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# Dissolved nitrogen form mediates phycocyanin content in cyanobacteria

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## Abstract

1. Chemically reduced nitrogen forms are increasing in aquatic systems and beginning to reach concentrations not previously measured. Despite this, little research has examined the potential of reduced nitrogen forms to encourage excess nitrogen storage and promote algal bloom longevity compared to oxidised forms.
2. A 2-week field, pulse-application experiment was carried out using 1,100-L plastic limnocorrals to examine cyanobacterial community response to three nitrogen forms, including nitrate, ammonium, and urea (added as 600 μg N/L). Cell pigments and counts were used to calculate cell-specific pigment concentrations, and cell-associated microcystin concentrations were also measured to examine toxin response to a shift in nitrogen source.
3. Results showed that, upon nitrogen introduction, extracellular nitrogen quickly decreased in accordance with an increase in cellular phycocyanin 72 hr after fertilisation. Ammonium and urea treatments had more phycocyanin/cell than nitrate or control treatments at 72 hr. After 72 hr, phycocyanin content quickly decreased, consistent with the use of nitrogen from phycobiliproteins. Despite the decrease in light-harvesting pigments, the total number of cyanobacterial cells increased in the ammonium and urea treatments after 2 weeks. Cyanobacterial particulate toxin (microcystin) quotas were not affected by nitrogen additions.
4. Results show that reduced nitrogen forms encourage greater nitrogen storage as pigments and increase bloom longevity compared to oxidised forms.
5. Findings support previous studies that suggest reduced nitrogen forms encourage greater cell density and algal bloom persistence. Results further point to excess nitrogen storage as another mechanism that allows cyanobacteria to dominate freshwater systems despite variable environmental conditions.

KEYWORDS
algal pigments, algal toxins, chlorophyll, eutrophication, microcystin

## 1 | INTRODUCTION

The impacts of nutrient management, in particular nitrogen and phosphorus, have become topics of discussion given their intense anthropogenic manipulation (Glibert et al., 2014; Vitousek et al., 1997). Global phosphorus cycles have been altered through mobilisation from mining for fertilisers (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 (Fowler et al., 2013; Vitousek et al., 1997). The intensive use of these nutrients has had measurable, and often negative, impacts on the natural resources of the planet, including freshwater systems (Battarbee et al., 2012; Howarth et al., 2000; Woodward et al., 2012).

Nitrogen and phosphorus are nutrients synonymous with algal blooms and are at the centre of policy and management practices related to their control (Anderson et al., 2002; Paerl et al., 2016; Schindler, 1974). Unlike phosphorus, bioavailable nitrogen commonly occurs in several forms, including dinitrogen gas ($N_2$), nitrate ($NO_3$), nitrite ($NO_2$), ammonium ($NH_4$), and urea ($CH_4N_2O$) (Flores & Herrero, 2005; Herrero et al., 2001). Dinitrogen gas is a less optimal form of nitrogen to utilise given the necessity for anoxic conditions, specific enzymes, and/or complex structures along with the energy required to fix it (i.e., 16 molecules of ATP require to fix one $N_2$ molecule; Flores & Herrero, 2005; Herrero et al., 2001). Nitrate requires biochemical reduction to ammonium, which is also energetically costly, but still more efficient than nitrogen fixation (Flores & Herrero, 2005; Glibert et al., 2016; Herrero et al., 2001; Syrett, 1956). 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 favourable to nitrate (Flores & Herrero, 2005; Herrero et al., 2001). Ammonium is a favourable form of nitrogen for cyanobacteria and its uptake can readily occur through ion pumps and be used by the cell, making it the most ideal compared to gaseous or oxidised nitrogen (Blomqvist et al., 1994; Flores & Herrero, 2005; Glibert et al., 2016; Herrero et al., 2001). The use of urea first requires the molecule to be hydrolysed by the enzyme urease, which requires energy to produce (Belisle et al., 2016; Donald et al., 2011). However, this process results in two ammonium molecules per urea molecule, making urea potentially as energetically beneficial as ammonium, if the cyanobacterium can synthesise urease (Belisle et al., 2016; Flores & Herrero, 2005; Herrero et al., 2001; Newell et al., 2019).

Phycocyanin is an accessory pigment found in cyanobacteria and some cryptophytes (Glazer & Wedemayer, 1995). This pigment is housed within the phycobilisome protein that is attached to the thylakoid membrane (Adir, 2021; Morançais et al., 2018). Phycocyanin and the phycobilisome can capture light energy that is around 624 nm (orange light) and funnel that energy towards chlorophyll-*a*, specifically photosystem II (Adir, 2021; Morançais et al., 2018). This process allows the cyanobacterial cell to take advantage of a wider array of the electromagnetic spectrum. Because phycocyanin and phycobilisomes are accessory pigments, they are not required for complete cell function. As a result, several lab experiments have reported cyanobacterial degrading phycobilisomes (and the phycocyanin within) as a nitrogen source when extracellular nitrogen is low (Elmorjani & Herdman, 1987; Li et al., 2018). These findings led Zhou et al. (2020) to conclude that phycocyanin concentrations probably fluctuate over the course of bloom development, peak, and senescence. Few works have quantified the capacity of cyanobacterial nitrogen storage as phycobilisomes or the factors that could influence whether nitrogen can be stored given the environmental conditions.

