Understanding how cyanobacterial communities respond to different dissolved nitrogen forms

by

Matthew F. Gladfelter

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Approved by

Alan Wilson, Chair, Professor of Fisheries and Allied Aquaculture, Auburn University
Matthew Wolak, Assistant Professor of Biological Sciences, Auburn University
Morgan Steffen, Associate Professor of Biological Sciences, James Madison University

Abstract

This thesis explored the ways in which cyanobacterial communities respond to three common nitrogen forms (i.e., nitrate, ammonium, and urea) via two field experiments. Chapter I describes a field experiment that demonstrated the preference of cyanobacterial communities to reduced forms of nitrogen (i.e., ammonium and urea) that was observed through high uptake and removal from the water column. Chapter II delves into how the cyanobacteria are using the nitrogen that was being introduced and showed that when excess concentrations of reduced nitrogen enter the water column, cyanobacteria quickly incorporate the nitrogen into phycocyanin pigments as a means of nitrogen storage. Then, once the environment is devoid of nitrogen, the cyanobacteria will begin degrading the phycocyanin pigments as a nitrogen source. Results also showed, on average, that reduced nitrogen forms promote more cells, a longer peak bloom period, and more toxins.

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Chapter 1

Nitrogen form dictates use and uptake rate in cyanobacterial communities

ASTRACT

The heavy use of nitrogen-based fertilizers and similar substances have made eutrophication and cyanobacterial blooms a regular occurrence. Cyanobacterial blooms are directly responsible for hundreds of millions of dollars in damage or lost revenue annually. While the complete prevention of excess nitrogen entering a system may be impractical, the next best solution may be to switch to nitrogen forms that do not promote cyanobacterial growth. A five-week field experiment tested this idea by subjecting cyanobacterial communities to three forms of nitrogen (i.e., nitrate, ammonium, and urea) at two levels of concentration (2 and 4-times ambient nitrogen concentration) to identify which, if any, nitrogen form promoted cyanobacterial growth or other harmful side effects like the production of toxic and taste and odor secondary metabolites. Although the treatments did not significantly affect cyanobacterial or total phytoplankton abundance throughout the experiment, large uptake differences were observed across the three different dissolved nitrogen forms. Urea and ammonium were removed at relatively equal rates during the experiment, while nitrate was removed at the lowest. This experiment provides a solid foundation for future experiments on the effects of different nitrogen forms on cyanobacterial communities.

INTRODUCTION

Harmful cyanobacterial blooms are an ongoing global issue. Cyanobacterial blooms are outgrowths of cyanobacterial biomass that have been increasing in occurrence due to eutrophication and global warming in freshwater systems (Hallegraeff, 1993; Glibert et al., 2005; Heisler et al., 2008; Paerl et al. 2016a). This increase in occurrence, and the accompanying

detriments to wildlife, livestock, pets, and even humans, have made harmful algal blooms (HABs) an annual talking point as these deleterious effects result in millions of dollars of lost revenue, medical expenses, and/or water management (Anderson et al., 2000). The toxic compounds that can be released into the water column upon cell death and/or lysing coupled with taste and odor compounds combine to make affected water bodies unpalatable as drinking water sources or even harmful as recreational sites (Dolah et al., 2001; Hillborn et al., 2014; Brooks et al., 2016). These effects are exacerbated in systems that have excessive nutrient loading as a result of heavy fertilizer use in urban and agricultural settings (Codd, 2000; Anderson et al., 2002; Shaw et al., 2003).

Fertilizers have been widely used in agricultural and urban areas since the early twentieth century (United States Environmental Protection Agency (USEPA), 2018). Since then, their use has steadily increased to the point where over 20 million tons of fertilizers are applied to crops annually in the U.S. alone (USEPA, 2018; United States Department of Agriculture (USDA), 2019). The growth benefits associated with fertilizer use are not confined to the area of application, however. Fertilizers can easily be washed out of soils before they are taken-in by the intended recipient plant and flow down the watershed into a waterbody (Nissen and Wander, 2003; Hubbard et al., 2004). This sudden pulse of nutrients, namely nitrogen and phosphorus, can cause increases in phytoplankton biomass and result in the aforementioned algal blooms (Anderson et al., 2002; Glibert et al., 2005).

Nitrogen, along with phosphorus, are two nutrients that are closely linked to algal blooms (Anderson et al., 2002) and are a common focus for bloom management. It is debated as to which is more important for phytoplankton growth (Schindler, 1974; Paerl et al., 2014; Schindler et al., 2016; Paerl et al., 2016b). Phosphorus is of course vital to the make-up of cell lipid

bilayers, production of coenzymes, and the synthesis of DNA, RNA and ATP and is only useful in its dissolved phosphate form (Kornberg et al., 1999). Nitrogen, likewise, is needed for amino acid and protein synthesis, pigment production, toxin production, and DNA replication, but can be useful to phytoplankton in several organic or inorganic forms and states of matter (Herrero et al., 2001; Flores and Herrero, 2005).

Although cyanobacteria can utilize different forms of nitrogen to meet their needs, the cells must convert the nitrogen into ammonium before it can be used in the various metabolic pathways (Herrero et al., 2001; Flores and Herrero, 2005). This fact has led to the assumption that different nitrogen forms may require more energy expended by the cell before it can be used, meaning that certain nitrogen forms could be 'favored' by cells due to their low energetic input requirements (Raven et al., 1992; Glibert et al., 2016; Erratt et al., 2018).

The five forms of bioavailable nitrogen that can be assimilated by cyanobacteria are gaseous nitrogen (N_2) which is only available to diazotrophic cyanobacteria capable of nitrogen fixation, nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+), and urea (CH_4N_2O) (Herrero et al., 2001; Flores and Herrero, 2005). Nitrogen fixation is the most energetically expensive nitrogen source due to the incredible amount of energy required to break the N-N triple bond. In optimal conditions, this process still requires 16 molecules of ATP to fix the dinitrogen gas molecule (Herrero et al., 2001; Flores and Herrero, 2005). For nitrate to be converted to a useful form, it must first be reduced to nitrite, then reduced again to ammonium. Although this requires several intermediary steps, this nitrogen form is still favorable and less energetically expensive than nitrogen fixation (Herrero et al., 2001; Flores and Herrero, 2005). Ammonium is energetically neutral as it can be taken in through ion pumps and then immediately used in metabolic pathways as needed and is less energetically expensive than nitrate or gaseous nitrogen (Herrero et al.,

2001; Flores and Herrero, 2005). Urea use in cells requires the molecule to be hydrolyzed by urease, resulting in two molecules of ammonium (Donald et al., 2011). While the cell requires energy to synthesize urease, the energetic gains of producing two ammonium molecules from one urea molecule make urea a potentially favorable nitrogen source. Furthermore, the hydrolysis of urea results in the release of CO_2 as a byproduct leading to the belief that this carbon could be used in photosynthesis (Glibert et al., 2014).

The potential benefits of a cyanobacterial cell using urea as a steady nitrogen source coupled with the fact that up to 60-80% of fertilizers used today are urea-based fertilizers (USDA, 2019) could point to an increase in the occurrence and severity of harmful cyanobacterial blooms moving forward. Previous studies (Raven et al., 1992; Blomqvist et al., 1994; Glibert et al., 2016; Erratt et al., 2018) have outline that reduced forms of nitrogen like ammonium and urea are typically preferred compared to oxidized forms like nitrate, but little else. Other hypotheses related to increased growth and toxicity have been offered, but with little replicable support.

In this field experiment, we sought to understand if different nitrogen forms are being taken in at higher rates relative to other nitrogen forms and if this is related to negative effects associated with harmful algal blooms (increased pigment concentration, increased toxin production, increased off-flavor production). We also wanted to test this hypothesis in a field setting where a more complete bloom community is present and results are truer to how a natural, functioning cyanobacterial bloom community with its associated bacterial community would respond to these changes. We hypothesized that (1) ammonium would be removed from the water column at the highest rate followed by urea then nitrate, (2) cyanobacterial pigment concentrations would be reflective of the uptake rates, with ammonium treatments having the

greatest concentration followed by urea and nitrate, following the quality over quantity hypothesis put forth by Erratt et al. (2018) and (3) ammonium and urea treatments would have the highest concentrations of toxins and off-flavors as a result of excess nitrogen being available for amino acid production etc.

METHODS

Site Description

The experiment was conducted from mid-July to mid-August of 2019 at pond S12 just north of the E.W. Shell Fisheries Center at Auburn University in Auburn, Alabama, USA (32°40'09.5"N, 85°30'24.2"W). Pond S12 is an earthen aquaculture production pond with a maximum depth of 2.7 m, a surface area of 9186 m^2, and a volume of 13198 m^3. The pond's nutrients fluctuated throughout 2018-2019 ranging from 883 µg/L total nitrogen (TN) and 128 µg/L total phosphorus (TP) during the winter (January-February) to 6390 µg/L TN and 714 µg/L TP during the summer (July-August) when catfish production is at its peak. S12 also had high primary productivity during the summer months with chlorophyll-a peaking at 503 µg/L and phycocyanin, a pigment protein found in cyanobacteria, peaking at 1126 µg/L.

Experimental design

This five-week nutrient manipulation experiment used 28 mesocosms made from clear polyethylene with a volume of 1640 L (diameter = 1.18 m, depth = 1.5 m). Mesocosms were sealed at the bottom, open at the top to the environment and suspended on floating PVC frames. All the enclosures were filled with water from pond S12 pumped through a 150um sieve to exclude large-bodied grazers, fish, leaves, and other debris. One week after set-up, all enclosures were sampled using an integrated tube sampler to a depth of 0.5 m. This sampled water was measured for total nitrogen (TN), total phosphorus (TP), chlorophyll, phycocyanin, nitrate,

ammonium, urea, toxins (microcystin), and taste and odor concentrations (2-methylisoborneol and geosmin). Two-days after the first sampling period, enclosures were fertilized with their respective nitrogen species at the appropriate concentration for each treatment. Each enclosure was randomly assigned a treatment in a controlled way that assured each treatment had two enclosures on the north side of the frames, two on the south side, no treatment had more than one enclosure on a corner, and replicates were at least two enclosures apart. The seven total treatments (control, nitrate 2x, nitrate 4x, ammonium 2x, ammonium 4x, urea 2x, urea 4x) each had four replicates. The 2x and 4x treatments refer to the nitrogen concentration with respect to the ambient total nitrogen measured in S12 prior to the beginning of the experiment (1500 µg/L) (Duarte et al., 2000). For example, in the nitrate 2x treatment, nitrate was added to the enclosure to reach a TN of double the ambient TN or 3000 µg/L. To prevent the cyanobacteria from being phosphorus-limited, phosphorus was also kept at a 10:1 TN:TP ratio with respect to each treatment. Nitrogen and phosphorus were added to the enclosures weekly to maintain treatment concentrations and monitor nitrogen removal rate from week to week. Nitrate was added as sodium nitrate ($NaNO_3$), ammonium was added as ammonium chloride (NH_4Cl), urea was added as urea (CH_4N_2O), and all phosphorus was added as potassium phosphate (K_2HPO_4).

