1990). Figure was redrawn from source.

APPENDIX II

CYANOBACTERIAL GROWTH AND NITROGEN FIXATION IN RESPONSE TO DEPTH, BICARBONATE SUPPLY, AND HOOP HOUSE COVERINGS IN OUTDOOR CULTURE

Preface

The productivity of cyanobacteria in raceways is often limited by light, C, and temperature. Growth and N-fixation can be increased by operation at a culture depth that allows for the most efficient use of sunlight, the addition of an inorganic C source, and by covering a raceway with a hoop house to avoid high light intensities and maintain heat during lower nighttime temperatures. The goal of this work was to assess methods for reducing C, light and temperature limitations by conducting three separate experiments in outdoor tanks. The first experiment compared the growth and N-fixation of batch cultures grown at two different depths (20-cm and 25-cm). The second experiment compared cyanobacterial growth and N-fixation in AA medium supplied with 0 mM (control), 0.2 mM (low treatment), and 2.0 mM (high treatment) of potassium bicarbonate ($KHCO_3$). The third experiment compared the growth and N-fixation of cultures grown under different hoop house plastics (Thermax, Luminance, Dura-film Super 4, and 4 mil Husky construction plastic) and a no-cover control.

For all experiments, biomass was estimated by optical density (OD) and chlorophyll content. N-fixation was estimated by net Total Kjeldahl Nitrogen (TKN). Dissolved oxygen (DO) content and pH were used to monitor photosynthetic activity. A xenic culture of cyanobacterium *Anabaena cylindrica* selectively cultured from a local lake was used in each

experiment. Experiments were conducted outdoors in 1.2-m (l) by 0.6-m (w) by 0.3-m (h)) metal tanks that were circulated with a submersible pump.

The selected culture depths did not have an effect on total growth or net N-fixation. It was hypothesized that the deeper depth (25-cm) was more productive at the beginning of the study due to less excessive light. Mid-way through the study, the shallow depth may have been more productive due to less light-limited culture volume. The reversal in yield advantage during the study may have resulted in no net difference between the two depths. There was no increase in growth or N-fixation due to addition of $KHCO_3$. It was concluded that inadequate $KHCO_3$ was added to significantly increase growth and that the addition of $NaHCO_3$ rather than $KHCO_3$ is necessary to assure adequate Na concentrations needed for maximal bicarbonate uptake. Finally, none of the covers increased the growth compared to the control. Zn slowly leached from the cultivation tanks, so that by the end of the cover study, zinc toxicity clouded the interpretation of results.

Introduction

Raceway production of microalgae is often limited by light, temperature, or nutrients (e.g., C) (Richmond 1986). However, there are measures to reduce the severity of these limiting factors, such as the addition of C to a C-limited raceway.

Light limitation in relation to culture depth

The major reason for low biomass production in raceway systems is the inefficient use of solar energy (Grobbelaar 2013). The rate of mixing, population density, and culture depth all affect the light intensity within a raceway (Richmond 1986). Particularly, the culture depth (also known as the optical-depth or light-path) is an important factor that determines microalgal growth. The downward transmitted light diminishes exponentially with depth, which results in

low biomass production (Grobbelaar 2013; Grobbelaar et al. 1990; Ritchie and Larkum 2012). In most raceways used for microalgal cultivation, culture depth varies from 10 to 50-cm with 15-cm as the most common (Grobbelaar 2013). As the cell density increases, less light penetrates to the bottom and causes poor solar energy conversion.

Net photosynthesis can be evaluated based on the three major layers within a raceway: the surface layer, the optimal depth, and the compensation depth. In the surface layer, sunlight saturates photosynthesis. In turn, photoinhibition can lead to sub-maximal net photosynthesis. Maximum net photosynthesis occurs below the surface layer, called the optimal depth (Ritchie and Larkum 2012). Below the optimal depth, respiratory losses increase and net photosynthesis declines. Available light continues to decline with increasing depth to the point that light levels reach 1% of its subsurface value and gross photosynthesis is equal to the loss from respiration, thus no net photosynthesis occurs —this is the compensation depth (Dubinsky 1986; Ritchie and Larkum 2012). Ideally, a raceway would be designed to operate at the optimal depth. However, this is not practically feasible due to other considerations such as maintaining paddle wheel efficiency, controlling temperature fluctuations, and minimizing evaporation. Alternatively, the culture depth can be maintained either at or above the compensation depth. This is feasible and will allow the most unproductive layer of a raceway to be eliminated.

In most raceway production, semi-continuous production allows operation at the optimum cell density. The optimum cell density varies with the depth of the culture. A deeper raceway must maintain a lower cell density to that of a shallow raceway to be productive (Ritchie and Larkum 2012). In this experiment, the raceways were operated as batch cultures rather than semi-continuous cultures, and therefore, the cell culture density varied throughout the experiment.

Carbon utilization and limitation

Cyanobacteria are photoautotrophic organisms and can utilize both CO_2 and bicarbonate (HCO_3^-) for C fixation. There are five transport systems for active inorganic C uptake, including two types of Na^+ dependent HCO_3^- transporters (BicA and SbtA), one Na^+ independent HCO_3^- transporter, which has only been physiologically characterized in *Synechococcus* PCC7942 (BCT1), and two CO_2 transporters (Price 2011).

The accumulation of intercellular C is determined by the external supply of inorganic C. As external inorganic C supply (CO_2 and HCO_3^-) in the environment is depleted, the internal cellular pool of C also begins to decline. In turn, the rate of photosynthesis decreases (Kaplan et al. 1980). Without the addition of C (e.g CO_2 or HCO_3^-) to raceways, severe C limitations can develop (Richmond 1999).

Under low CO_2 conditions, many cyanobacterial species are dependent on HCO_3^- uptake for photosynthesis (Bloye et al. 1992; Maeso et al. 1987). The dissociation of a HCO_3^- source (e.g. $KHCO_3$) is dependent on the pH of the medium solution. Between pH 6.4 and 10.2, $KHCO_3$ dissociates into HCO_3^- and K^+ (Lindsay 1979). The growth medium used in this study had a pH of 7.5, thus HCO_3^- was supplied by the addition of $KHCO_3$. HCO_3^- was selected as a C supplementation over CO_2 because it is less expensive and requires no extra infrastructure like that of CO_2 sparging.

High light irradiance with no hoop house cover

High light intensity in cyanobacterial cultures can cause photoinhibition, damaging the PSII system and in turn decreasing photosynthetic capability (Grobbelaar 2007). In microalgae production, some raceways are operated under polyethylene plastic hoop houses to decrease damaging light intensity. Various studies have been conducted to assess the growth effect on

cultures grown in raceways with and without a cover. Richmond et al. (1990) concluded that the productivity of *Spirulina* in a raceway with a polyethelene cover was higher than without a cover due to protection from high mid-day light intensity, causing less photoinhibition.

There are additional advantages to covered raceways. Less evaporation occurs in a closed system compared to an uncovered raceway, and a cover such as a hoop house can elevate temperatures up to 6 to 8 $^{\circ}$C above outdoor temperatures. Finally, a cover keeps dirt and insects out of the raceway culture (Richmond 1986).

In this study, three experiments were conducted to assess methods for minimizing light, C, and low nighttime temperature limitations. The objective of each study is detailed below:

(1) To determine if cyanobacterial yield is dependent on the culture depth.

(2) To determine if a supply of C ($KHCO_3$) will increase growth and N-fixation.

(3) To evaluate light and temperature control provided by different hoop house coverings and determine which cover promotes the most growth and N-fixation.