The purpose of this 2-week field experiment was to rigorously test the hypothesis of increased pigment content in response to nitrogen supplementation. Specifically, we wanted to test whether reduced nitrogen forms (e.g., ammonium and urea) result in increased cellular pigment content in response to increased biomass. We also sought to examine other important ecological traits that could be affected by these nitrogen forms, namely total cyanobacterial biovolume and toxin production. We hypothesised that: (1) urea and ammonium would perform comparably due to their reduced states and have more phycocyanin/cell than nitrate (Erratt et al., 2018); (2) cyanobacterial biomass would be highest in urea and ammonium treatments due to energy saved through growth on these nutrients (Chaffin and Bridgeman, 2014); and (3) urea and ammonium treatments would have the highest toxin concentrations as resources saved from nitrogen assimilation can be used towards secondary metabolite production (Finlay et al., 2010).

## 2 | METHODS

### 2.1 | Study site

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, U.S.A. (32°40′8.929″N, 85°30′31.464″W). Pond S10 is an earthen aquaculture production pond with a maximum depth of 3.35 m (average depth *c*. 1.83), a surface area of 13,233 $m^2$, and a volume of 22,819 $m^3$ (Boyd & Shelton, 1984). The pond is categorised as hypereutrophic with total nitrogen (TN) reaching 5,000 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 and 60 μg/L, respectively, because of reduced additions of fish feed. Pond S10 is also highly productive, with peak chlorophyll-*a* and phycocyanin often reaching 650 and 1,000 μg/L, respectively, during the summer (A. E. Wilson, unpublished data).

### 2.2 | Study design

This 2-week, nutrient addition experiment used 24 mesocosms made from clear polyethylene with a volume of 1,100 L (diameter = 1.18m, depth = 1 m). Enclosures were sealed at the bottom, open at the top to the environment, and suspended on floating PVC

frames (Buley et al., 2021; Chislock et al., 2021; Olsen et al., 2017). All enclosures were filled with water from pond S10 pumped through window screen (*c*. 500-μm mesh) to exclude any fish and large debris. Enclosures were assigned to one of four treatments (nitrate, ammonium, urea, or no nitrogen addition control) in a randomised block design resulting in six replicates of 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 procedure was done 10 times to ensure that the entire mesocosm was well-mixed for a representative water sample. Three days after installation, all enclosures were sampled using an integrated tube sampler (inside diameter of 5.1 cm) to a depth of 0.5 m. Water samples were poured into 4-L containers and kept in a cooler on ice until transported to the lab for filtration. Sampled water was collected on filters (Pall A/E glass fibre, 1 μm pore size; Pall Corporation) for algal pigments (i.e., chlorophyll-*a* and phycocyanin) or toxins (i.e., particulate microcystin). Filtrate was stored at 4°C until used for measurement of dissolved nitrogen forms (i.e., nitrate, total ammonia/ammonium nitrogen, and urea). Reported concentrations reflect any changes driven by microbial activity between sample collection and analysis, but these effects were minimised by transporting whole-water samples on ice in a dark cooler and conducting analyses within 48 hr.

Lastly, whole water was collected into 120-ml 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 0 hr), enclosures were fertilised 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). This pulse-addition was used to mimic run-off that could be reasonably expected from a freshwater system whose catchment includes intensive agriculture where the addition of nutrients/fertilisers would be expected. Potassium phosphate was also added to all enclosures to increase TP by 100 μg P/L to combat phosphorus limitation. Enclosures were then sampled 2 hr, 24 hr, 48 hr, 72 hr, 1 week, and 2 weeks after fertilisation.

Mesocosms were first fertilised at 10:00; therefore, the 0-h period was sampled before 10:00 and the 2-hr period was sampled starting at 12:00. All other periods were sampled starting at 10:00. Along with water samples, a Hydrolab YSI multisonde (YSI Incorporated) was used to measure temperature (°C), pH, and dissolved oxygen (mg/L). Once collected, all water samples were brought back to the lab for processing. Nutrient samples were all measured colorimetrically (PerkinElmer Lambda 25 UV/VIS spectrometer, PerkinElmer). Dissolved nitrogen was measured from water filtered through Pall® A/E glass fibre filters (nominal 1 μm pore size). Nitrate was measured using the Szechrome NAS method according to Polysciences Incorporated (2011). Total ammonia/ammonium nitrogen (TAN) was measured using the salicylate catalyst method according to Reardon et al. (1966). Urea was measured using diacetyl-monoxime, thiosemicarbozide, and iron (III) sulfate according to Chen et al. (2015). Chlorophyll-*a* concentrations were measured fluorometrically (Turner Designs Trilogy fluorometer; Turner Designs) after A/E glass fibre filters were subjected to 23 hr cold-extraction in 90% aqueous ethanol and allowed to warm to room temperature for 1 hr (Sartory & Grobbelaar, 1984). Phycocyanin concentrations were measured fluorometrically after grinding A/E filters and a 4-hr dark, room-temperature extraction in 50 mM phosphate buffer (Kasinak et al., 2015). Cell-associated (particulate) microcystin toxin concentrations were determined by twice extracting toxins from cells collected on an A/E glass fibre filters using acidic 75% aqueous methanol, pooling and filtering the extract before removing the solvent by evaporation, redissolving the extract in 5 ml of a phosphate buffer, and finally measuring microcystin using enzyme-linked immunosorbent assay kits (Beacon Analytical Systems Inc.; Yang et al., 2018). Preserved phytoplankton samples were settled by pipetting a 2 ml sub-sample into a 10 ml Hydro-bios® chamber, filling the chamber with DI water, and allowing the sample to settle for at least 24 hr. Phytoplankton were then enumerated and measured ($n = 10$/species) by counting the number of cells of each taxon observed in 25 fields under 200× or 400× magnification (Nikon® Eclipse Ti2 inverted compound microscope; Nikon). Phytoplankton were identified to genus using a combination of pictorial and dichotomous keys (Smith, 1950; Whitford & Schumacher, 1983).