Enclosures were sampled weekly between 7am-9am for the above-mentioned water quality parameters along with temperature (ºC) and dissolved oxygen with the use of a Hydrolab multisonde. Once collected, all samples were brought back to the lab. Total nitrogen and total phosphorus were measured spectrophotometrically according to Gross and Boyd (1998). Nitrate, ammonia, and urea were measured colorimetrically according to Polysciences Incorporated (2011), Reardon et al. (1966) and Chen et al. (2015), respectively. Chlorophyll-a and phycocyanin were measured fluorescently according to Kasinak et al. (2015). Off-flavor

compounds (geosmin and 2-methyisoborneol) were measured using solid-phase microextraction (SPME) gas chromatography/mass spectrometry (GC/MS) (Schrader and Tucker, 2012), and toxin compounds were measured using enzyme-linked immunosorbent assay (ELISA) kits (Yang et al., 2018).

Data analysis

All statistical analyses were conducted using RStudio version 3.6.0. A series of two-way repeated measures analysis of variance (RM-ANOVA) were completed using the **nlme** package (Pinheiro et al., 2020) to compare pigment concentration, nutrient concentration, toxin and off-flavor concentration between treatments over time. Models first looked for the effects of treatment, time, and the interaction between treatment and time. When appropriate, data following a quadratic curve will be fit to a model to account for this pattern. However, if the quadratic model fails to significantly improve the fit of the data then the simpler RM-ANOVA will be used. If any significant differences ($p < 0.05$) were observed in the model a pair-wise multiple comparisons post-hoc test using the **emmeans** package (Lenth et al., 2020) was used to identify when and between what treatments were differences observed (see supplemental materials for example of code used). Please note that the **emmeans** package automatically adjusts for multiple consecutive comparisons.

RESULTS

Nitrogen Removal

All nitrogen additions resulted in increased amounts of total nitrogen (Figure 1). The only difference in total nitrogen concentrations were seen during week 1 and between the nitrate 4x and ammonium and urea 4x. The nitrate 4x treatment was significantly higher than the expected threshold of 6000 µg/L.

The nutrient additions resulted in increased dissolved nitrogen concentrations for each treatment (Figure 2A). These dissolved nitrogen measurements did not directly coincide with the total nitrogen measurements particularly when the values were converted into the amount of nitrogen removed. There was a significant treatment effect (RM-ANOVA, $F_{5, 18} = 31.71$, $p < 0.0001$), no significant effect of time (RM-ANOVA, $F_{3, 54} = 2.695$, $p = 0.0550$) and a significant interaction of time and treatment (RM-ANOVA, $F_{15, 54} = 3.723$, $p = 0.0002$, Figure 2B). As Figure 2B shows, most dissolved nitrogen is removed at a relatively stable rate throughout the experiment. The only major exception is the ammonium 4x treatment which steadily increased in the amount of nitrogen removed as the experiment progressed. In fact, if it were not for that ammonium 4x treatment, there would not be an observed effect of time on the amount of nitrogen removed, as the post-hoc comparisons showed. The ammonium 4x treatment was not the only treatment to see significant differences over the course of the experiment; this treatment had significantly more nitrogen removed than all other treatments from weeks 2-3 and after. Most differences were observed earlier in the experiment and became less pronounced as the experiment progressed. The urea 2x treatment had an average of 1708 µg/L more nitrogen removed than the nitrate 2x treatment between weeks 1 and 2 ($p = 0.0041$). The urea 4x treatment also had an average of 2012 µg/L more nitrogen removed than the nitrate 2x treatment between this same time period ($p = 0.0008$). These differences would persist from weeks 2 to 3 where the urea 2x treatments had an average of 1322 µg/L more nitrogen removed than the nitrate 2x treatment ($p = 0.0326$). Similarly, the urea 4x treatment had an average of 1364 µg/L more nitrogen removed than the nitrate 2x treatment ($p = 0.0261$). However, these differences would not persist through the rest of the experiment.

Algal Pigments

Because the pigment data appeared to follow a quadratic curve, the data were fit to models to account for this pattern. However, the quadratic models showed no significant change in the F statistics or p-values and the simpler RM-ANOVA was used. All treatments supported phytoplankton growth, but there was no significant effect of the nutrient treatment on the chlorophyll concentration (RM-ANOVA, $F_{6, 21} = 0.6129$, $p = 0.7175$, Figure 3A). None of the treatments were significantly different from each other as a result, with respect to chlorophyll concentration. There was a significant effect due to time, however (RM-ANOVA, $F_{4, 84} = 8.468$, $p < 0.0001$). Lastly, there was no significant interaction between treatment and time (RM-ANOVA, $F_{24, 84} = 0.6908$, $p = 0.8471$). The multiple comparisons post-hoc test showed that the significant effect due to time is attributed to three pairs of measurements. The NH_3 2x treatment had 52.83 µg/L more chlorophyll in week 4 compared to week 5, on average ($p = 0.0101$). The urea 2x treatment had 47.15 µg/L more chlorophyll in week 3 compared to week 1, on average ($p = 0.0286$). The urea 2x treatment also had 49.66 µg/L more chlorophyll in week 3, on average, compared to week 5 ($p = 0.0183$).

All nutrient treatments supported cyanobacterial growth, but there was no significant effect of the nutrient treatments on the phycocyanin concentration (RM-ANOVA, $F_{6, 21} = 0.7285$, $p = 0.6319$, Figure 3B). There was a significant effect of time on the phycocyanin concentration (RM-ANOVA, $F_{4, 84} = 4.685$, $p = 0.0018$). There was no significant interaction between nutrient treatment and time (RM-ANOVA, $F_{24, 84} = 0.7350$, $p = 0.8017$). The multiple comparisons post-hoc test showed that the significant effect due to time was a result of a single pair-wise difference; the phycocyanin concentration of the NH_3 4x treatment was 116 µg/L higher in week 3, on average, compared to week 1.

Toxins and Off-flavors

No detectable amounts of toxins were observed in the enclosures during community growth, peak, or death and were not included in statistical analyses. Common taste and odor compounds associated with cyanobacterial blooms were detected, but not consistently across a particular treatment or nutrient type. There were no significant treatment effects on MIB (RM-ANOVA, $F_{6, 21} = 1.607$, $p = 0.1945$, Supplemental Figure S1A) or geosmin concentration (RM-ANOVA, $F_{6, 21} = 1.479$, $p = 0.2332$, Supplemental Figure S1B).

DISCUSSION

Both nitrogen and phosphorus require careful regulation to mitigate the harmful effects of cyanobacterial blooms. Yet phosphorus is only useful to phytoplankton as dissolved phosphate (Kornberg et al., 1999), while nitrogen can be used by phytoplankton in multiple different forms and states (Herrero et al., 2001; Flores and Herrero, 2005). Mitigation efforts could be aided by targeting specific nitrogen forms that give harmful algal species a competitive advantage or encourage production of toxic or off-flavor compounds. This experiment looked to identify those nitrogen forms that may give harmful cyanobacteria a competitive advantage.

The primary way that cyanobacteria could benefit from the use of nitrogen forms, like ammonium and urea, according to Erratt et al. (2018), Li et al. (2018) and Zhou (2020) is photosynthetic capacity. These experiments found that, under controlled conditions in a lab setting, cyanobacterial cultures grown on urea had higher concentrations of both chlorophyll and phycocyanin, with phycocyanin experiencing a profoundly greater increase in concentration. This significantly greater concentration of pigments, particularly phycocyanin, lead the authors to theorize that resources and/or the energy saved/gained by utilizing reduced nitrogen forms were not being put towards growth and cell replication, but rather towards pigment production

and increased photosynthetic capacity. This adaptation could certainly give cyanobacterial species an advantage over other phytoplankton in low-light conditions, like those found in dense bloom communities. However, as Figure 1 shows, there is no significant difference in the chlorophyll or phycocyanin concentration in these communities. Further, there is a great deal of within-treatment variation which does not provide support for our hypothesis that efficient nitrogen forms like ammonium and urea would result in increased growth and pigment concentration as a result. This observation in the field does not necessarily refute the findings of previous studies. Indeed, it is likely that reduced nitrogen forms encourage higher pigment content in cyanobacterial cells, but those differences become muted and less pronounced in a complex phytoplankton community where multiple different taxa are vying for nitrogen at once. Future experiments should expand on these ideas and examine the uptake of nitrogen in different phytoplankton, not solely cyanobacteria, to add context to the results presented here and the differences between these and those presented by lab-based experiments.

The rate at which phytoplankton remove nitrogen from the water column can be a clue as to which nitrogen forms are more useful or more efficient for the cell to take-in for protein synthesis and other metabolic processes. Presently, there have been few projects to examine nitrogen efficiency or preference in cyanobacterial bloom communities (but see Glibert et al., 2014; Erratt et al., 2018), particularly urea. We found that, when standardized for concentration and time, urea and ammonium at low concentrations seemed to be removed at higher rates than nitrate. This finding aligns with our initial hypothesis and suggests that ammonium and urea are not necessarily preferred over each other in these systems. This would agree with Blomqvist et al. (1994) who hypothesized that reduced forms of nitrogen (i.e., ammonium and urea) are preferred by prokaryotes while eukaryotes are more adept to use oxidized forms of nitrogen (i.e.,

nitrate and nitrite). However, when the nitrogen removal data are compared to the pigment data it is difficult to see how the cells are using ammonium and urea. Trends in the phycocyanin concentrations could point to the quality over quantity hypothesis put forth by Erratt et al. (2018), but further field experimentation is required due to the high variability observed in this study. Future projects should take a more molecular approach and uncover mechanisms for urea uptake regulation and look to explain why this molecule is taken-in so rapidly and where exactly the nitrogen is used and by which organisms.

Some interesting patterns can be observed when comparing Figures 2B and 3B. Primarily, how the urea 4x treatment saw such rapid removal of TN from week 1 to week 3 while the removal of dissolved urea stayed relatively steady throughout the experiment. The urea 4x treatment did not experience particularly high values of phycocyanin, which could suggest that the urea was too concentrated (i.e., toxic) and the cyanobacteria were unable to grow as a result. This observation coincides with previous studies that suggest high concentrations ($\geq$ 3.6mmol-N/L, but can be species-specific) or even prolonged cultivation on urea is toxic to cyanobacteria and inhibits growth (Collier et al., 1999; Wu et al., 2015). This lack of growth would have resulted in the urea either settling out of the water column or being used by denitrifying bacteria. The opposite can be said about the ammonium 4x treatment which experienced such excessive dissolved nitrogen removal compared to other treatments. One explanation is that the ammonium was being rapidly taken-in by the phytoplankton and stored in the cell. Then, once ammonium became too concentrated and the pH began to rise, the cells died and settled out. The resulting ammonia from the elevated pH may then have been lost to the atmosphere. This could also explain why ammonium/ammonia was being removed, but phycocyanin quickly crashed in that treatment.