Methods

Microorganism

A xenic culture of *Anabaena cylindrica* was used for this study. The heterocystous, N-fixing cyanobacteria were originally cultured in Allen and Arnon medium from sediment collected from Richard's Lake in Fort Collins, CO in October 2009. DNA was extracted from the enriched cyanobacterial culture as described by Morin et al. (2010). Sequence analysis of a 446 bp region of 16S DNA was amplified with the primers and conditions described in Nubel et al. (1997). The region was cloned and sequenced. Clones shared 99% similarity with *Anabaena cylindrica*.

Field site and tank operation

The experiments were conducted during the summer of 2012 at the Colorado State University (CSU) Horticulture Research Center in Fort Collins, CO. Galvanized steel oval tanks (1.2-m (l) by 0.6-m (w) by 0.3-m (h)) were used as mini production ponds. To provide mixing and aeration during the study, each tank contained one SEIO Prop Super Flow pump and one airstone with air delivered by a Top Fin Air pump. No supplemental CO_2 was added to the tanks. To prevent Zn leaching from the galvanized steel, each tank was coated with Rust-Oleum Leak Seal Flexible Rubber Coating Black. For all studies, each tank was inoculated with *Anabaena cylindrica* into Allen and Arnon (AA) medium at a 1:10 dilution ratio. Municipal water was used as the water source for AA medium, and evaporative water loss was replenished daily with AA medium before measurements.

Depth study

The study was conducted from June 29, 2012 through July 14, 2012 inside the hoop house described above. Cyanobacteria were grown in the tanks in a replicated RBD with three replicates and two treatments: 20-cm and 25-cm culture depths.

Bicarbonate study

The study was conducted in a 16-m by 6-m hoop house from July 23, 2012 through August 6, 2012. Cyanobacteria were grown in tanks in a replicated randomized block design (RBD) with three replicates and three treatments: high bicarbonate (2.0 mM $KHCO_3$), low bicarbonate (0.2 mM $KHCO_3$), and control (0 mM $KHCO_3$). $KHCO_3$ was added to the AA medium before cyanobacterial inoculation. No additional $KHCO_3$ was provided throughout the study. The low $KHCO_3$ concentration was modeled after the 0.15 mM $NaHCO_3$ concentration in COMBO, Fraquil, and WC media (Andersen et al. 2005). The low $KHCO_3$ treatment was

multiplied by 10 in order to get the high $KHCO_3$ treatment. Culture volume was maintained at 106.7 L, which corresponded to a 20-cm culture depth.

Cover study

The study was conducted outside of the hoop house from August 11, 2012 through August 25, 2012. Cyanobacteria were grown in tanks in a replicated RBD with three replicates and five cover treatments: four plastic covers and one control (no cover). The plastics included: Dura-Film Super 4 (AT Film, Inc.), Thermax (AT Films, Inc.), Luminance (AT Films, Inc.), and 4 mil Husky construction plastic. A mini hoop house was constructed for each cover treatment (Fig. 5AII) and placed over the tanks. Culture volume was maintained at 106.7 L, which corresponded to a culture depth of 20-cm.

Measurements

Triplicate samples from each tank were analyzed daily for optical density (OD) and chlorophyll content as estimators of biomass concentration. In the cover study and bicarbonate study, chlorophyll samples were taken on days 0, 5, 9, and 14. Samples were immediately stored at 4 °C and analyzed the same day. The OD was determined spectrophotometrically at 665 nm with a microplate reader (Model 680, Bio Rad Laboratories, Inc., Japan). The *ln* of the daily OD was calculated to identify the exponential and linear growth phase in each experiment. The slope of each growth phase was calculated to determine the growth rate for each phase. Chlorophyll was extracted from samples as described by Charpy et al. (2010), and the chlorophyll content was determined using Eq. (1) (Montana State University n.d):

$$C = \frac{D}{(d * a)} \quad \text{where} \tag{1}$$

C = chlorophyll *a* concentration (mM)
D = optical density of chlorophyll extract

d = inside path length of spectrophotometer (0.26-cm)
a = extinction coefficient for chlorophyll *a* (75.05 L/mmol-cm)

One initial and two final samples from each tank were frozen and analyzed at the CSU Soil, Water, and Plant Testing Lab for Total Kjeldahl Nitrogen (TKN) and total solids (TS). Net TKN was used as an estimator of net N-fixation. Net TKN and net TS were calculated using Eq. (2):

$$\frac{(\text{Final}_1 - \text{Initial}) + (\text{Final}_2 - \text{Initial})}{2} \quad (2)$$

The areal yield for chlorophyll content, net TS, and net TKN were analyzed in the depth study (Eq. 3).

$$Y = \frac{C * V_d}{1\ \text{m}^2} \quad \text{where} \quad (3)$$

Y = areal yield (net mmol chlorophyll m^{-2}, net mg TKN m^{-2}, net mg TS m^{-2})
C = concentration (mM chlorophyll, net TKN mg L^{-1}, net TS mg L^{-1})
V_d = the volume of the tank (V_{20cm} = 106.7 L or V_{25cm} =133.4 L)

The volumetric yield for chlorophyll content, net TS, and net TKN were also analyzed in the depth study. Units were already in the form of volumetric yield and no further calculation was needed. In the bicarbonate and cover studies, all treatments were maintained at a volume of 106.7 L; therefore, only volumetric yield was analyzed.

Triplicate measurements of dissolved oxygen (DO) in mg L^{-1}, pH, and culture temperature (°C) of each tank were determined with an Orion 5 Star portable meter (Thermo Fisher Scientific Inc., Beverly, MA, USA) three times a day, at 8AM, 12PM and 4PM. The pH meter was calibrated once to twice a week. For the cover study, the light transmission of the hoop house plastics was recorded during the same time as the pH and DO measurements. A CEM DT-1309 Auto Ranging Light Meter was used to record the kLux inside and outside each

plastic cover. The percent light transmission was calculated by dividing the inside kLux over the outside kLux. Microscopic analyses were performed periodically to qualitatively monitor health.

The Rust-Oleum Leak Seal Flexible Rubber Coating Black applied to each tank was not adequate to prevent Zn leaching during the bicarbonate and cover studies. To account for this, final samples were analyzed for dissolved Zn at the CSU Soil, Water, and Plant Testing Lab.

Statistical analysis

Data were analyzed using SAS 9.3 (SAS Institute Inc., Cary, NC). Significance was defined as $\alpha = 0.05$ for all statistical analyses. For the depth study, replicate was not found to be significant evidenced by $p>0.05$ for replicate effect in two-way ANOVA analyses. Thus, all two-way ANOVA analyses were run as one-way ANOVA in order to exclude replicate in the model. Differences between depth treatments for net TKN, exponential and linear growth rate, and net total solids were obtained from a one-way ANOVA (PROC MIXED). For the bicarbonate and cover studies, the Zn leaching from the galvanized tanks affected growth of cultures. Zn was used as a covariate in the ANCOVA (PROC MIXED in SAS 9.3) test for net TKN, exponential and linear growth rate, and net total solids, and chlorophyll content in bicarbonate and cover studies. A repeated measures analysis was used to analyze daily treatment differences in pH, DO, culture temperature, light cover transmission, and chlorophyll content in the cover study. Once the specific days with treatment differences for these parameters were identified with the repeated measures analysis, Tukey's test for mean separation (PROC GLM) was used.