### 2.3 | Data analysis

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 likelihood (REML) method were completed using the *nlme* package (Pinheiro et al., 2020) to compare pigments per cell, toxin cell quotas, 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). The *emmeans* package automatically corrects for multiple comparisons within groups (see Searle et al., 1980).

## 3 | RESULTS

### 3.1 | Total nitrogen, TP, TN:TP ratio, and soluble reactive phosphorus

Total nitrogen increased in the nitrogen treatments in response to nutrient additions to a concentration of about 2,600 μg N/L (Figure 1a). All three nitrogen treatments followed similar patterns with no difference in TN concentration throughout the course of the experiment ($p \geq 0.420$). Total nitrogen decreased

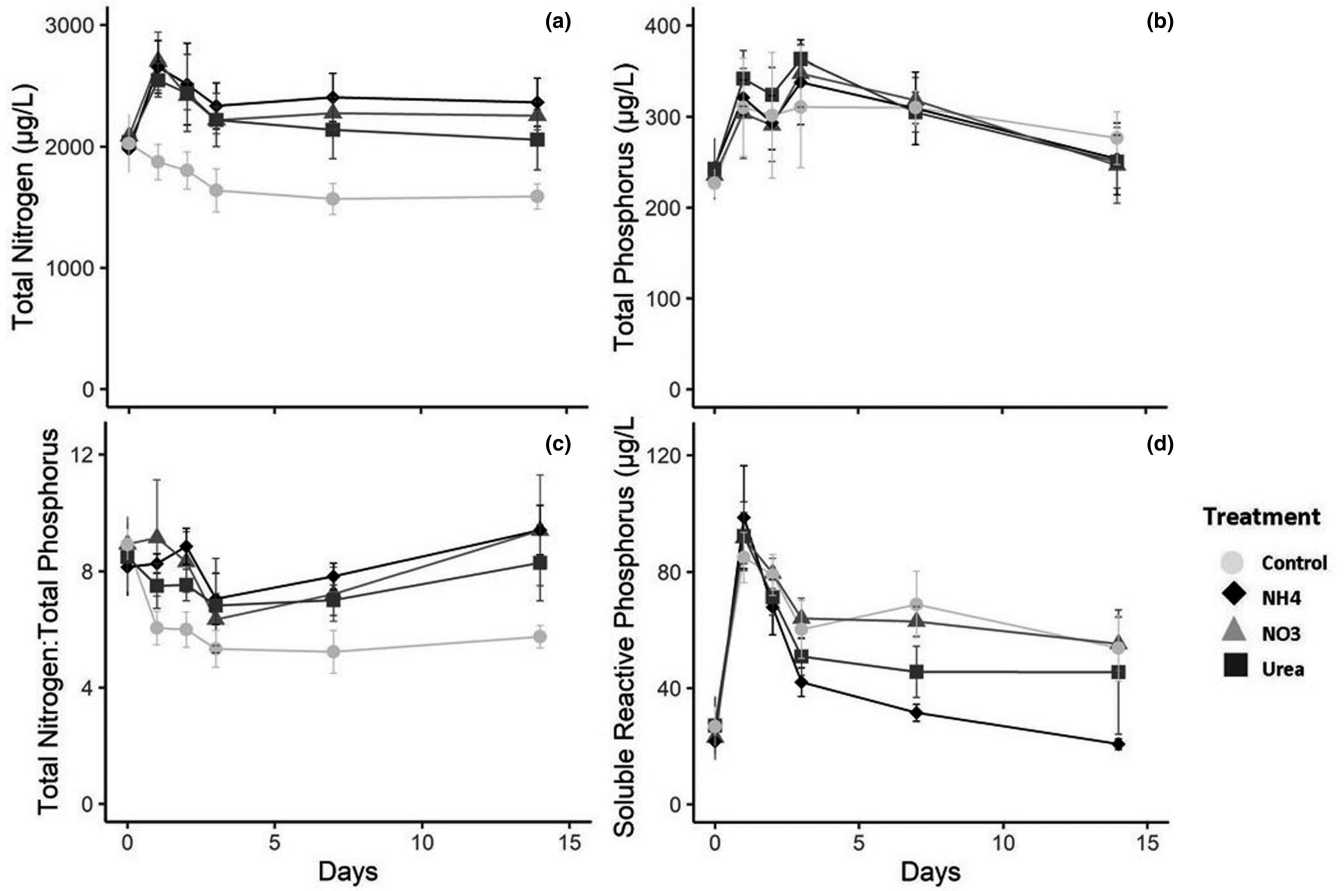


FIGURE 1 Average (a) total nitrogen (TN), (b) total phosphorus (TP), (c) TN:TP ratio, and (d) soluble reactive phosphorus (SRP) for each treatment ± *SD* over time

in nitrogen addition treatments by the end of the experiment to about 2,200 µg N/L.