The way that organisms can cope with their surroundings and manipulate them to their benefit is incredibly fascinating, particularly for microscopic organisms. Erratt et al. (2018) found that when cultures of *Dolichospermum sp., Microcystis sp.,* and *Synechococcus sp.* were grown with urea as the sole nitrogen source the concentration of urea steadily decreased as the cyanobacteria were consuming it, but ammonium began to increase, suggesting that the cyanobacteria were importing urea, hydrolyzing it, and then exporting one or both of the ammonium molecules back out of the cell. This contradicts most of what is expected out of microbes, which are notoriously efficient in terms of energy expenditures. However, these results were not necessarily new. Mackerras and Smith (1986) found that *Anabaena cylindrica* will hydrolyze urea in excess of cellular requirements. Similar conditions were observed in this field experiment (Figure 4) showing that this is something that cyanobacteria are well-adapted to. In this experiment, nitrogen did not exceed 400 µM (5600 µg/L) nitrogen in the urea 4x treatments, while Erratt et al. (2018) subjected their cultures to 3000 µM (42000 µg/L) and 7000 µM (98000 µg/L) nitrogen. It was in the 7000µM treatment where they observed the urea 'gluttony'; however, this mechanism was observed in the field at the lower concentrations. Further experimentation is required to fully understand this mechanism, but one hypothesis that we put forth is that because urea in high concentrations is toxic and inhibits cyanobacterial growth the cyanobacteria are transforming the toxic urea into a less toxic nitrogen form (i.e., ammonium). Again, further experimentation is required to fully understand what is occurring here.

CONCLUSIONS

Harmful cyanobacterial blooms will continue to be an issue in freshwater systems if excessive nutrient pollution persists. It should be noted that cyanobacteria must not be regarded as immediately harmful or nuisance species if they are identified in drinking water, recreation, or

livestock systems. Cyanobacteria, like all phytoplankton give off oxygen during photosynthesis, and are a part of a healthy ecosystem. Issues arise when excess nutrients encourage growth in ways that give cyanobacteria a competitive advantage to the point where they dominate an ecosystem and make the water dangerous to drink or come in contact with. Limiting both nitrogen and phosphorus should still be a major target in mitigating harmful cyanobacterial blooms. This experiment focused on nitrogen because of its various forms and states in which it can be useful to cyanobacteria. We found that urea and ammonium were removed at higher rates than nitrate, but neither ammonium nor urea seemed to be preferred. This high removal rate did not coincide with increased pigment concentration as chlorophyll and phycocyanin did not differ significantly between treatments throughout the experiment. Phycocyanin was highly variable and trends may suggest that urea and ammonia promote high phycocyanin concentrations, but more experiments in the field are required.

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FIGURE LEGENDS

Figure 1. Average total nitrogen (A) and total nitrogen removed from week to week (B) for each treatment over time ± SEM. Nutrient additions had a significant effect on the total nitrogen concentration ($p < 0.001$) over time. There was also a significant treatment effect for the amount of total nitrogen lost from the water column ($p = 0.0001$).

Figure 2. Average dissolved nitrogen (A) and amount of dissolved nitrogen removed (B) for each treatment over time ± SEM. Nutrient additions had a significant effect on the dissolved nitrogen concentration ($p < 0.0001$). There was also a significant treatment effect for the amount of dissolved nitrogen removed from the water column ($p < 0.0001$).

Figure 3. Average chlorophyll-a (A) and phycocyanin (B) concentrations for each treatment over time ± SEM. Treatment effects were not observed in either the concentration of chlorophyll ($p = 0.7175$) or phycocyanin ($p = 0.6319$).

Figure 4. Average urea and ammonium concentration for the urea 2x (A) and the urea 4x (B) treatments ± SEM. Only urea was added to these enclosures. Cyanobacteria appear to be hydrolyzing urea in excess of metabolic requirements.

Figure 1.1

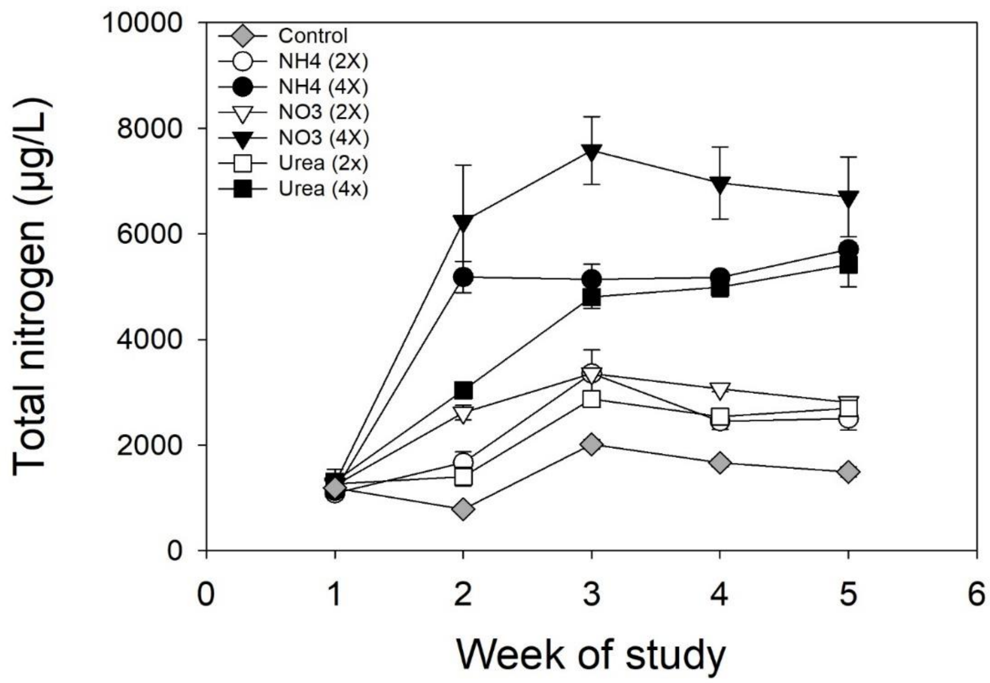
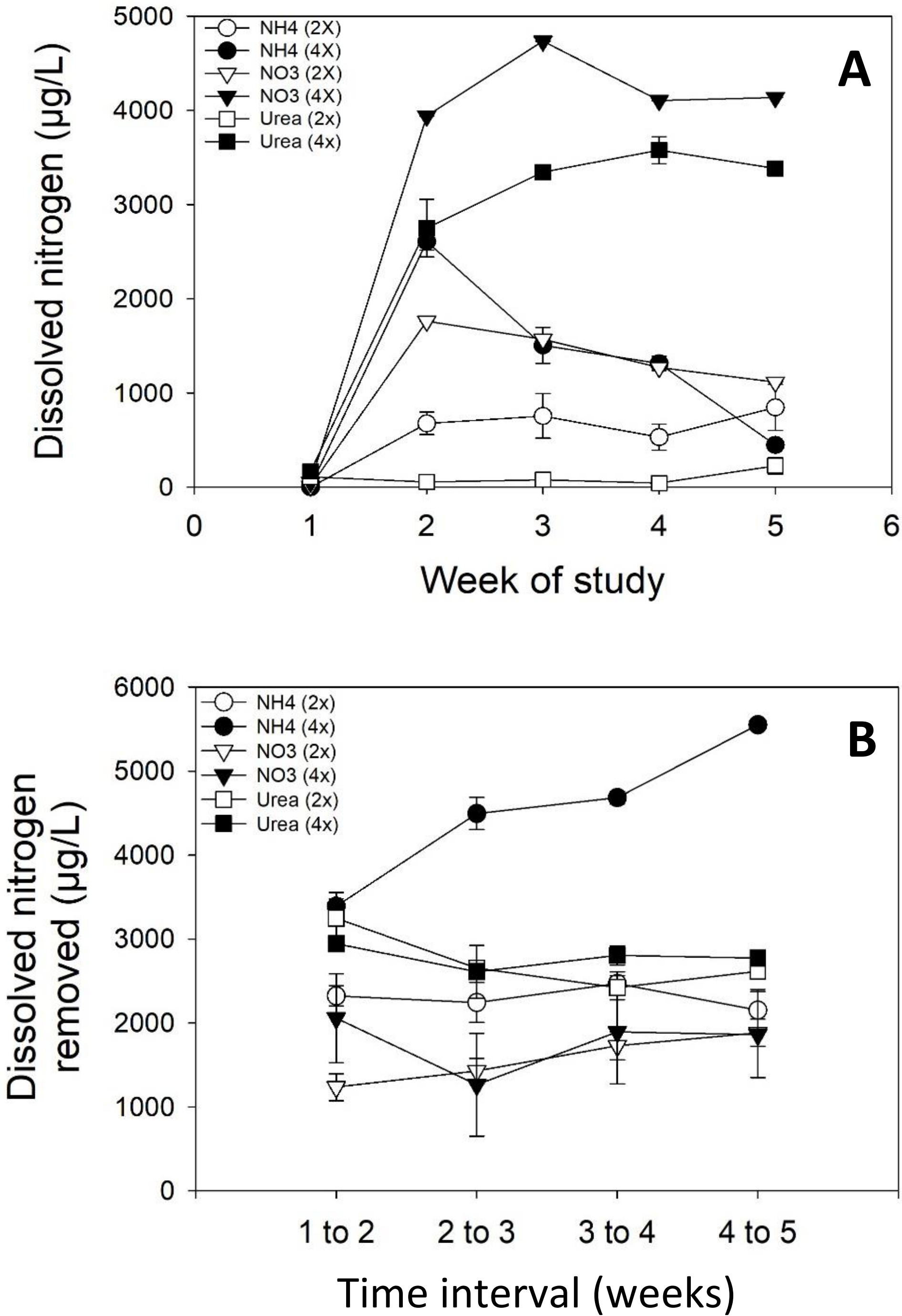