Results and discussion

Depth study

When the OD data were *ln* transformed, two bacterial growth phases were evident: the exponential growth from day 0 to 6 and linear growth from day 6 to 14 (Fig. 6AII). There was no

treatment difference in the exponential or the linear growth rates. There was no treatment difference in the volumetric and areal yield for the following measurements: 14 days of chlorophyll content (data not shown), final net total solids (mg L^{-1}), and final net TKN (mg L^{-1}) (Table 2AII). When each day of the OD data was analyzed for treatment differences, the 20-cm treatment was significantly higher in volumetric OD on days 11, 13, 14, and 15 than the 25-cm treatment ($p<0.05$). The 25-cm treatment was significantly higher in areal OD on days 3, 7, 8, and 10 ($p<0.05$).

Analysis of treatment differences in pH, DO, and temperature was divided into morning (8AM), mid-morning (10AM), noon (12PM), and afternoon (4PM). There was no difference between treatments in pH, DO (Fig. 7AII), and temperature (data not shown) in each analysis of morning, mid-morning, noon, and afternoon periods One treatment did not appear to be more fragmented or contaminated than another, as noted with microscopic analysis. Towards the end of the study, all filaments developed akinetes.

The lack of significant difference between treatments in terms of growth rate, chlorophyll, final net TS, and final net TKN may be explained by the effect of depth and culture density on net photosynthesis within a batch culture raceway. The biomass production at a specific depth depends on the cell density within that culture. A comparison of annual *Phaeodactylum tricornutum* productivity at different culture depths as cell biomass increased from below to above optimum density is presented by Slegers et al. (Fig. 8AII) (2013). The 20-cm and 30-cm depths are closest to our study depths (20-cm and 25-cm). Three points are clear from this graph: (1) a shallower culture depth has a higher optimum cell density than a deeper depth; (2) when 20-cm and 30-cm depths are at an equal cell density that is below their respective optimum cell density, the deeper depth is more productive than the shallower depth;

(3) When both depths are at an equal cell density that is above their respective optimum cell density, the shallower depth is more productive than the deeper depth. At low cell densities, a shallower depth results in unutilized, excessive light, which lowers the productivity and can cause photoinhibition (Ritchie and Larkum 2012). At high cell densities above the optimum cell density, both culture depths develop a compensation depth. A hypothetical 10-cm compensation depth in both the 20-cm and 30-cm treatment would result in less cyanobacteria below the compensation depth in the shallower depth compared to the deeper depth. This will encourage less loss to respiration by cells (Ritchie and Larkum 2012). Essentially, a reversal in productivity for a shallower depth and deeper depth occurs according to whether the cell density is above or below optimum cell density.

A reversal effect would predict that at the beginning of our study, the 25-cm depth would most efficiently utilize the high light transmitted through the low cell density culture and would be more productive than the 20-cm depth. As cell density increased, the 25-cm depth would first hit its specific optimum cell density and peak in productivity. As this was occurring, the 20-cm depth would still be below its optimum cell density and progressively increase in productivity until reaching optimum cell density. Once both treatments' cell density increased over their optimum cell density, the 20-cm tanks would remain at a higher productivity than the 25-cm depth due to less volume below the compensation depth. Greater production in the deeper depth at the beginning of the study and more production in the shallower depth at the end of the study could explain the lack of difference in total yield. However, greater production in the deeper treatment at the beginning of the study and vice versa for the shallower depth should be reflected in a higher exponential growth rate, and daily areal and volumetric OD of the deeper (25-cm) treatment in the first week, followed by significantly higher daily areal and volumetric OD in the

shallower (20-cm) during the second week (Moheimani and Borowitzka 2007). This was not consistently observed. Moreno et al. (1987) found that there was no difference in productivity (g dry weight m^{-2} day^{-1}) in a SC culture maintained at a 2 mg chlorophyll L^{-1} in 0.25-m^2 outdoor tanks when operated at 20-cm versus 25-cm. No explanation was given for results.

There are advantages to maintaining the culture at a shallower depth in this batch scenario. Less paddlewheel energy is needed to maintain sufficient turbulence for homogeneity (Ritchie and Larkum 2012). Also, the greater surface:volume ratio facilitates greater dissipation of dissolved oxygen from the culture (Richmond and Grobbelaar 1986). High oxygen concentrations can lead to photorespiration, in turn negatively affecting productivity (Moheimani and Borowitzka 2007).

Depth study conclusions

The purpose of the depth study was to determine whether two operational depths (20-cm and 25-cm) under batch cultivation would result in biomass and N-fixation yield differences. There were no treatment differences in exponential growth rate, linear growth rate, daily pH and DO, volumetric and areal yield for daily chlorophyll content, final net TS, and final net TKN. Other studies under SC rather than B operation in outdoor tanks at these two depths found similar results, although no explanation was given for results (Moreno et al. 1987). Although not fully supported by our data, one possible reason for the lack of treatment differences was the deeper depth offering greater protection from excessive light at the beginning of the study when the light cell density was prone to photoinhibition, in turn promoting higher growth. As cell density increased over the optimum cell density, the shallower depth then became more productive due to less volume below the compensation depth. Measurement of light intensity

within the culture at 1-cm increments over the study would allow for further evaluation of this hypothesis.

Bicarbonate study

Cyanobacterial growth and N-fixation

When OD data were *ln* transformed, two bacterial growth phases were evident: the exponential phase, which occurred from day 0 to 4; and the linear phase, which occurred from day 4 to 14 (Fig. 9AII). There was no difference in the exponential growth rate of the control treatment and the low bicarbonate treatment. Exponential growth was not evident in the high bicarbonate treatment. The linear growth rates of the 3 treatments were not statistically different. The initial chlorophyll content was the same for all treatments. There was no difference in chlorophyll content between the control and low bicarbonate treatment on days 5, 9, and 14, while the high bicarbonate treatment had significantly lower chlorophyll content (data not shown). There was no difference in the net TKN between control (15.81 mg L^{-1}) and low bicarbonate treatment (19.89 mg L^{-1}), while the high bicarbonate treatment had significantly lower net TKN (3.59 mg L^{-1}). Neither the low or high bicarbonate treatment increased growth or N-fixation. Additionally, the high bicarbonate treatment reduced cyanobacterial growth and N-fixation.

pH and DO

The pH and DO are both indicators of photosynthetic activity. Lower DO and pH values indicate less photosynthetic activity due to a stressed culture (Richmond 1986). During the first week of the study, the high bicarbonate treatment was significantly lower in DO than both the low bicarbonate treatment and the control (Fig. 10AII). During the second week, there was no difference in DO among the three treatments. For the majority of the experiment, the low and

control were not statistically different in pH, while the high treatment had a significantly lower pH. Clearly, the high bicarbonate treatment reduced cyanobacterial growth, as seen by the lower photosynthetic activity, lower growth, and lower N-fixation.

Microscopy and color changes

In all 3 replicates of the high bicarbonate treatment, the filaments were severely fragmented compared to the control and low bicarbonate treatment, and the culture was heavily contaminated with protozoa within 3 days of inoculation. Within one week of inoculation, the high treatment was brown in color, whereas the low and control treatments were green.

The below optimum $KHCO_3$ concentration and the limited Na^+ concentration needed for optimal HCO_3^- uptake may explain why increased growth and N-fixation in the low and high bicarbonate treatments did not occur. In a 12-day lab study, 20 mM $KHCO_3$ was the optimum concentration for highest *Anabaena* sp. (ARM 629) growth (Jaiswal and Kashyap 2002). Above or below this concentration, the specific growth rate declined. The high bicarbonate (2 mM $KHCO_3$) and low bicarbonate (0.2 mM $KHCO_3$) treatments were 90% and 99% below the optimum lab concentration, respectively. Due to the low $KHCO_3$ concentrations used in this study, it is likely that the minor additions of HCO_3^- were insufficient to increase growth compared to the control.