Similarly, TP increased in all treatments to about 320 µg P/L because of phosphorus fertilisation (Figure 1b). All treatments, likewise, followed similar trends in TP concentration with no differences in phosphorus concentration (RMANOVA, $F_{3,20} = 0.485$, $p = 0.6967$). TP decreased in all treatments by the end of the experiment to a concentration of about 250 µg P/L.

The TN:TP ratios varied slightly among treatments, with the control having a lower TN:TP ratio than the nitrogen treatments after 72 hr ($p \leq 0.0181$; Figure 1c). TN:TP ratio was 7–10 for the three nitrogen treatments and did not differ throughout the experiment ($p > 0.05$). As productivity increased, TN:TP decreased in all three nitrogen treatments to about 6, but then increased again to around 9 by the end of the experiment.

Soluble reactive phosphorus (SRP) increased to about 100 µg/L in all enclosures as a result of nutrient additions (Figure 1d). All treatments had similar SRP concentrations up to 48 hr ($p \geq 0.0871$). After 48 hr, treatments began to diverge and, on day 7, ammonium (31 µg/L) enclosures had less SRP then nitrate and control enclosures (*c.* 65 µg/L; $p = 0.0001$; $p < 0.0001$, respectively) and urea (45 µg/L) treatments had less SRP than nitrate and the control (*c.* 65 µg/L) as well ($p = 0.0231$; $p = 0.0022$, respectively). By the conclusion of the experiment, SRP in ammonium treatments was close to 20 µg/L, while urea, nitrate, and control enclosures were all close to 50 µg/L.

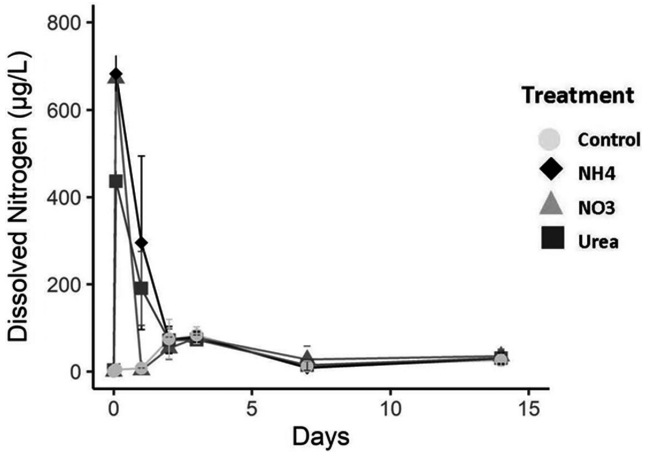


FIGURE 2 Average total dissolved nitrogen (TDN µg/L) for each treatment ± *SD* overtime. TDN was calculated as the sum of ($NO_3$ + TAN + urea). The dissolved nitrogen form that was introduced after time 0 hr accounted for 99% of all nitrogen at time 2 hr and anywhere from 0% to 90% by 72 hr

### 3.2 | Dissolved nitrogen

All enclosures within this study received nitrogen additions that were weighed prior to the start of the experiment and verified to equal the desired 600 μg N/L. However, nitrogen measurements from the urea treatments appear to have registered about 450 μg N/L while nitrate and ammonium received closer to 680 μg N/L (Figure 2). The difference in nutrients added is attributed to issues with sample storage that resulted in urea loss before analysis. Dissolved urea samples were intended to be analysed within 48 hr but were instead analysed between 72 and 96 hr due to our repeated sampling at the start of the experiment. Examination of TN data (Figure 1a) verifies that the appropriate amount of urea was added to the enclosures.

As expected, total dissolved nitrogen concentrations spiked because of nutrient additions (Figure 2). Dissolved nitrogen decreased in all treatments by 60% of urea-N, 70% of ammonium-N, and 99% of nitrate-N after only 24 hr. Most dissolved nitrogen was removed from all treatments by 72 hr and remained low or zero throughout the remainder of the experiment.

### 3.3 | 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 taxon over time can be seen in Figures S3 and S4, respectively.

Overall, there was a strong effect of nutrient treatments (RM-ANOVA, $F_{3,20} = 30.46$, $p < 0.0001$), time (RM-ANOVA, $F_{3,60} = 4.37$, $p = 0.0076$), and their interaction (RM-ANOVA, $F_{9,60} = 5.46$, $p < 0.0001$, Figure 3a) on the average number of phytoplankton cells. As hypothesised, phytoplankton cell concentrations were higher in ammonium enclosures, on average, than any other treatment at any point during the experiment, and nearly double the total number of cells in the control enclosures at day 7 ($p < 0.0001$). Phytoplankton in the urea treatment showed a slower growth response to the nutrient addition but supported a similar number of cells to the ammonium treatment after 1 week and continued into week 2 ($p \geq 0.1963$). Urea treatment cell densities were also nearly double to that of the control by day 7 ($p < 0.0001$). Phytoplankton abundance in the nitrate treatment was high initially but quickly decreased to concentrations less than the ammonium ($p \leq 0.0096$) and urea ($p \leq 0.0396$) treatments, but greater than the control ($p \leq 0.0195$). Phytoplankton abundance in the control treatment was lowest at day 7 compared to the start of the experiment ($p = 0.0277$). The total number of cells in the control was not different from day 0 to day 14 ($p = 0.1146$).