Figure 1.2



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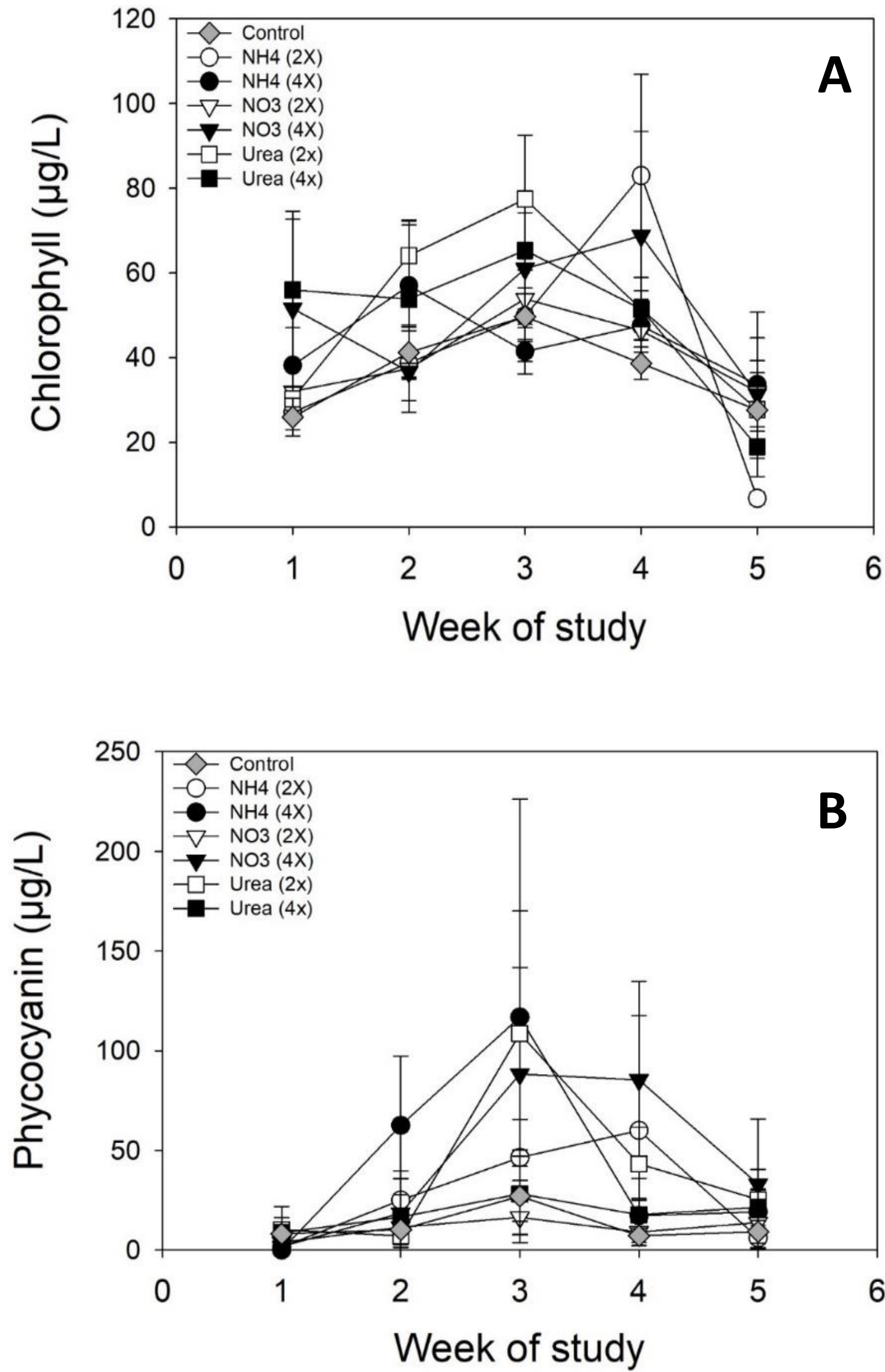
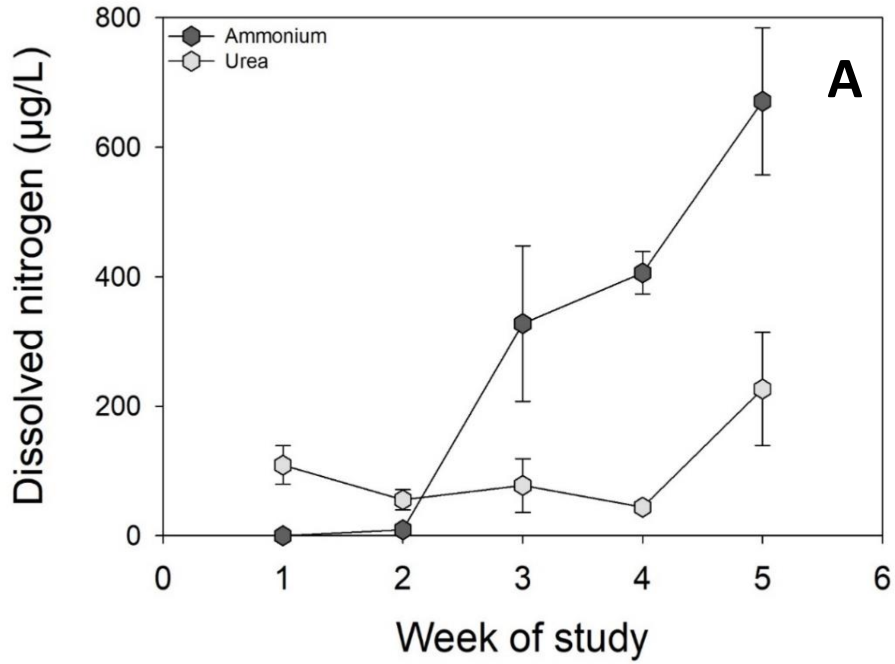
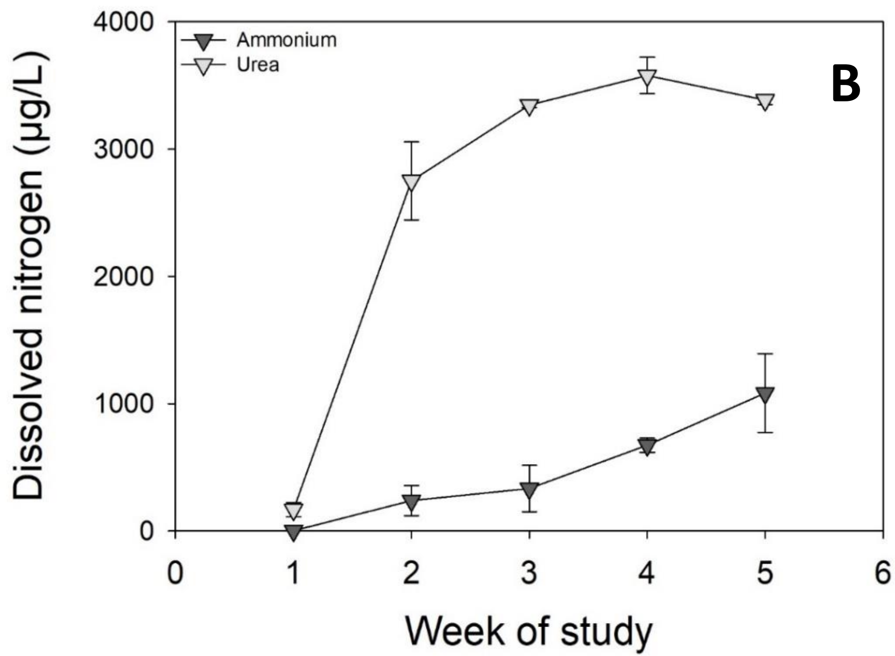


Figure 1.4





Chapter 2

Available dissolved nitrogen form mediates phycocyanin content in cyanobacteria

ABSTRACT

A two-week field experiment was carried out using 24, 1100L plastic limnocorrals to examine how different forms of dissolved nitrogen (i.e., nitrate, ammonium, and urea) impact the phycocyanin pigment content of cyanobacterial cells. Results showed that upon nitrogen introduction, cells quickly incorporated the nitrogen into excess phycocyanin as a possible means of nitrogen storage, peaking at 72h after fertilization. Ammonium and urea treatments had significantly more phycocyanin per cell than nitrate or control treatments at 72h. After 72h, phycocyanin content quickly decreased as cells degraded the pigment as a source of nitrogen. Despite the decrease in light-harvesting pigments, total number of cyanobacterial cells increased in the ammonium and urea treatments after two weeks. Nitrate treatment caused an initial growth of cyanobacteria followed by a rapid decline to starting concentrations. Cyanobacterial toxins (microcystins) peaked 72h after nitrogen fertilization. Urea and ammonium treatments resulted in the most toxic communities and had significantly more toxins than the control, however the nitrate toxin concentrations were not different from any other treatment. Results from this experiment add to the growing understanding of the advantages that cyanobacteria gain from growth on reduced forms of nitrogen (e.g., ammonium and urea). Results also suggest that simple pigment extractions may be under-estimating the amount of cyanobacteria present during a bloom depending on ambient dissolved nitrogen concentrations.

INTRODUCTION

The impacts of nutrient management, in particular nitrogen and phosphorus, have become areas of concern given their intense anthropogenic manipulation (Vitousek et al., 1997; Glibert et al., 2014). Global phosphorus cycles have been altered through mobilization from mining for fertilizers (Yuan et al., 2018), and the industrial fixation of nitrogen, alone, has increased fixed nitrogen loads from < 10 Tg/year in 1950 to > 120 Tg/year today (Vitousek et al., 1997; Fowler et al., 2013). The intensive use of these nutrients has had measurable, and oftentimes negative, impacts on the resources of the planet, perhaps affecting aquatic systems the most (Howarth et al., 2000; Battarbee et al., 2012; Woodward et al., 2012).

Freshwater ecosystems are very sensitive to terrestrial nutrient manipulations (Mason, 2002). An influx of bioavailable limiting nutrients, such as nitrogen and/or phosphorus, can encourage a rapid increase in primary productivity (Schindler, 1974; Paerl et al., 2001; Anderson et al., 2002). The negative impacts related to sudden growth of phytoplankton biomass (e.g., algal blooms) have been well documented up to this point (Anderson et al., 2000; Hallegraeff, 2003; Sellner et al., 2003; Lewitus et al., 2012). What is less understood is how these microbes utilize the different nutrients and how that gives nuisance organisms, like cyanobacteria, a competitive advantage.