Use of the optimum $KHCO_3$ concentration must be accompanied by an adequate supply of Na^+ for HCO_3^- uptake. Two Na^+ dependent HCO_3^- uptake systems, BicA and SbtA, are used for HCO_3^- uptake in cyanobacteria. Na^+ strongly promotes HCO_3^- uptake, whereas K^+ has no effect on it (Reinhold et al. 1984). As the Na^+ concentration increases, more HCO_3^- is taken up via the Na^+ dependent HCO_3^- systems until maximal HCO_3^- uptake is reached (Reinhold et al. 1984). Once maximal HCO_3^- uptake is reached, increasing Na^+ concentrations does not increase

bicarbonate uptake (Fig. 11AII) (Kaplan et al. 1984). Due to the requirement of Na^+ in the cyanobacterial HCO_3^- transport system, the use of $NaHCO_3$ supports higher growth in comparison to $KHCO_3$ (Jaiswal and Kashyap 2002). The use of $NaHCO_3$ increases Na^+ concentrations that in turn promote higher HCO_3^- uptake and higher growth compared to $KHCO_3$. Without additions of Na^+ to AA medium by use of $NaHCO_3$, the AA concentration of Na^+ may have limited full uptake of HCO_3^-. Maximal bicarbonate uptake in the low bicarbonate treatment would require approximately 40 mM Na^+ (Kaplan et al. 1984). This requirement is roughly 36 times more Na^+ than the theoretical AA 1.1 mM Na^+ concentration. The low AA Na^+ concentration limited HCO_3^- uptake, which may explain the lack of increased growth due to C supplementation.

Reasons for lower growth in high treatment

The low Na^+ concentration may have affected the cells' ability to regulate the internal pH during the minimal HCO_3^- uptake that did occur. This could offer an explanation to the lower growth and filament fragmentation in the high dosage. In addition to the Na^+ requirement for Na^+ dependent HCO_3^- transport systems, Na^+ is also needed for regulation of the intracellular pH during HCO_3^- uptake. As HCO_3^- is taken up and located to the carboxysome, an OH^- ion is released from the HCO_3^-, contributing to the alkaline pH of cyanobacteria (Kaplan et al. 1984). To avoid high alkalization of the cytoplasm, a Na^+-H^+ exchange mechanism pumps protons into the cytoplasm. The limited Na^+ concentration could have limited Na^+-H^+ exchange mechanisms, reducing the amount of regulatory H^+ pumped. Assuming there was a greater uptake of HCO_3^- in the high dosage compared to the low dosage, this would cause a greater amount of OH^- released from the HCO_3^-. This coupled with a lower quantity of cytoplasm protons could cause cells to

become too alkaline. Alkalization of cells has been shown to reduce growth and cause fragmentation (Kaplan et al. 1984).

Bicarbonate study conclusions

The addition of $KHCO_3$ was designed to reduce the C limitation in the raceways. A high and fluctuating pH is indicative of a C limitation (Richmond 1999). Both the control and low bicarbonate treatments rose from 8.5 to a maximum of 10.4 pH, indicating that there was C limitation in the low bicarbonate treatment despite the addition of $KHCO_3$ (Fig. 10AII). Neither the low or high bicarbonate treatment increased cyanobacterial growth nor N-fixation compared to the control. The reason for this is two-fold: due to treatment concentrations 90 to 99% lower than the optimum $KHCO_3$ concentration for maximal growth and the low Na^+ concentration of AA medium which limited maximal HCO_3^- uptake. The optimal concentration of bicarbonate should also be supplied with enough Na^+ to support maximal uptake. The use of $NaHCO_3$ will assure adequate Na^+ supply. However, there would be a greater growth advantage to using CO_2 over HCO_3^- since the *Anabaena* genus prefers CO_2 over HCO_3^-, and in turn, higher growth may be achieved with the use of CO_2 compared to HCO_3^- (Jaiswal and Kashyap 2002). Additionally, the high Na requirement for maximal HCO_3^- uptake (40mM) would increase Na levels in soils during cyanobacterial application. Elevated concentrations of Na^+ in soils cause soils to become sodic, having poor drainage due to the dispersion of soil particles (Davis et al. 2012).

Cover study

Light transmission and culture temperature

The CEM DT-1309 Auto Ranging Light Meter measures light wavelength within the range of 480 nm to 620 nm, which corresponds to the photosynthetically active radiation (PAR) range (400-700 nm). Both Thermax and Dura-Film Super 4 had significantly higher light

transmission than Luminance and the construction plastic (Table 3AII). Thermax and Dura-Film also had the highest reported PAR transmission, followed by Luminance (Table 3AII).

The optimum temperature range for growth of *Anabaena* genus is 30-35 °C (Fontes et al. 1987). For most of the experiment, the culture temperature was maintained below the optimum temperature range for all treatments (Fig. 12AII). Therefore, it would be advantageous to select a cover with the highest daily minimum (8AM measurement) and maximum (4PM measurement) culture temperature. The daily maximum temperature of Thermax was significantly higher than that of both Luminance (on average by 1 ± 0.26 ºC) and Construction (on average by 2.65 ± 0.36 ºC) for 4 out of the 6 days measured, while Dura-Film Super 4 was not significantly different from Thermax or Luminance and was significantly higher than the construction plastic (data not shown). When observing values distinct from statistical analysis, Thermax had the highest maximum temperature, followed by the control, Dura-Film Super 4, Luminance, and Construction. There was no difference among all treatments in daily minimum temperature.

Due to the higher measured light transmission, advertised PAR transmission in both the Thermax and Dura-Film Super 4 and the higher maximum temperature in Thermax, it was hypothesized that these covers would promote the highest cyanobacterial growth and N-fixation. One potential advantage of Thermax compared to Dura-Film Super 4 is that it is designed to retain nighttime heat better than Dura-Film Super 4. Heat from the sun that is transmitted through hoop house plastics can be absorbed by the soil and material objects in the hoop house (e.g. raceway frame). During the night, this absorbed heat is released as long wave infrared radiation (IR). Hoop house plastic is specifically designed to absorb released long wave IR and reflect this energy. In turn, this heat is retained in the hoop house and night temperatures are higher in the hoop house than ambient temperatures (AT Films, Inc. 2013). Thermicity is a

measure of how well a hoop house plastic retains long wavelength IR. A plastic with a lower thermicity means more long wavelength IR is retained, allowing more heat to be retained in the hoop house during the night (BGS, Inc. 2013). Thermax has the lowest thermicity and is designed to retain more long wavelength IR than Dura-Film Super 4 and Luminance (Table 3AII). In turn, the night temperatures are likely higher in Thermax than the other tested plastics. Although night temperatures were not recorded, an early morning culture temperature may be indicative of nighttime temperatures. For example, at 6 AM on August 15, 2012 the culture temperature of Thermax and Dura-Film Super 4 was 17.5 °C and 16.7 °C, respectively. Thermax may result in increased nighttime temperatures and may better sustain cyanobacterial growth compared to Dura-Film Super 4. The heat retained by Thermax plastic can provide additional protection against colder nights and may promote higher daytime temperatures.

Thermax plastic and Dura-Film Super 4 transmitted the highest % PAR light. Thermax additionally had a higher culture temperature maxima and the lowest thermicity %. Thus, it would be expected that Thermax and Dura-Film Super 4 would promote the most growth and N-fixation.