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 will be summarised. All trends were like those of the total phytoplankton cells/ml (Figure 3b). Nutrient treatments (RM-ANOVA, $F_{3,20} = 32.58$, $p < 0.0001$), time ($F_{3,20} = 8.67$, $p = 0.0001$), and their interaction ($F_{3,60} = 5.73$, $p < 0.0001$) all exhibited strong effects on cyanobacterial counts.

### 3.4 | Cell pigment content

Nutrient additions had a weak effect on the average amount of chlorophyll-*a*/cell (RM-ANOVA, $F_{3,20} = 2.14$, $p = 0.1273$, Figure 4a).

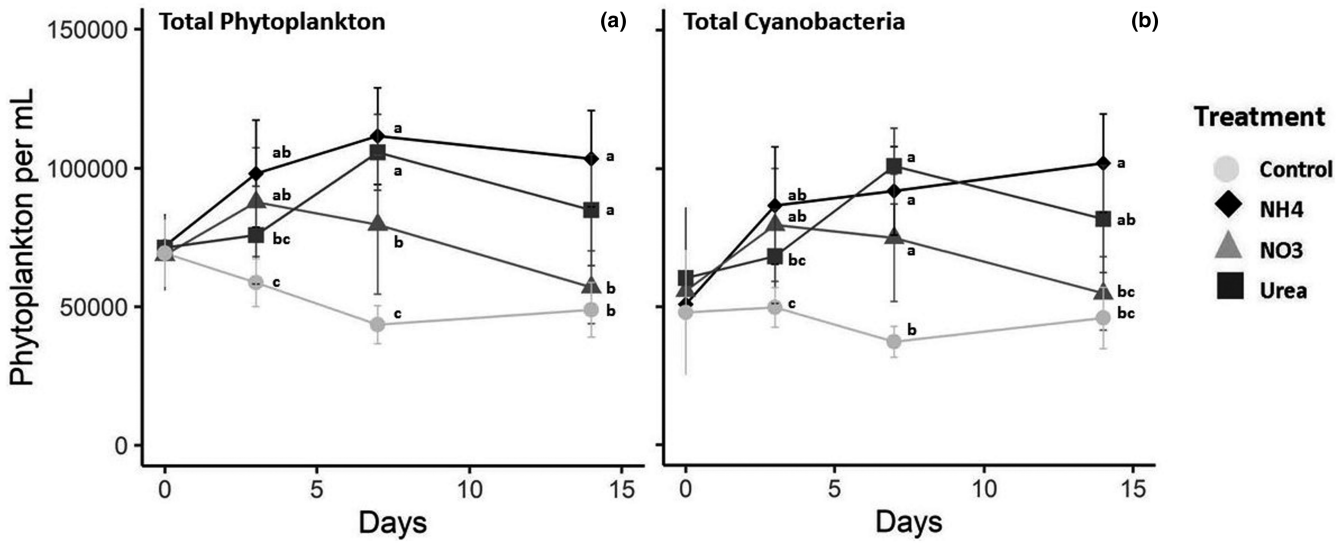


FIGURE 3 Average total number (cells/ml) of (a) phytoplankton or (b) cyanobacteria for each treatment ± *SD* over time. Lower case letters indicate statistical significance. Treatment sharing the same letter are not significantly different and treatments with different letters are significantly different. Ammonium and nitrate encouraged rapid growth in the first 72 hr. After 72 hr 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 2 weeks