Nitrogen and phosphorus are nutrients synonymous with algal blooms and are at the center of policy and management practices related to their control (Schindler, 1974; Anderson et al., 2002; Paerl et al., 2012). Despite this, nitrogen has been studied far more often than phosphorus due to the different forms of nitrogen that are bioavailable to cyanobacteria. These forms include dinitrogen gas (N_2), nitrate (NO_3), nitrite (NO_2), ammonium (NH_4), and urea (CH_4N_2O) (Herrero et al., 2001; Flores and Herrero, 2005). Dinitrogen gas is only available to diazotrophic cyanobacteria that have the necessary enzymes and hypoxic compartments to

perform nitrogen fixation, and even then, the process requires immense amounts of energy (16 molecules of ATP require to fix N_2; Herrero et al., 2001; Flores and Herrero, 2005). Nitrate requires biochemical reduction to ammonium, which is also energetically costly, but still more efficient than nitrogen fixation (Syrett, 1956; Herrero et al., 2001; Flores and Herrero, 2005, Glibert et al., 2016). Dissolved nitrite is usually scarce in typical aquatic systems and follows the same reduction pathway as nitrate, but requires one less step, thus being slightly more efficient, and therefore favorable to nitrate (Herrero et al., 2001; Flores and Herrero, 2005). Ammonium is a favorable form of nitrogen for cyanobacteria and can be readily taken-in through ion pumps and used by the cell, making it most favorable compared to gaseous or oxidized nitrogen (Blomqvist et al., 1994; Herrero et al., 2001; Flores and Herrero, 2005; Glibert et al., 2016). The use of urea first requires the molecule to be hydrolyzed by the enzyme, urease, which requires energy to make (Donald et al., 2011). However, this process results in two ammonium molecules per one urea molecule, making it potentially as energetically beneficial as ammonium, if the cyanobacterium can synthesize urease (Herrero et al., 2001; Flores and Herrero, 2005). Furthermore, given the incorporation of carbon in the molecular structure of urea it has been suggested that urea could also serve as a partial carbon (CO_2) source when pH is high and carbon is limiting (Flores and Herrero, 2005).

The potential benefits associated with cyanobacterial growth on more favorable forms of nitrogen are poorly understood. Past (Syrett, 1956; Blomqvist et al., 1994) and recent (Glibert et al., 2014; Glibert et al., 2016; Erratt et al., 2018) studies have concluded that reduced nitrogen forms (e.g. ammonium and urea) are generally favored by cyanobacteria over oxidized forms (e.g. nitrate and nitrite), and usually lead to steeper growth curves, but little else. Recent experiments (Erratt et al, 2018; Li et al., 2018; Zhou et al., 2020) have found that cyanobacterial

cultures grown on reduced nitrogen forms tended to have higher pigment concentrations per cell than oxidized forms. The excess pigments were believed to allow for extra light energy to be funneled to photosystem II. This led to a hypothesis that excess nitrogen was being allocated towards pigment proteins as a potential strategy to increase photosynthetic capacity referred to by the authors as "higher quality" cells (Erratt et al., 2018). Accordingly, previous experiments have highlighted the differential expression of cyanobacterial genomes in response to various nitrogen forms and concentrations (Harke and Gobler, 2013; Steffen et al., 2014; Harke et al., 2017). Briefly, these experiments found that when nitrogen is low, genes related to nitrogen uptake were up-regulated while genes related to photosynthesis and toxin production were down-regulated (Harke and Gobler, 2013). Furthermore, expression differences were observed both in response to different nitrogen forms (nitrate, ammonium, or urea) and between different species exhibiting that genomic responses to environmental conditions are not as universal as once thought (Steffen et al., 2014; Harke et al., 2017).

The purpose of this two-week field experiment was to more rigorously test the hypothesis of increased pigment content in response to nitrogen supplementation. Specifically, we wanted to test whether reduced nitrogen forms result in increased cellular pigment content in response to increased biomass. We also sought out to examine other traits that could be affected by these nitrogen forms, namely total biomass and toxin production. We conducted this experiment in a field setting as a means of examining how a more complete bloom community would respond to these various nitrogen introductions. We hypothesized that (1) urea and ammonium would perform comparably due to their reduced states and have more pigments per cell than nitrate, (2) cyanobacterial biomass would be highest in urea and ammonium treatments due to energy saved through growth on these nutrients, and (3) urea and ammonium treatments would have the

highest toxin concentrations as energy saved from nitrogen assimilation can be used towards secondary metabolite production.

METHODS

Site Description

The experiment was conducted from late-September to early-October of 2020 at pond S10, north of the E.W. Shell Fisheries Center at Auburn University in Auburn, Alabama, USA (32º40'8.929"N, 85º30'31.464"W). Pond S10 is an earthen aquaculture production pond with a maximum depth of 3.35 m, a surface area of 13233 m^2, and a volume of 22819 m^3 (Boyd and Shelton, 1984). The pond is categorized as hypereutrophic with total nitrogen (TN) reaching 5000 µg/L and total phosphorus (TP) peaking at 600 µg/L during the summer (July-August). During the winter (December-February) TN and TP fall close to 600 µg/L and 60 µg/L, respectively, because of reduced feeding. Pond S10 is also highly productive with chlorophyll reaching 650 µg/L and phycocyanin, a pigment protein found in cyanobacteria, peaking around 1000 µg/L during the summer (Wilson, unpublished data).

Experimental Design

This two-week nutrient addition experiment used 24 mesocosms made from clear polyethylene with a volume of 1100L (diameter = 1.18m, depth = 1m). Mesocosms were sealed at the bottom, open at the top to the environment, and suspended on floating PVC frames (Olsen et al., 2017; Buley et al., 2021; Chislock et al., 2021). All the enclosures were filled with water from pond S10 pumped through a window screen (500 µm mesh size) to exclude any fish and large debris. Enclosures were assigned one of four treatments (nitrate, ammonium, urea, or no nitrogen addition control) in a randomized block design resulting in six replicates for each

treatment. Enclosures were all mixed before each sample was taken by dropping a Secchi disk to the bottom of the enclosure and quickly pulling up. This was done 10x to ensure the entire mesocosm was well-mixed for a representative water sample. Three days after set-up, all enclosures were sampled using an integrated tube sampler (inside diameter of 5.1 cm) to a depth of 0.5 m. Collected water was measured for dissolved nitrogen (i.e., nitrate, total ammonia/ammonium nitrogen, and urea), algal pigments (i.e., chlorophyll and phycocyanin), toxins (i.e., microcystin), and sub-samples were put into 150mL glass bottles and preserved with Lugol's iodine solution to a concentration of 1% for phytoplankton enumeration. Immediately after the first samples were collected (time 0h), enclosures were fertilized with either sodium nitrate ($NaNO_3$), ammonium chloride (NH_4Cl), or urea (CH_4N_2O) to increase the total nitrogen concentration by 600 µg N/L (about 33% of the total nitrogen in S10 at the start of the experiment). Potassium phosphate was also added to nutrient treatments to increase TP by 100 µg P/L in order to combat phosphorus limitation. Enclosures were then sampled 2h, 24h, 48h, 72h, 1-week, and 2-weeks after fertilization.

Mesocosms were first fertilized at 10am, therefore, other than the 2h period, enclosures were sampled between 10:00am and 12:30pm. Along with water samples, a Hydrolab YSI multisonde (YSI®) was used to measure temperature (ºC), pH, dissolved oxygen (mg/L), and conductivity (µS/cm). Once collected, all water samples were brought back to the lab for processing. Nutrient samples were all measured colorimetrically. Dissolved nitrogen was measured from water filtered through Pall® A/E glass fiber filters (nominal 1µm pore size). Nitrate was measured using the szechrome NAS method according to Polysciences Incorporated (2011). Total ammonia/ammonium nitrogen was measured using the salicylate catalyst method according to Reardon et al. (1966). Urea was measured using diaceytl-monoxime,

thiosemicarbozide, and iron (III) sulfate according to Chen et al. (2015). Chlorophyll concentrations were measured fluorometrically (Turner Designs Trilogy fluorometer) after A/E glass fiber filters were subjected to 23h cold-extraction in 90% aqueous ethanol (Sartory and Grobbelaar, 1984). Phycocyanin concentrations were measured fluorometrically after grinding A/E filters and a 4h dark, room-temperature extraction in 50mM phosphate buffer (Kasinak et al., 2015). Intracellular microcystin toxin concentrations were determined by twice extracting toxins from cells collected on an A/E glass fiber filter using 75% aqueous methanol then measured using enzyme-linked immunosorbent assay (ELISA) kits (Beacon Analytical Systems Inc.; Yang et al., 2018). Preserved phytoplankton samples were settled by pipetting a 2mL sub-sample into a 10mL Hydro-bios® chamber, filling chamber with DI water, and allowing the sample to settle for 24h. Phytoplankton were then enumerated and measured (n = 10) by counting and the number of cells of each taxon observed in 25 fields under 200x or 400x magnification (Nikon® Eclipse Ti2 inverted compound microscope). Phytoplankton were identified to genus using *Freshwater Algae of the United States* (Smith, 1950) in combination with *A Manual of Fresh-water Algae* (Whitford and Schumacher, 1983).

Data Analyses

All statistical analyses were conducted using RStudio version 4.0.1. A series of two-way repeated measures analysis of variance (RM-ANOVA) using a restricted maximum log-likelihood (REML) method were completed using the *nlme* package (Pinheiro et al., 2020) to compare pigments per cell, toxin concentrations, and total cell concentrations among treatments over time. Models first looked for the effects of treatment, time, and the interaction between treatment and time. If any significant differences ($p < 0.05$) were observed in the model, a pair-wise multiple comparison post-hoc test using the *emmeans* package (Lenth et al., 2020) was used

to identify when and among what treatments were differences observed (see supplemental materials for example of code used). Please note that the *emmeans* package automatically accounts for multiple consecutive comparisons.

RESULTS

Dissolved Nitrogen

We would first like to acknowledge that dissolved nitrogen concentrations were not equal to the desired 600 µg N/L that was intended for these enclosures. Urea treatments received about 450 µg N/L while nitrate and ammonium received closer to 680 µg N/L (Figure 1). The difference in nutrients added is attributed to differences in bag volume that were more or less than 1100L as a result of over or under filling the enclosures and/or issues with sample storage that resulted in urea loss before analysis.

As expected, total dissolved nitrogen concentrations spiked as a result of nutrient additions (Figure 1). All three treatments experienced rapid nitrogen removal as 60% of urea-N, 70% of ammonium-N, and 99% of nitrate-N were removed after only 24h. Almost all dissolved nitrogen was removed from all treatments by 72h and remained low or zero throughout the remainder of the experiment.