Growth and N-fixation

There was no significant difference in the exponential growth rate among all five treatments, including the control. Luminance had a significantly higher linear growth rate compared to Construction and control, while there was no difference in linear growth rate among Luminance, Thermax, and Dura-Film Super 4. There was no significant difference in the net TKN among all treatments, including the control. Finally, all four covers were not significantly different in chlorophyll content. Only Luminance and Thermax were significantly higher in chlorophyll content compared to the control on two of the four days analyzed. Thermax and

Dura-Film Super 4 did not promote the highest growth and N-fixation compared to the other covers and the control.

Zinc as a covariate

There was a significant Zn effect on the final net TKN and chlorophyll content for days 9 and 14 (p <0.05). The average Zn concentration of the AA medium on the final day was 0.44 mg L^{-1}, over 90% higher than the zinc concentration of the AA medium before being dispensed into the steel tanks. Elevated Zn concentrations are toxic to cyanobacteria due to adverse effects on growth, cell division, photosynthesis, and destruction of primary metabolites (Tang et al. 2013). In a lab study, a 76% reduction in *Anabaena* sp. biomass was observed when cultivated in BG-11 medium with 0.5 mg L^{-1} Zn concentration compared to the regular BG-11 medium Zn concentration (Tang et al. 2013). The 0.44 mg L^{-1} Zn concentration was inhibitory to growth in this study and more than likely reduced growth in all treatments and, in turn, inhibited a treatment effect.

Cover study conclusions

It was hypothesized that the two covers with the highest PAR light transmission and maximum daily temperatures (Thermax and Dura-Film Super 4) would produce the highest growth. All covers (Thermax, Dura-Film Super 4, Luminance, and Construction plastic) were not different from the no-cover control for the exponential growth rate and net TKN. There was no significant difference in chlorophyll content among Thermax, Dura-Film Super 4, Luminance, and Construction plastic. Zn leached from the galvanized steel tanks used in the experiment. An ANCOVA analysis with Zn as the covariate verified that there was a significant Zn effect on the chlorophyll content and net TKN. This may explain why no consistent treatment differences among the four plastics were detected.

Appendix II conclusions

There were no treatment differences in biomass and N-fixation yield in batch cultures operated at 20-cm and 25- cm depths. One possible reason for no treatment differences was the reversal in productivity during the study, with the deeper depth offering greater protection from excessive light at the beginning of the study when the light cell density was prone to photoinhibition, in turn, promoting higher growth. As cell density increased over the optimum cell density, the shallower depth then became more productive due to less volume below the compensation depth. However, a higher exponential growth rate and daily areal and volumetric OD in the 25-cm treatment at the beginning of the study to support this hypothesis was not observed.

In the bicarbonate study, neither the low nor high bicarbonate treatments increased cyanobacterial growth or N-fixation compared to the control. The treatment concentrations were below the optimum $KHCO_3$ concentration needed for maximal growth. Additionally, the low Na^+ concentration of AA medium limited maximal HCO_3^- uptake. *Anabaena* genus prefers CO_2 over HCO_3^-, and in turn, higher growth may be achieved with the use of CO_2 compared to HCO_3^- (Jaiswal and Kashyap 2002).

In the cover study it was hypothesized that Thermax and Dura-Film Super 4 would support higher growth and N-fixation compared to Luminance and Construction plastic due to higher light transmittance and maintenance of higher hoop house temperatures. However, no one cover excelled over the other covers, as there was no consistent difference in chlorophyll content, exponential and linear growth rate, and net TKN among the four covers. The Zn concentrations in the AA medium towards the end of the study were found to significantly affect chlorophyll

and net TKN, and it was concluded that Zn negatively affected growth and N-fixation in all treatments and clouded interpretation of results.

TABLES

Table 2AII. The areal and volumetric net total solids (TS) and Total Kiejdahl Nitrogen (TKN) of a xenic culture of *Anabaena cylindrica* cultured at two depths (20-cm and 25-cm) in outdoor tanks (n=3, mean ± standard deviation). One replicate of treatment 25-cm crashed during the study and was not included in analysis. Same letters indicate no statistical difference as determined by one-way ANOVA (p<0.05).

Treatment	Final Net TS		Final Net TKN	
	Areal (mg m^{-2})	Volumetric (mg L^{-1})	Areal (mg m^{-2})	Volumetric (mg L^{-1})
20-cm	26075.3 ± 1924.9 A	244.2 ± 18.0 A	1243.3 ± 388.3 A	11.7 ± 3.64 A
25-cm	31148.9 ± 16318.8 A	233.5 ± 122.3 A	1014.0 ± 446.2 A	7.6 ± 3.3 A

Table 3AII. The measured light transmission percentage of four hoop house plastic covers as estimated by a CEM DT-1309 Auto Ranging Light Meter light meter. The light transmission was averaged over time periods (8AM, 12PM and 4PM) and day with three replications per treatment. Distinct letters indicate statistical difference as determined by Tukey adjusted mean separation (p<0.05). The PAR transmission and thermicity percentages are values reported by the specific cover company (AT Films, Inc. 2013). Thermicity is a measure of how well a hoop house plastic retains long wavelength infrared radiation (IR). A lower thermicity signifies that more long wavelength IR is retained.

Treatment	**Light Transmission(%)**	**PAR Transmission(%)**	**Thermicity(%)**
Control	100.00 A	---	---
Dura-film Super 4	72.65 B	91	55
Thermax	71.86 B	91	28
Luminance	60.20 C	87	85
Construction	36.23 D	---	---

FIGURES

Figure 5AII. Set up of cover study. Five treatments were tested that included: Dura-Film Super 4 (AT Films, Inc.), Thermax (AT Films, Inc.), Luminance (AT Films, Inc.), 4 mil Husky construction plastic and a no cover control. Three replications per treatment were used.