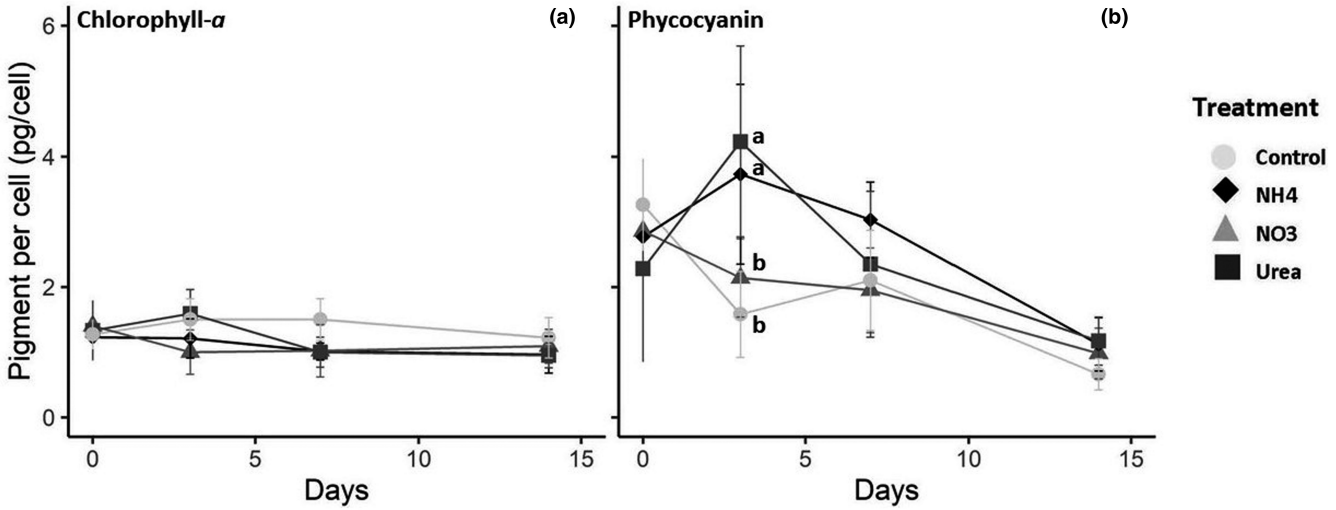


FIGURE 4 Average amount of (a) chlorophyll or (b) phycocyanin/cell (pg) for each treatment ± *SD* over time. Lower case letters indicate statistical significance. Treatment sharing the same letter are not significantly different and treatments with different letters are significantly different. The average amount of phycocyanin/cell was only different between treatments at 72 hr. At 72 hr, the urea and ammonium treatments had the most phycocyanin/cell and the nitrate and control were not significantly different. The average amount of chlorophyll/cell did not differ significantly between treatments at any point in the experiment

However, there was a moderate effect of time on the average chlorophyll-*a* content (RM-ANOVA, $F_{3,60} = 3.39$, $p = 0.0236$) along with a moderate treatment-time interaction (RM-ANOVA, $F_{9,60} = 2.32$, $p = 0.0261$). Average chlorophyll-*a* content steadily decreased in all treatments over the course of the experiment. Only chlorophyll-*a* content in the urea and control treatments showed no change or slight increase at the 72-hr interval. After 72 hr, chlorophyll-*a* content in all treatments showed either a decrease or no change for the duration of the experiment.

The average amount of phycocyanin/cell was affected by nutrient additions (RM-ANOVA, $F_{3,20} = 3.87$, $p = 0.0173$, Figure 4b), time ($F_{3,60} = 27.43$, $p < 0.0001$), and their interaction ($F_{9,60} = 2.63$, $p = 0.0069$). There was also a strong effect of time on the average phycocyanin content (RM-ANOVA, $F_{3,60} = 27.43$, $p < 0.0001$), along with a moderate interaction of treatments and time (RM-ANOVA, $F_{9,60} = 2.63$, $p = 0.0069$). Ammonium additions caused a spike in the average phycocyanin content/cell, increasing by 34% in 72 hr and had more than twice the amount of phycocyanin/cell than control enclosures. After 72 hr, however, phycocyanin decreased rapidly and led to a net loss of 60% of the original amount of phycocyanin/cell by the end of the experiment. The urea treatment also resulted in an 85% spike in average phycocyanin/cell and was also double the phycocyanin content of the control, but quickly decreased to 51% of the original concentration by the end of the experiment. Nitrate additions resulted in a 25% loss of phycocyanin content after 72 hr. Average phycocyanin/cell in the nitrate treatment continued to decrease to 34% of the original value by the conclusion of the experiment and was never statistically different from the control, on average ($p \geq 0.7879$). Phycocyanin declined rapidly in the control treatment, and differences in phycocyanin/cell were only observed at 72 hr, respectively. 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/cell at the 72-hr time interval, after which all treatments had similar phycocyanin content, on average, through the rest of the experiment.

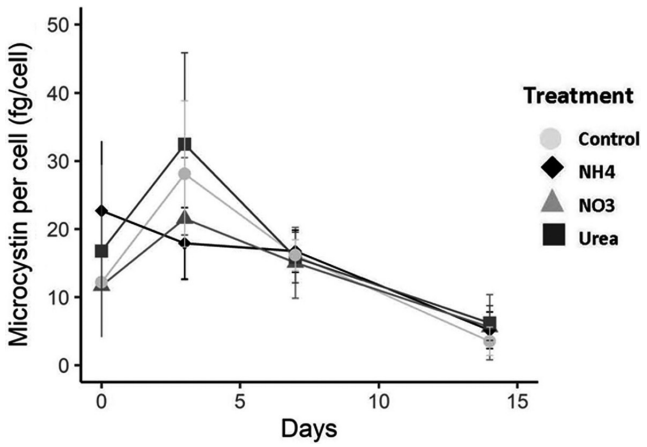


FIGURE 5 Average amount (fg) of microcystin/cell for each treatment ± *SD* over time. Nitrogen form had no significant effect on average concentration of toxins/cell. Time had a significant effect on the internal toxin concentration/cell

## 3.5 | Cell-associated toxins

Nitrogen additions had no effect on the average concentration of microcystins/cyanobacterial cell ($p = 0.3368$, $F_{3,20} = 1.1957$, Figure 5). However, there was a strong effect of time ($p < 0.0001$, $F_{3,60} = 24.2236$). Cell-associated toxins seemingly increased by 72 hr in all treatments aside from the ammonium treatment, then

decreased below the original starting concentrations. However, the effect of time was seen only between 0-hr and 2-week intervals.