Phytoplankton Enumerations

Enclosure communities were dominated by the cyanobacterium, *Microcystis* sp., which accounted for approximately 70% of the total number of cells at the start of the experiment and as much as 99% by the end. Other taxa that were commonly seen were chlorophytes, including *Staurastrum* sp., *Desmodesmus* sp., *Franceia* sp., *Pediastrum* sp., and the cyanobacterium, *Dolichospermum* sp. Total biovolume and percent composition of each taxa over time can be

seen in figures 2 and 3, respectively. Overall, there was a significant effect of nutrient treatments (RM-ANOVA, $F_{3,20} = 30.46$, $p < 0.0001$), effect of time (RM-ANOVA, $F_{3,60} = 4.37$, $p = 0.0076$), and interaction of treatment and time effects (RM-ANOVA, $F_{9,60} = 5.46$, $p < 0.0001$, Figure 4A) on the average number of phytoplankton cells. The ammonium treatment supported the most phytoplankton cells/mL, on average, than any other treatment at any point during the experiment. The urea treatment showed a slower growth response to the nutrient addition but supported similar cells/mL to the ammonium treatment after one week and continued into week 2. The nitrate treatment exhibited strong initial growth but quickly decreased to concentrations comparable to the control, resulting in a slight net loss of total cells by the end of the experiment. The control treatment saw a net loss of cells/mL, on average, over the course of the experiment. All three nutrient treatments supported cell growth in the first 72h. At the 72h time point, the ammonium and nitrate treatments were not significantly different from each other ($p = 0.6681$), on average, but both were significantly higher than the control and urea treatments; the urea and control were not significantly different from one another ($p = 0.2530$). However, after 7d the nitrate treatment began crashing, resulting in the treatment having significantly less cells/mL, on average than the ammonium ($p = 0.0096$) and urea ($p = 0.0396$) treatments, but still significantly more cells/mL than the control ($p = 0.0034$). Ammonium and urea had the highest cells/mL concentration after 7d and were not significantly different from one another ($p = 0.9129$), on average. At the end of the experiment (14d), the ammonium and urea treatments both showed a net gain in the average number of cells/mL, and had significantly more cells/mL, on average than the nitrate treatment and control, but were not significantly different from one another ($p = 0.1963$). The nitrate treatment experienced a net loss in average cells/mL by the end of the

experiment, resulting in no significant difference in the average number of cells/mL from the control ($p = 0.8000$).

Because cyanobacteria comprised an overwhelming majority of the total number of cells (up to 99% at certain points), the results of the total number of cyanobacterial cells/mL will be summarized. All trends were similar to those of the total phytoplankton cells/mL (Figure 4B). There was a significant effect of the nutrient treatments (RM-ANOVA, $F_{3,20} = 32.58$, $p < 0.0001$), a significant effect of time (RM-ANOVA, $F_{3,60} = 8.67$, $p = 0.0001$), and a significant interaction of the treatments and time (RM-ANOVA, $F_{9,60} = 5.7293$, $p < 0.0001$).

Cell Pigment Content

Nutrient additions had no significant effect on the average amount of chlorophyll per cell (RM-ANOVA, $F_{3,20} = 2.14$, $p = 0.1273$, Figure 5B). However, there was a significant effect of time on the average chlorophyll content (RM-ANOVA, $F_{3,60} = 3.39$, $p = 0.0236$) along with a significant treatment-time interaction (RM-ANOVA, $F_{9,60} = 2.32$, $p = 0.0261$). Average chlorophyll content steadily decreased in all treatments over the course of the experiment. Only the urea and control treatments showed no change or slight increase at the 72h interval. After 72h all treatments showed either a decrease or no change in average chlorophyll content per cell for the duration of the experiment.

Nutrient additions had a significant effect on the average amount of phycocyanin per cell (RM-ANOVA, $F_{3,20} = 3.87$, $p = 0.0173$, Figure 5A). There was also a significant effect of time on the average phycocyanin content (RM-ANOVA, $F_{3,60} = 27.43$, $p < 0.0001$), along with a significant interaction of treatments and time (RM-ANOVA, $F_{9,60} = 2.63$, $p = 0.0069$). Ammonium additions caused an immediate spike in the average phycocyanin content per cell,

increasing by 34% in 72h. After 72h, however, phycocyanin was quickly degraded and led to a net loss of 60% of the original amount of phycocyanin per cell by the end of the experiment. The urea treatment also resulted in an 85% spike in average phycocyanin per cell, but quickly crashed to 51% of the original concentration by the end of the experiment. Nitrate additions resulted in a 25% loss of phycocyanin content after 72h. Average phycocyanin per cell in the nitrate treatment continued to decrease to 34% of the original value by the conclusion of the experiment. The no-nutrient control saw a rapid decline in phycocyanin per cell by 52% after 72h. The phycocyanin content continued to decrease in the control to 20% of the starting value. All treatments only saw differences in the average amount of phycocyanin per cell at the 72h time interval, after which all treatments had similar phycocyanin content, on average, throughout the experiment. At the 72h interval the urea and ammonium treatments had the highest average amount of phycocyanin per cell. Both had similar average phycocyanin content ($p = 0.8560$) and were significantly higher than the nitrate and control treatments. The average amount of phycocyanin per cell in the nitrate and control treatments were not significantly different from each other ($p = 0.7879$).

Intracellular Toxins

Intracellular toxin concentrations were significantly affected by nutrient additions (RM-ANOVA, $F_{3,20} = 9.498$, $p = 0.0002$, Figure 6). There was also a significant effect of time (RM-ANOVA, $F_{3,60} = 44.073$, $p < 0.0001$), but no significant treatment-time interaction (RM-ANOVA, $F_{9,60} = 1.829$, $p = 0.0635$). All treatments had similar initial toxin concentrations. However, after 72h all treatments including the no-nutrient control experienced a spike in intracellular toxins. Urea treatments had the highest toxin concentrations, increasing by 121%, on average, and were the only ones to be significantly different from the control at the 72h interval ($p = 0.0497$). All other treatments were not different from the control or the urea

treatment. Ammonium and nitrate toxins increased by 16% and 149%, respectively. The control increased by 85%. After 7 days, all treatments experienced a decrease in intra-cellular toxins except for ammonium which saw a continued rise in average toxin concentration. Ammonium and urea treatments had the highest toxin values and were not significantly different from each other ($p = 0.9148$), but were significantly different from the control ($p = 0.0006$; $p = 0.0039$, respectively) at the 7d period. Nitrate had significantly less toxins than the ammonium treatment ($p = 0.0463$), and not was different from the urea ($p = 0.2233$) or control ($p = 0.3131$). After 14 days, all treatments saw a net loss in intra-cellular toxins from the start of the experiment and no treatments were significantly different from each other, on average.

DISCUSSION

Nitrogen and phosphorus are both integral in the function and formation of any cyanobacterial community. The thoughtful management of both nutrients is required for proper mitigation of future cyanobacterial blooms. This experiment focused solely on the differential effects of various nitrogen forms on the phytoplankton community because of the array of chemical structures that are bioavailable to phytoplankton and the limited number of prior experiments carried out to explore those effects. This experiment sought to examine potential advantages that cyanobacteria gain from growth on reduced forms of nitrogen (i.e., ammonium and urea). Specifically, we wanted to test the prediction that reduced nitrogen forms promote more phycocyanin content per cell than oxidized forms, like nitrate. We secondarily assessed how the total number of algal cells and toxin production were affected by the nutrients.

The plasticity of cyanobacterial pigments in response to changes in dissolved nitrogen concentrations has been examined in previous, laboratory experiments, but not directly. Elmorjani and Herdman (1987) noticed that Synechocystis phycocyanin content decreased by

50% in the first 24hr after being transferred to no-nitrogen media. This finding was supported when Li et al. (2018) concluded that phycocyanin content is much more plastic in response to nitrogen starvation and Zhou et al. (2020) who, similarly, found that phycocyanin content in cyanobacteria began to decrease as the dissolved nitrogen concentration in the culture media decreased. Upon further molecular examination, Zhou et al. (2020) were able to uncover that it was not phycocyanin, specifically, that was being used as a means of nitrogen storage. The entire phycocyanin molecule only incorporates four nitrogen atoms. It was the entire phycobilisome protein that was being synthesized and degraded in response to nitrogen excess and starvation. The results of our field experiment align with Elmorjani and Herdman (1987), Erratt et al. (2018), Li et al. (2018), and Zhou et al. (2020). Our results show that phycocyanin content spikes in response to the introduction of dissolved nitrogen and slowly decreases as the dissolved nitrogen is exhausted. However, this apparently only applies to reduced forms of nitrogen as the nitrate treatment was seemingly unable to store any excess nitrate as phycobilisomes. We can only speculate that energy expended to assimilate the nitrogen was too much to then synthesize extra phycobilisomes (Syrett, 1956). As a result, the energetic demand for continued growth was exhausted. It is possible that a greater nitrate addition relative to ammonium and urea would have yielded comparable longevity of the bloom. These results partially aligned with our hypothesis in that urea and ammonium had higher phycocyanin per cell, but we were not expecting the lack of increase in the nitrate treatments. This experiment is novel in documenting both the increase in phycocyanin in response to the dissolved nitrogen and subsequent decrease as a result of nitrogen starvation. These results further point to potential under-estimations of algal bloom severity based on the pigment content alone. Future studies should examine if these under-estimations

could have any real-world implications or if differences between chlorophyll guidelines and cells/mL guidelines are negligible for management advisement.

Average chlorophyll concentration per cell did not differ significantly among treatments during the experiment. These findings further support the previous hypothesis put forth by Zhou et al. (2020) that phycobilisome proteins are the main nitrogen storage vessels when nitrogen is in excess. Cyanobacteria appear adapted to synthesize and degrade phycobilisomes in response to extra-cellular nitrogen. It may be likely that this is done because chlorophyll is the major major site for photosynthesis as opposed to phycobilisomes or that phycobilisomes are simpler to construct and break down. Future studies should examine this phenomenon to uncover why phycobilisomes are preferentially synthesized and degraded.

Cyanobacterial bloom communities can vary in their size, severity, and composition in response to a number of variables including geographical region, temperature, salinity, water clarity, water flow, and the available nutrients. We found that nitrogen form did have an effect on the bloom community. In particular, nitrate and ammonium encouraged rapid cell replication while urea resulted in more bloom growth than the control, but less than ammonium and nitrate in the short-term. However, as the experiment progressed, urea and ammonium were similar in their cell concentrations while nitrate cell concentrations slowly declined. These results align with previous experiments that have found higher growth on reduced nitrogen forms when compared to oxidized forms (Raven et al., 1992; Herndon and Cochlan, 2007; Solomon et al., 2010). However, it should be noted that these patterns are not completely agreed upon in the scientific community (Glibert et al., 2016 summarizes). Interestingly, when comparing the results for cell growth and pigment content, one can notice that the excess nitrogen saved in the urea and ammonium treatments was enough to sustain or continue bloom growth for nearly 10-

days following dissolved nitrogen depletion, while the lack of nitrogen stores in the nitrate treatment seem to encourage the crash. Community composition did not differ greatly between treatments, but it should be noted that community comparisons are less meaningful in this instance due to the overwhelming dominance of *Microcystis* by the end of the experiment. That being said, cyanobacteria, specifically *Microcystis*, dominated all treatments for the entirety of the experiment. By the end of the 14-days most enclosures, regardless of treatment, saw 2 – 4 taxa practically extirpated from the enclosures (taxa present in samples, but not captured in 25 random fields). This result is common in eutrophic settings where *Microcystis* is already dominant and temperature and nutrient concentrations are high (Yamomoto and Nakahara, 2006; Paerl and Huisman, 2008; Alvarez et al., 2016; Wilhelm et al., 2020).