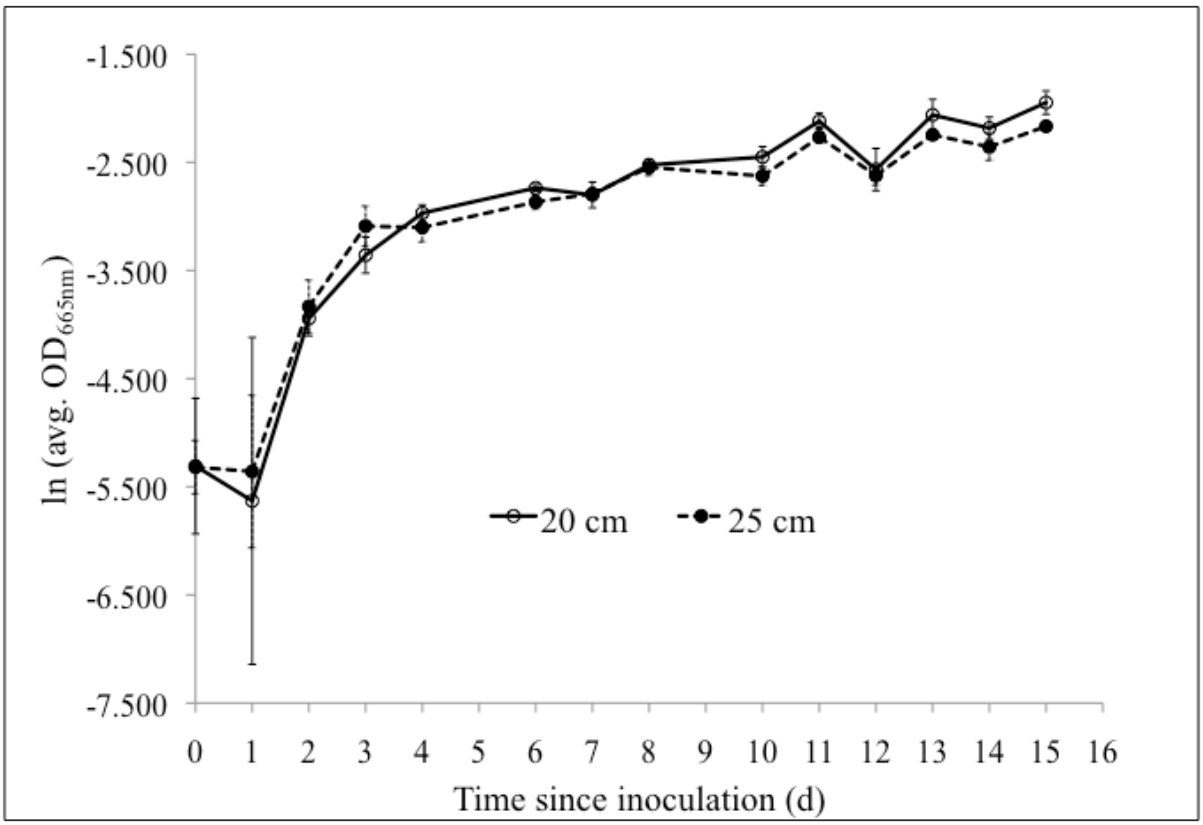

Figure 6AII. The natural log transformed optical density (OD) of a xenic culture of *Anabaena cylindrica* cultivated in outdoor tanks under two operational depths: 20-cm and 25-cm. Exponential growth occurred from day 0 to 6 and linear growth from day 6 to 14. The slope of each growth phase was calculated to determine the growth rate for each phase. There was no treatment difference in the exponential or the linear growth rates as determined by one-way ANOVA (p<0.05). Error bars represent standard deviation (n=3).

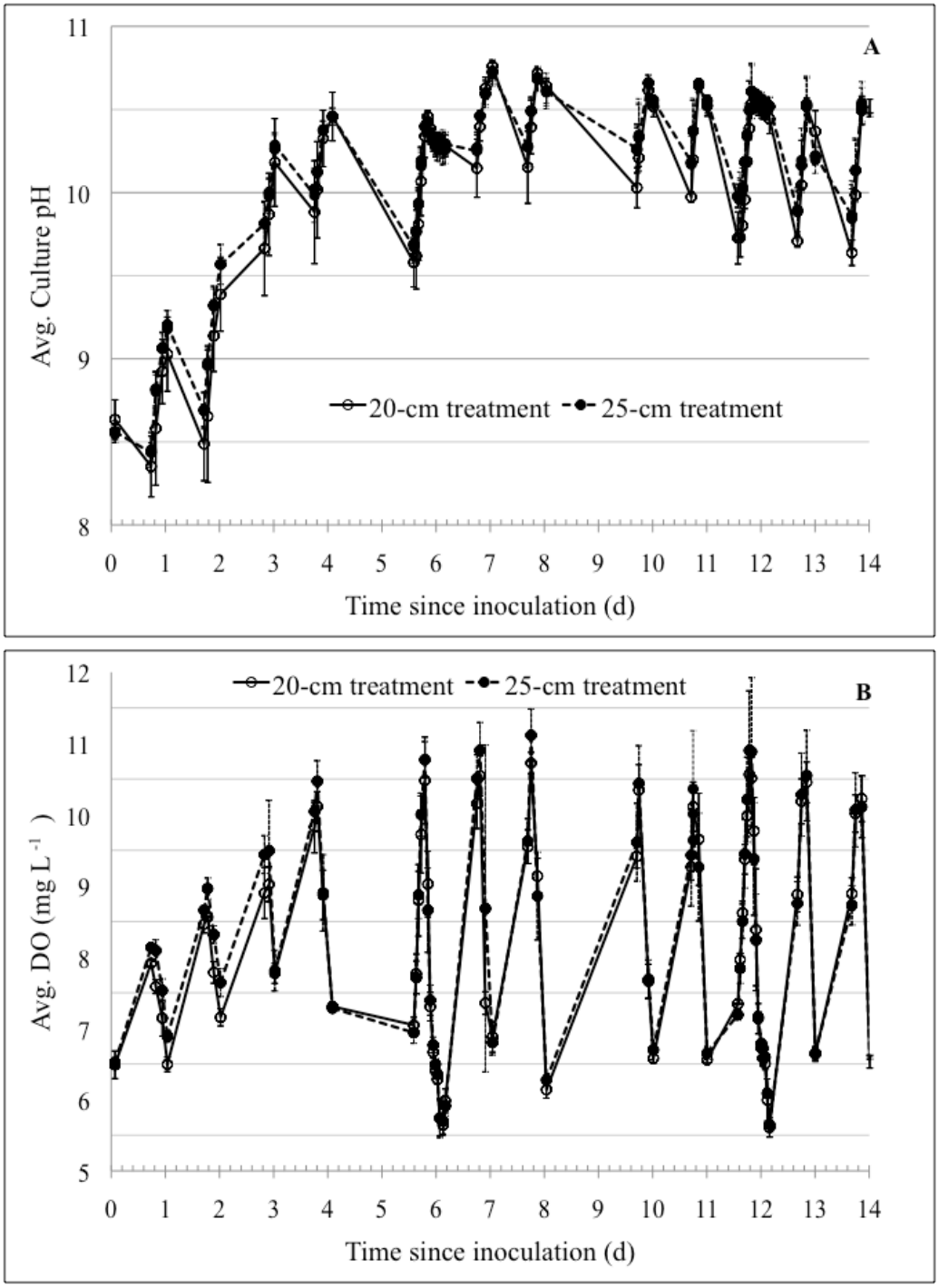

Figure 7AII. The pH (A) and dissolved oxygen (DO) concentration (B) of a xenic culture of *Anabaena cylindrica* cultivated in outdoor tanks under two operational depths: 20-cm and 25-cm. pH and DO were measured at 8AM, 10AM, 12PM and 4PM. Maxima on pH graph occur at 4PM. Maxima on DO graph occur at 10AM on days 0,1,7,9,10, and 11 and at 12PM on days 3, 5,6,12, and 13.). No treatment differences in pH and DO were determined in the repeated measures analysis ($p<0.05$). Error bars represent standard deviation ($n=3$).

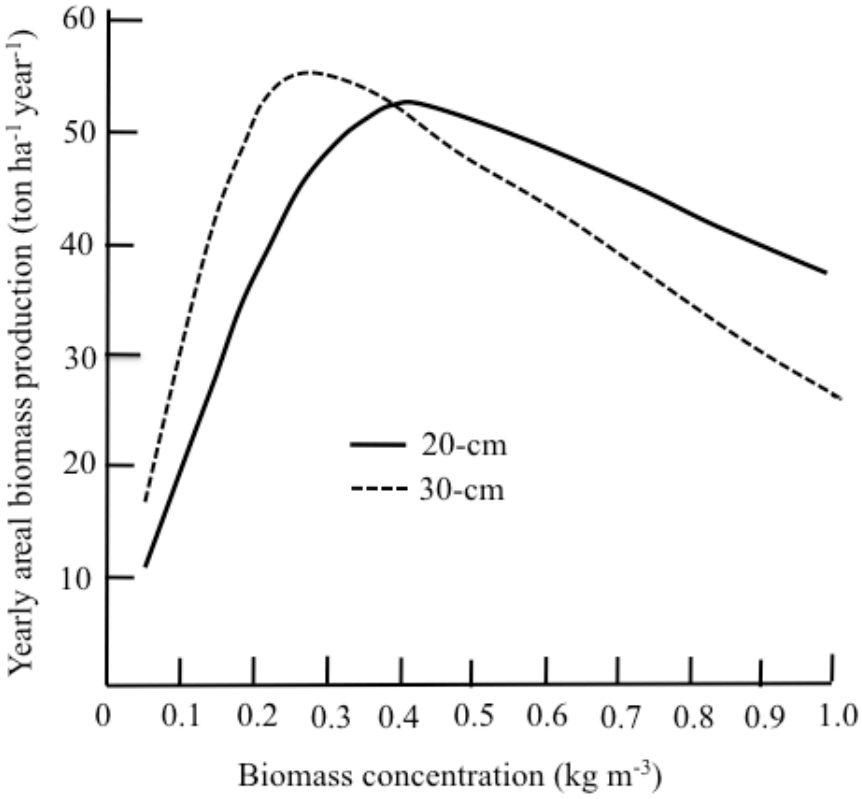

Figure 8AII. The annual areal biomass production of *Phaeodactylum tricornutum* as a function of biomass concentration and pond depth (modified from Slegers et al. 2013).