## 4 | DISCUSSION

This experiment examined potential advantages that cyanobacteria, particularly *Microcystis*, can gain from growth on different dissolved nitrogen forms. Specifically, we examined the effect of reduced and oxidised nitrogen forms on the pigment content of cyanobacterial cells. We found that reduced nitrogen forms (i.e., ammonium/ammonia and urea) encouraged more cells/ml and more phycocyanin/cell than nitrate. Increases in phycocyanin/cell during nitrogen excess and subsequent declines during nitrogen removal suggest phycocyanin and the phycobilisome as preferred nitrogen storage structures. The results of this experiment encapsulate the capability of phycocyanin as a nitrogen storage mechanism and the potential for a single pulse of nutrients, particularly reduced nitrogen, to sustain a bloom community for an extended period of time. These results serve as further evidence for the need for reduced nitrogen control and increased efficiency of global nutrients sources, such as agricultural fertiliser application. The pulses applied in this experiment (600 μgN/L and 100 μgP/L) have been recorded globally (Boesch et al., 2002; Ham & DeSutter, 1999; Kato et al., 2009; Steinfeld et al., 2006).

Average chlorophyll-*a* concentration/cell did not differ among treatments during the experiment. This result was expected based on current knowledge of nitrogen storage and accessory pigments in cyanobacteria. Chlorophyll-*a* is the major contributor to photosynthesis in cyanobacteria, and chlorophyll content in cyanobacteria can change, but rarely in a significant amount given ample light (Geider, 1987; Riemann et al., 1989). Geider (1987) found that chlorophyll-*a* content in microalgae changes drastically in response to changes in light intensity or extreme temperatures. Additionally, Riemann et al. (1989) found that chlorophyll-*a* content of natural and cultured phytoplankton changed only by 1.22%–6.08% under nitrogen and/or phosphorus deficiency. In contrast, secondary pigments, such as phycocyanin, improve photosynthetic efficiency by absorbing light energy from a different region of the electromagnetic spectrum, but are not required for photosynthesis; therefore, cellular content of secondary pigments can vary greatly. Boussiba and Richmond (1980) found that *Spirulina platensis* phycocyanin decreased by 30%–50% under low-nitrogen conditions. Similarly, Zhou et al. (2020) found that *Microcystis aeruginosa* phycocyanin content decreased by 60% of starting concentrations 10 days after being placed in nitrogen-free media. Our results align with the above findings and add that reduced nitrogen forms encourage more nitrogen storage and can further sustain cyanobacterial growth long after initial nitrogen addition.

We found that phycocyanin content increased in response to additions of reduced nitrogen, while inputs of oxidised nitrogen elicited no noticeable amount of nitrogen storage as pigments. The flexibility of cyanobacterial pigments in response to changes in dissolved nitrogen concentrations has been examined previously in laboratory experiments. Elmorjani and Herdman (1987) noticed that *Synechocystis* phycocyanin content decreased by 50% in the first 24 hr after being transferred to no-nitrogen media. Phycocyanin content varied in response to nitrogen starvation (Li et al., 2018), and phycocyanin content decreased with dissolved nitrogen concentrations in lab cultures (Zhou et al., 2020). Molecular examination revealed that the entire phycobilisome protein, not just phycocyanin, was synthesised and degraded in response to nitrogen excess and stress, respectively (Zhou et al., 2020).

Our results show that cyanobacteria are capable of storing reduced forms of nitrogen more readily than oxidised forms. Previous experiments have recorded changes in cellular phycocyanin content in response to changes in nutrient levels (Elmorjani & Herdman, 1987; Erratt et al., 2018; Li et al., 2018; Zhou et al., 2020), but our results add that cyanobacteria can store reduced nitrogen more readily, allowing for a greater intracellular nitrogen pool than expected from growth on oxidised nitrogen. This finding points to the potential for continued bloom growth far beyond the exhaustion of extracellular nitrogen and farther than when oxidised nitrogen is the original source. Another molecule, cyanophycin, is ubiquitous among cyanobacteria and has been characterised as a nitrogen storage structure. Further research should target the preference and dynamics for nitrogen storage and breakdown in phycobilisomes versus cyanophycin.

Nitrogen form had a strong effect on the *Microcystis* blooms in this experiment. Phytoplankton counts showed that *Microcystis* rapidly increased in response to reduced nitrogen and continued to increase through the conclusion of the experiment, far outlasting the nitrate treatment in number of cells and length of peak community growth. This result is in agreement with similar findings in the field (see Deschoenmaeker et al., 2017; Khazi et al., 2018; Richardson et al., 2001), but not others (see An et al., 2020; Erratt et al., 2018; Kim et al., 2016; Rückert and Giani, 2004). These conflicting results can probably be attributed to the paradoxical nature of ammonium/ammonia as a nitrogen source (Glibert et al., 2016).