Cyanobacterial toxins are an oft-debated topic in relation to cyanobacterial ecology. Studies are continually carried out to understand why cyanobacteria produce toxins and under what specific conditions. In this study, intracellular microcystin concentrations peaked 72h after fertilization, which aligned with peak pigment concentrations and total removal of dissolved nitrogen. These findings coincide with Pimentel and Giani (2014), Beversdorf et al. (2015), Qian et al. (2019), and Zhou et al. (2020) who found that microcystin concentrations increased under nitrogen starvation. The authors appear to agree that this may indicate that microcystins could be used as a mechanism to ease oxidative stress after a bloom peaks. However, there is also substantial evidence that this may not be the case. For example, Vezie et al. (2002) found that intracellular microcystins in four different cultured *Microcystis* strains increased when both nitrogen and phosphorus were high in the media, not correlating with growth or nitrogen starvation. Monchamp et al. (2014) were able to correlate total microcystin concentrations with *Microcystis* biomass, and Song et al. (2006) found that *Microcystis viridis* communities produced

the most toxins under high light intensity and high (9.2) and low (7.0) pH. In short, the true role of microcystins is still in question, but toxin production under nitrogen stress appears to be most common in *Microcystis*. More research is required to affirm or contest this assertion and further uncover the true role of microcystins in cyanobacterial cells.

As stated in the results section, we acknowledge that the urea treatment did not appear to reach the 600 µg/L treatment level that was intended. However, we contend that these differences did not have significant effects on the final results as seen in the phycocyanin per cell and total cell concentrations. Urea performed comparably to ammonium treatments, as expected, despite apparently receiving less nitrogen, on average.

CONCLUSIONS

This experiment set out to examine potential advantages that cyanobacteria, particularly *Microcystis*, can gain from growth on different dissolved nitrogen forms. Specifically, we examined the effect of reduced and oxidized nitrogen forms on the pigment content of cyanobacterial cells. We found that reduced nitrogen forms (i.e., ammonium and urea) encouraged more cells per mL and more phycocyanin per cell than nitrate. Increases in phycocyanin per cell during nitrogen excess and subsequent declines during nitrogen removal point to phycocyanin and the phycobilisome as preferred nitrogen storage structures. We also found that intra-cellular microcystins peaked shortly after dissolved nitrogen was removed from the water column, seemingly coinciding with nitrogen starvation and a response to cell stress. The results of this experiment encapsulate the fluidity of phycocyanin as a nitrogen storage mechanism and support previous experiments that suggest microcystin production is a mechanism in response to oxidative stress during nitrogen starvation.

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FIGURE LEGENDS

Figure 1. Average total dissolved nitrogen (µg/L) for each treatment ± SD, over time. TDN was calculated as the sum of (NO_3 + NH_4 + urea). The dissolved nitrogen form that was introduced after time 0h accounted for 99% of all nitrogen at time 2h and anywhere from 0% to 90% by 72h.

Figure 2. Average total biovolume that each taxa represents at time 0h (A), 72h (B), 7d (C), and 14d (D). Biovolume measurements were obtained from 10 individual cells before being averaged and then multiplied by the total number of cells/mL to obtain biovolume/mL. Cells were measured using a Nikon Eclipse Ti2 inverted compound microscope.

Figure 3. Average proportion of the total number of phytoplankton cells that each taxa represents at time 0h (A), 72h (B), 7d (C), and 14d (D). *Microcystis sp.* dominated the mesocosms from the beginning of the experiment and that trend continued from the entire two-weeks. Cells were enumerated by counting cells of each taxa within 25 random fields using a Nikon Eclipse Ti2 inverted compound microscope.

Figure 4. Average total number (cells/mL) of (A) phytoplankton or (B) cyanobacteria for each treatment ± SD, over time. Lower case letters indicate statistical significance. Treatments sharing the same letter are not significantly different and treatments with different letters are significantly different. Ammonium and nitrate encouraged rapid growth in the first 72h. After 72h the urea and ammonium treatments were not significantly different and the nitrate treatment became comparable to the no-nutrient control by the end of the two week. Phytoplankton were

enumerated by counting all taxa in 25 random fields at either 200x or 400x magnification using a Nikon Eclipse Ti2 inverted compound microscope.

Figure 5. Average amount of (A) phycocyanin or (B) chlorophyll per cell (pg) for each treatment ± SD, over time. Lower case letters indicate statistical significance. Treatments sharing the same letter are not significantly different and treatments with different letters are significantly different. The average amount of phycocyanin per cell was only different between treatments at time 72h. At 72h the urea and ammonium treatments had the most phycocyanin per cell and the nitrate and control were not significantly different. The average amount of chlorophyll per cell did not differ significantly between treatments at any point in the experiment.

Figure 6. Average amount of intracellular microcystin (µg/L) for each treatment ± SD, over time. Lower case letters indicate statistical significance. Treatments sharing the same letter are not significantly different and treatments with different letters are significantly different. The average amount of intra-cellular microcystin peaks 72h after initial fertilization. Ammonium and urea treatments had the highest toxin concentrations at 72h and 7 days. All treatments experienced a net loss in average microcystin concentration by the end of the 14 days.