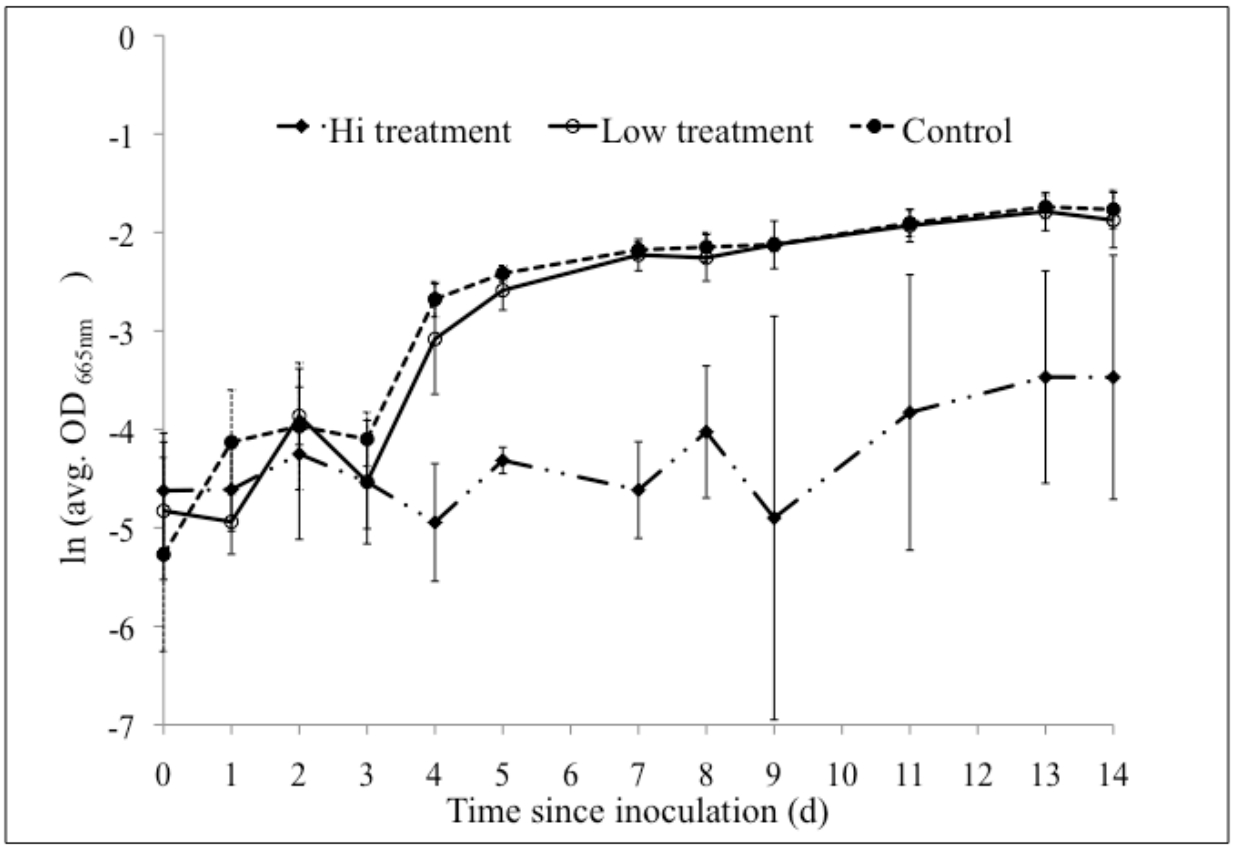

Figure 9AII. The natural log transformed optical density (OD) of a xenic culture of *Anabaena cylindrica* cultivated in outdoor tanks supplied with initial concentration of 0 mM (control), 0.2 mM (low treatment), 2.0 mM (high treatment) $KHCO_3$ added to AA medium. Exponential growth for the control and low treatment occurred from day 0 to 4 while no exponential growth was evident in the high treatment. Linear growth for all treatments occurred from day 4 to 14. The slope of each growth phase was calculated to determine the growth rate for each phase. There was no difference in the exponential growth rate of the control treatment and the low bicarbonate treatment as determined by an ANCOVA (p<0.05). The linear growth rates of the 3 treatments were not statistically different. Standard deviation is represented by error bars (n=3).