Internal nitrogen regeneration is an important subject to address with any experiment examining the long-term impacts of nitrogen pulses on phytoplankton. In our experiment, internal nitrogen regeneration was not measured, so it is difficult to quantify the impact that regeneration had on phytoplankton growth in this experiment. Numerous experiments have summarised internal nitrogen regeneration (see Bronk et al., 1994 and references within) and examined it in natural settings (see Axler et al., 1981; Hampel et al., 2018, 2019; Klawonn et al., 2019 and references within). Internal nitrogen regeneration can be adequate to maintain primary productivity over a range of algal densities (see Axler et al., 1981; Hampel et al., 2018, 2019; Klawonn et al., 2019). Future experiments should use targeted techniques, such as nitrogen isotope tracing, to examine the importance of nitrogen regeneration during different stages of bloom development.

We found that nitrogen form influenced the bloom community and nutrient additions encouraged the dominance of *Microcystis*. Even

after dissolved nitrogen was gone from the enclosures, *Microcystis* continued to dominate all other taxa through the conclusion of the experiment. This result is somewhat unexpected based on what is known about non-$N_2$-fixing cyanobacteria when dissolved nitrogen is low and nitrogen-fixing taxa are present. Once nitrogen concentrations in the water column are low and there is enough phosphorus to support primary productivity, one would expect $N_2$-fixing taxa, such as *Dolichospermum*, to dominate. However, results from experiments, such as Paerl et al. (2014), have found that $N_2$-fixing taxa may increase in abundance, but do not replace non-$N_2$-fixing cyanobacteria like *Microcystis*. Meanwhile, Chislock et al. (2014) found that *Cylindrospermopsis raciborskii* dominated in eutrophic mesocosms no matter the N:P ratio, and Newell et al. (2019) observed that reduced nitrogen forms encourage non-$N_2$-fixing taxa in western Lake Erie. In this experiment, *Microcystis* accounted for >75% of relative abundance and *c*. 50% of total biovolume at the start of the experiment. Furthermore, *Dolichospermum* was unable to compete with *Microcystis* and green algae, such as *Staurastrum*, in the control treatments. This overwhelming dominance of *Microcystis* from the start of the experiment is probably why the $N_2$-fixing *Dolichospermum* was unable to compete with *Microcystis* to such an extent that it could not be found in week 2 samples. This result is common in eutrophic settings where *Microcystis* is already dominant and temperature and nutrient concentrations are high (Álvarez et al., 2016; Paerl & Huisman, 2008; Wilhelm et al., 2020; Yamamoto & Nakahara, 2005). Community composition results further accentuate the competitive abilities of *Microcystis* in a dynamic system.

Cyanobacterial communities treated with reduced nitrogen forms were seemingly able to store more nitrogen as excess, resulting in greater bloom longevity when compared to the nitrate and control treatments. Even 14 days after nutrient introduction, the ammonium treatment continued to have a positive trajectory in the total numbers of cells, the urea treatment was beginning to level-off, and the nitrate and control treatments had negative trends. These findings align with what is known about how these nitrogen forms are used once they enter the cell. For nitrate to be used as a nitrogen source for protein synthesis it must be first be reduced to nitrite by nitrate reductase, then further reduced from nitrite to ammonium through nitrite reductase. From this point, the ammonium can be used for amino acid assembly and protein synthesis (Flores & Herrero, 2005; Herrero et al., 2001). These steps require more energy and resources expended along with more time to allow for these reactions to occur. These differences align with our findings that cyanobacterial growth is greater with reduced nitrogen sources as opposed to oxidised sources. Previous experiments have identified greater cell numbers in response to reduced nitrogen additions compared to oxidised inputs (Herndon & Cochlan, 2007; Raven et al., 1992; Solomon et al., 2010). However, whether the form of nitrogen can influence bloom longevity in the field appears understudied.

Cell-associated microcystins were detected in the enclosures of all treatments at concentrations below U.S. Environmental Protection Agency guidelines (8 μg/L; US EPA, 2018). In this study, cell-associated microcystin concentrations peaked 72 hr after fertilisation, but statistical results showed no effect of nitrogen additions on toxin cell quotas ($F_{3,20} = 0.8485$; $p = 0.4836$). Furthermore, although microcystin concentrations seem to have increased in all but the ammonium treatment, statistical differences were only noted between the start and endpoints of the experiment. Future experiments should continue to uncover the environmental triggers for microcystin production.

The findings from this experiment provide more support that cyanobacteria can store excess nitrogen as phycocyanin and other related structures. Furthermore, our results show that these nitrogen stores are potentially apt for sustaining cyanobacterial growth for a significant amount of time beyond an initial pulse of nutrients and long after extracellular dissolved nitrogen concentrations are diminished. This could explain the generally inconclusive correlations between cyanobacterial abundance and nitrogen in field studies (Poste et al., 2013; Song et al., 1998; Xue et al., 2016; Yang et al., 2006; Yen et al., 2012; Yu et al., 2014). This potentially key detail should be considered when conducting such studies.

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## AUTHOR CONTRIBUTIONS

M.F.G. and A.E.W. conceived of the project idea and designed the experiment. All authors contributed to experimental setup. M.F.G. collected all field samples at the specified intervals. All authors contributed to processing field samples in the lab. M.F.G. carried out all statistical analysis and all authors verified the statistical analyses and aided in data visualisation. M.F.G. prepared the manuscript and all authors contributed to editing the final version.

## DATA AVAILABILITY STATEMENT

Data are available from the authors upon reasonable request.

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