Figure 2.1

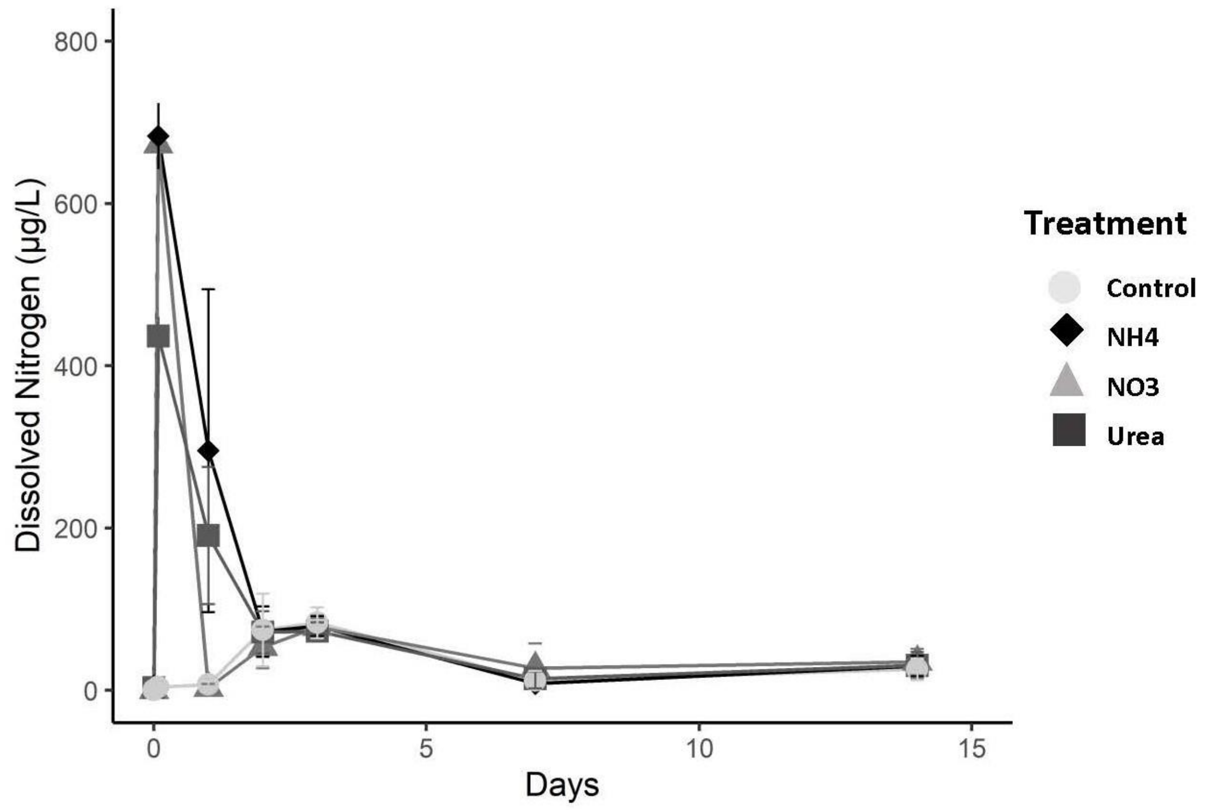


Figure 2.2

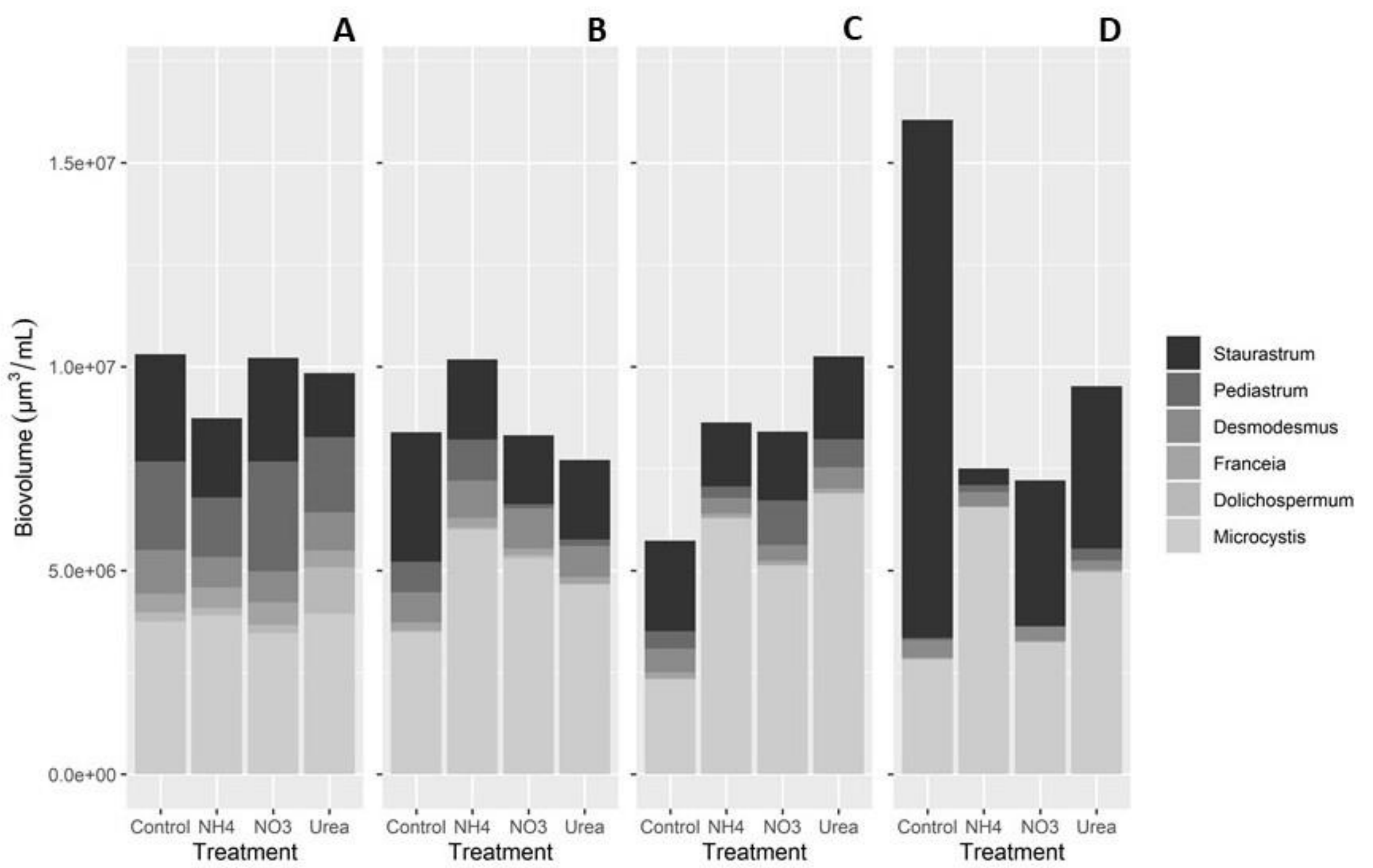
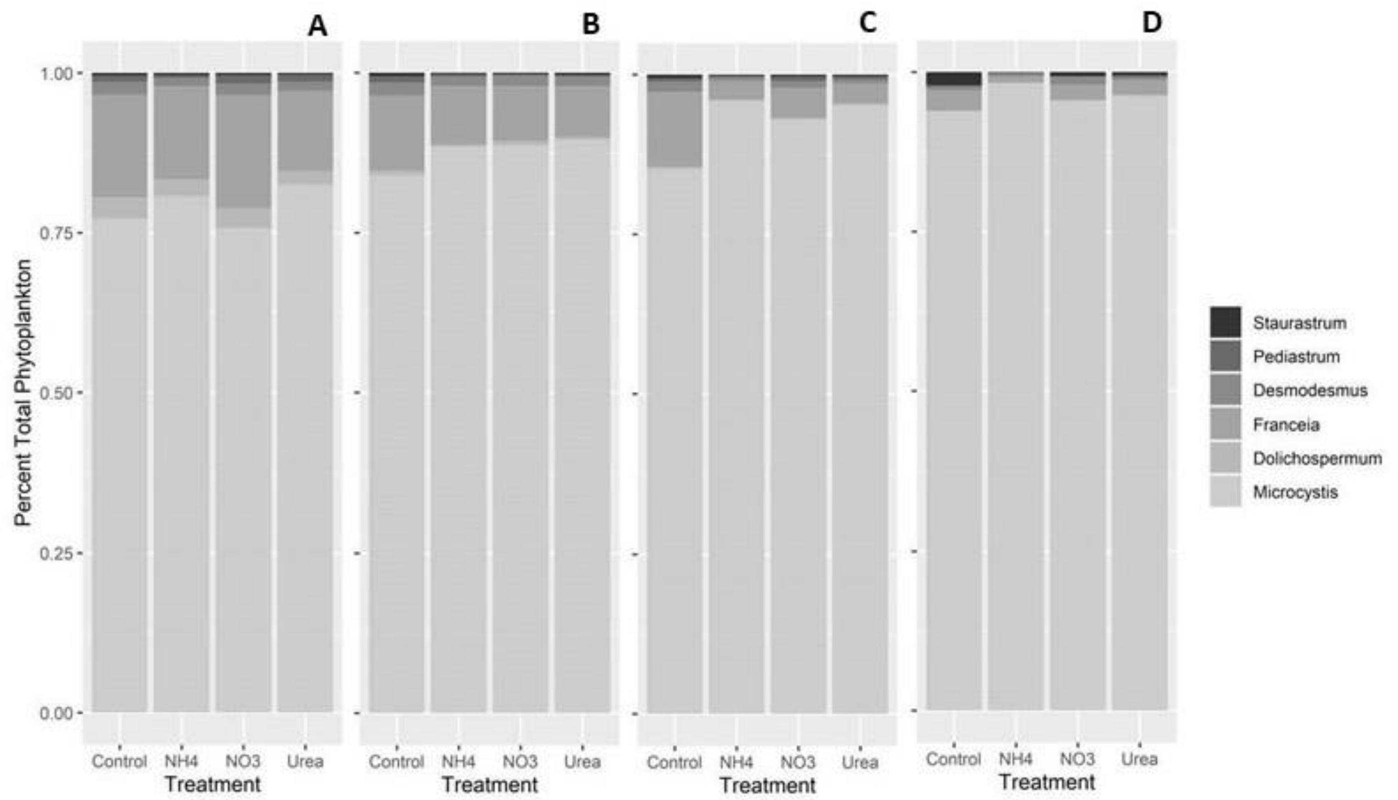


Figure 2.3



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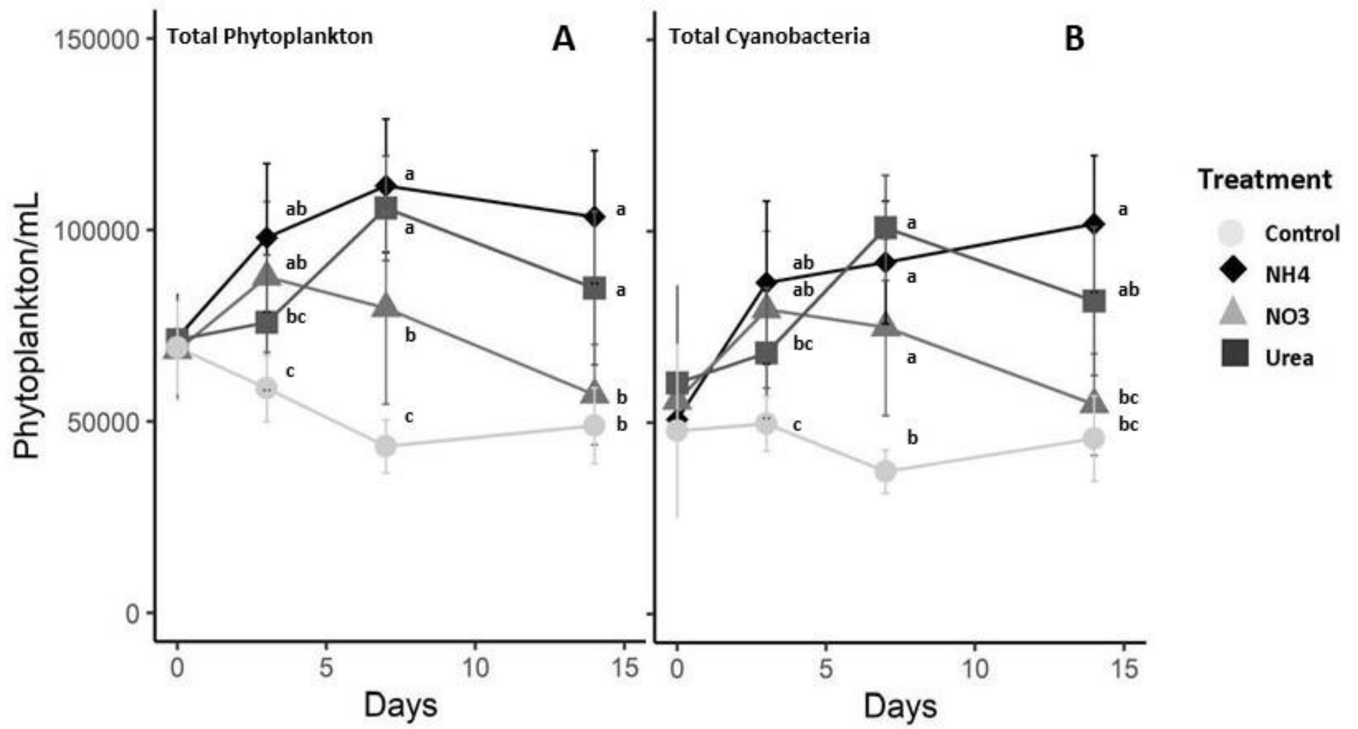

Figure 2.5

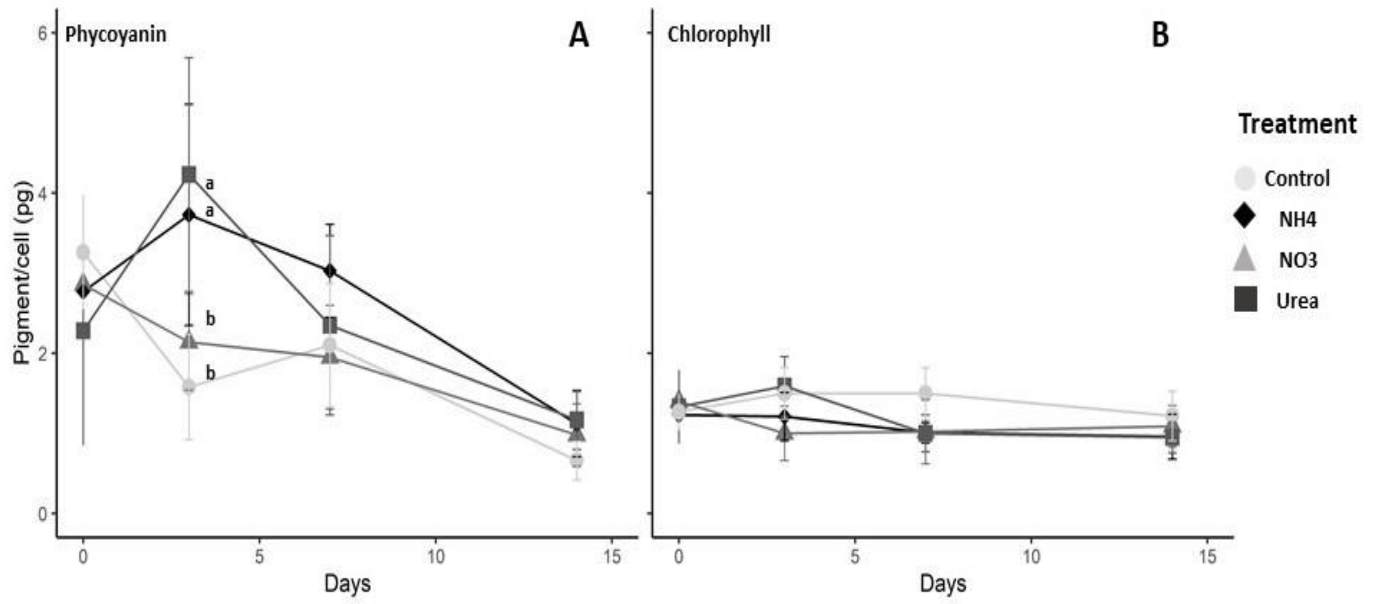

Figure 2.6