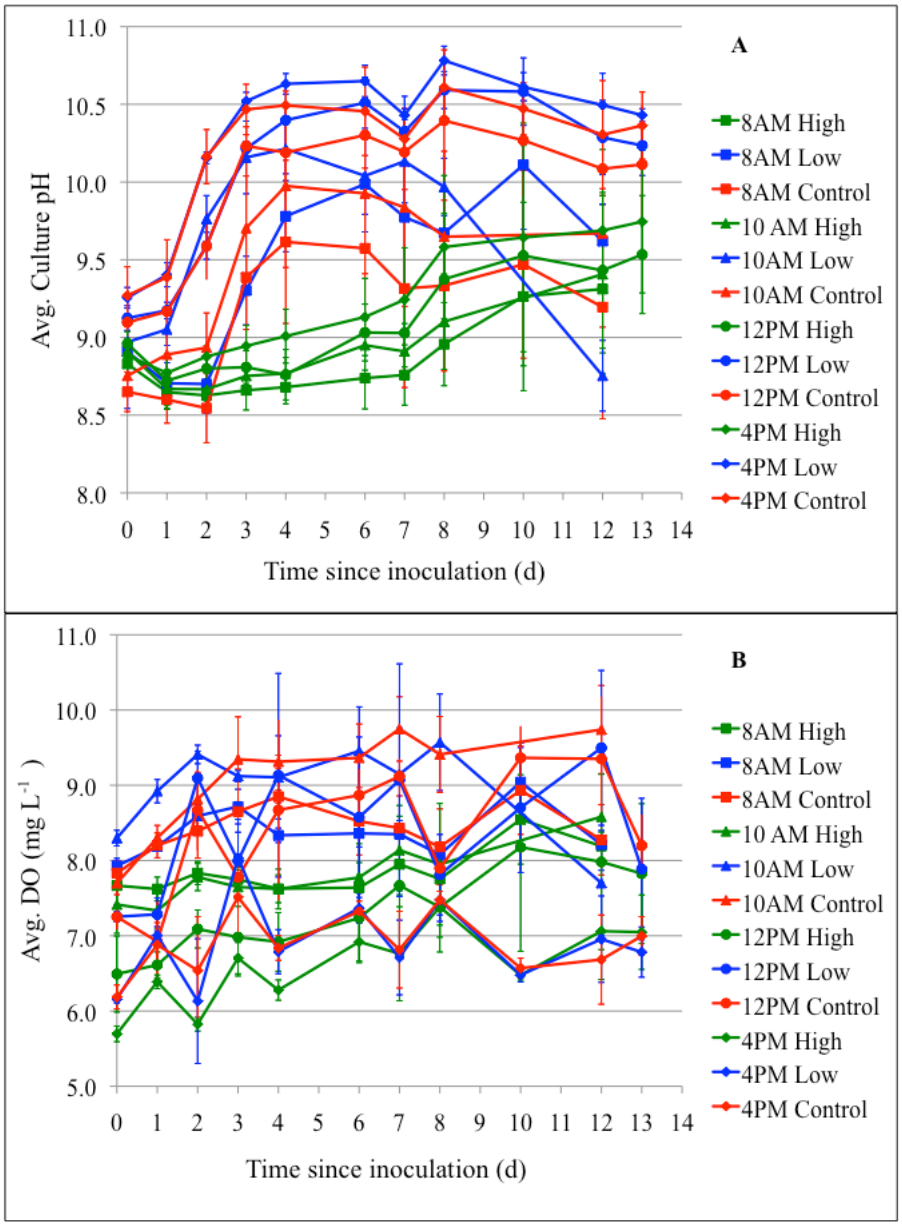

Figure 10AII. The pH (A) and dissolved oxygen (DO) concentration (B) of a xenic culture of *Anabaena cylindrica* cultivated in outdoor tanks supplied with an initial concentration of 0 mM (control), 0.2 mM (low treatment), 2.0 mM (high treatment) $KHCO_3$ added to the AA medium. pH and DO were measured at 8AM, 10AM, 12PM and 4PM. The standard deviation is represented by error bars (n=3). On 8AM and 4PM DO measurements, from day 1 to day 7, the control and low treatment were significantly higher than the high treatment, and from day 8 to 14, there was no significant difference in DO among the three treatments. On 10AM and 12PM DO measurements, from day 1 to day 14 the control and low treatment were significantly higher than the high treatment. On 12PM and 4PM pH measurements, beginning on day 2, control and low treatments were both significantly higher than the high treatment (p<0.05). Significance was determined with a repeated measures analysis.

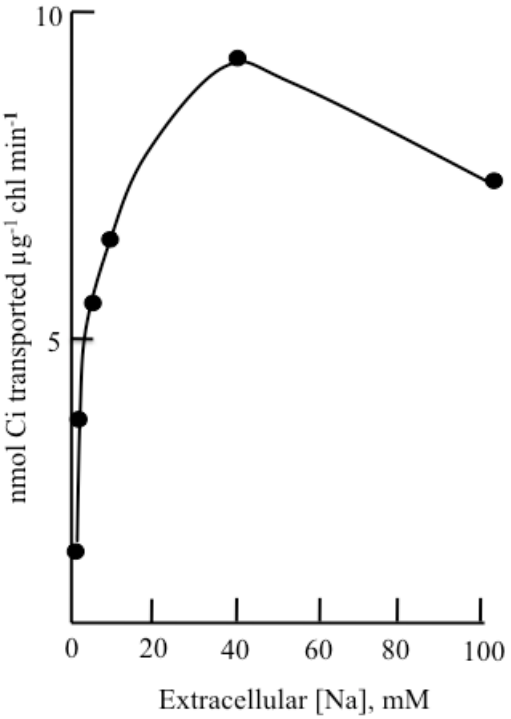

Figure 11AII. The dependence of extracellular Na concentrations on uptake of HCO_3^- by *Anabaena variabilis* cells. Cells were supplied with 0.15 mM $NaHCO_3$ for 5 s (modified from Kaplan et al. 1984).

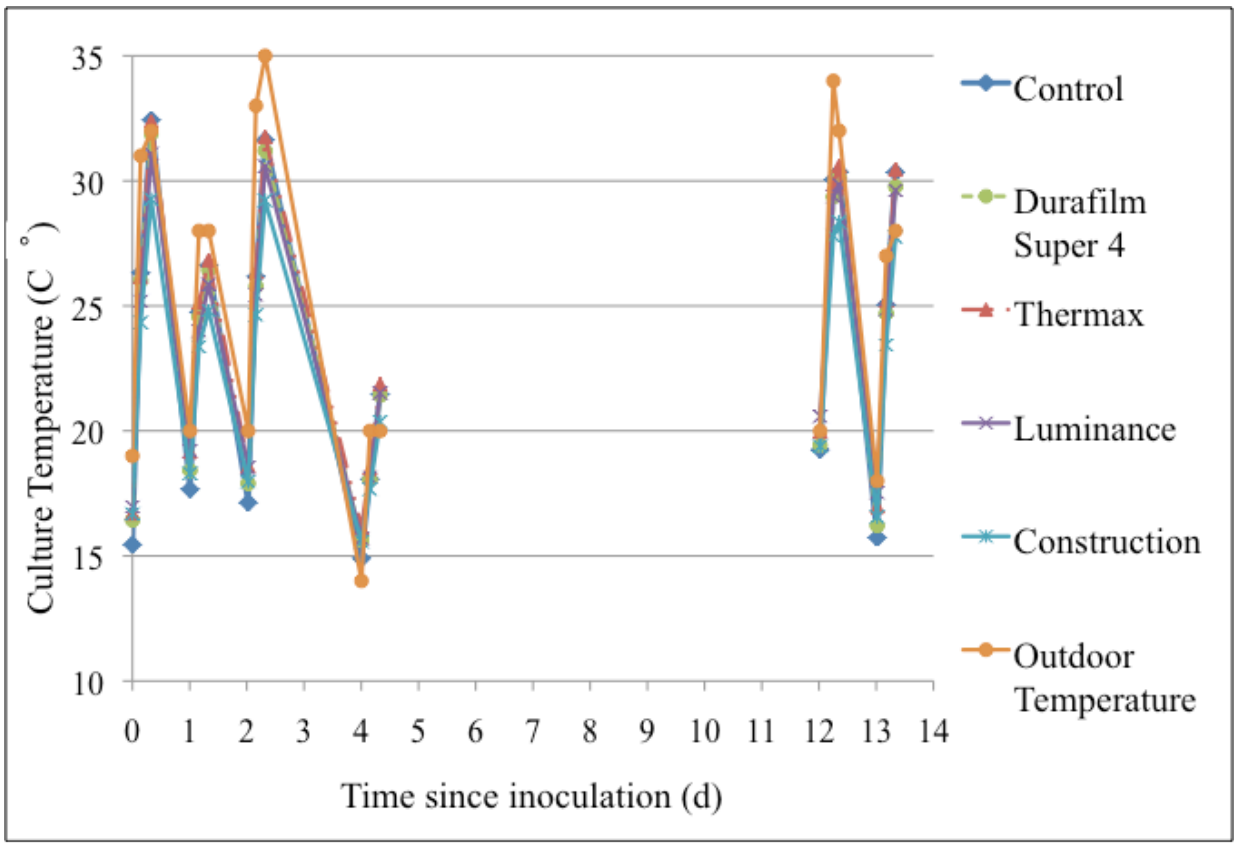

Figure 12AII. The culture temperature at 8AM, 12PM, and 4PM of a xenic culture of *Anabaena cylindrica* cultivated in outdoor tanks under different hoop house covers. Data was missing from 8/17/12 to 8/23/12. Treatment differences occurred on 4PM measurements, where Thermax was significantly higher than Luminance and construction plastic on days 1, 3, 13, and 14, while Dura-Film Super 4 was not significantly different from Thermax or Luminance and significantly higher than the construction plastic as determined by repeated measures analysis ($p<0.05$).

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