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# Nitrogen form, concentration, and micronutrient availability affect microcystin production in cyanobacterial blooms

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## Abstract

Harmful algal blooms (HABs) are increasing in magnitude, frequency, and duration caused by anthropogenic factors such as eutrophication and altered climatic regimes. While the concentrations and ratios of nitrogen (N) and phosphorus are correlated with bloom biomass and cyanotoxin production, there is less known about how N forms and micronutrients (MN) interact to regulate HABs and cyanotoxin production. Here, we used two separate approaches to examine how N and MN supply affects cyanobacteria biomass and cyanotoxin production. First, we used a *Microcystis* laboratory culture to examine how N and MN concentration and N form affected the biomass, particulate N, and microcystin-LR concentration and cell quotas. Then, we monitored the N, iron, molybdenum, and total microcystin concentrations from a hypereutrophic reservoir. From this hypereutrophic reservoir, we performed a community HAB bioassay to examine how N and MN addition affected the biomass, particulate N, and microcystin concentration. *Microcystis* laboratory cultures grown in high urea and MN conditions produced more biomass, particulate N,

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

and had similar C:N stoichiometry, but lower microcystin-LR concentrations and cell quotas when compared to high nitrate and MN conditions. Our community HAB bioassay revealed no interactions between N concentration and MN addition caused by non-limiting MN background concentrations. Biomass, particulate N, and microcystin concentration increased with N addition. The community HAB amended with MN resulted in greater microcystin-LA concentration compared to non-MN amended community HABs. Our results highlight the complexity of how abiotic variables control biomass and cyanotoxin production in both laboratory cultures of *Microcystis* and community HABs.

**Keywords**

Harmful algae blooms; ecological stoichiometry; iron; zinc; nitrate; urea; *Microcystis*; Nitrogen species

## I. Introduction

The magnitude, frequency, and duration of harmful algal blooms (HABs) are increasing, in part caused by anthropogenic activities such as eutrophication and global climate change (Paerl and Barnard, 2020). Harmful algal blooms are one of the greatest threats to water quality because of the difficulty in predicting the multifaceted nature of bloom formation and toxin production (Brooks et al., 2016). A common cyanobacteria HAB-forming genus, *Microcystis*, has been identified on every continent except Antarctica (Harke et al., 2016), and can produce a variety of microcystin congeners. Chronic exposure to low microcystin concentrations can cause non-alcoholic liver disease directly through proinflammatory events (Sarkar et al., 2020) and indirectly by altering the intestinal microbiome (Sarkar et al., 2019). Thus, understanding what factors control HABs and cyanotoxin production will provide management solutions to improve water quality (Wilhelm et al., 2020) and human health.

Macronutrients, nitrogen (N) and phosphorus (P), often limit primary production (Elser et al., 2007), including HABs (Gobler et al., 2016). Microcystin concentration is positively correlated with chlorophyll a, total N, and total P concentrations at continental scales (Yuan et al., 2014; Yuan and Pollard, 2017). Given that HAB biomass and cyanotoxin production are sensitive to macronutrient concentration, ecological stoichiometry is an ideal framework to investigate the growth and toxin production of HABs. Ecological stoichiometry predicts N-rich cyanotoxins (e.g., microcystins, cylindrospermopsins, and saxitoxins) production is regulated by the N:P supply, with increased production of N-rich cyanotoxins occurring when N:P supply is high relative to demand (van de Waal et al., 2014). This prediction is supported by the positive relationship between N:P supply and microcystin concentrations in *Microcystis* strains (Brandenburg et al., 2020; van de Waal et al., 2014; Wagner et al., 2019). The biomass and stoichiometry of *Microcystis* populations is controlled by the concentrations and bioavailability of P, N, and N turnover rates (Hampel et al., 2019), while microcystin cell quotas are controlled by the cellular C:N in a stoichiometric explicit manner (Wagner et al., 2019).

Depending on the surrounding land use, N inputs into lakes and reservoirs are predominantly in the form of nitrate ($NO_3^-$), ammonium ($NH_4^+$), or dissolved organic N (Donald et al., 2011). Non-point source N input from agricultural fertilization has largely switched from inorganic N (i.e., ammonia nitrate) to urea (Glibert et al., 2014, 2006). Thus, urea loading can potentially become a source of N especially in watersheds that are dominated by agricultural land use. Cyanobacteria have multiple pathways to utilize different N forms (Erratt et al., 2018; Flores et al., 2005). The most energetically favorable N form is $NH_4^+$ because it can diffuse across the cell membrane and be directly incorporated into amino acids and protein synthesis (Finlay et al., 2010; Herrero et al., 2001). Urea is also capable of diffusion but requires slightly more energy for assimilation because it requires the enzyme urease to convert it into two $NH_4^+$ and one carbon dioxide (Erratt et al., 2018; Finlay et al., 2010; Herrero et al., 2001). Nitrate assimilation is more energetically costly than urea because $NO_3^-$ requires active transport through the cell membrane after which it is reduced to nitrite by nitrate reductase and then further reduced to $NH_4^+$ by nitrite reductase (Flores et al., 2005). The enzymes required to assimilate urea and $NO_3^-$ contain metal cofactors; therefore, trace metal concentrations can affect N assimilation.

Metal cofactors are essential in many enzymatic processes that control nutrient acquisition, gene transcription, and growth. Nitrate reductase has an iron-sulfur cluster that interacts with ferredoxin to obtain two electrons needed for the molybdenum cofactor to reduce nitrate to nitrite (Flores et al., 2005). Nitrite reductase has an iron-sulfur cluster that interacts with ferredoxin to obtain the six electrons needed to reduce nitrite to $NH_4^+$ (Flores et al., 2005). Urease contains two nickel cofactors that are responsible for converting urea to two $NH_4^+$ molecules (Glass et al., 2009). In addition to the metals required for N assimilation, other metals such as cobalt, copper, manganese, and zinc are required for growth in many cyanobacteria species (Facey et al., 2019). Cyanobacteria typically have a higher trace-metal cell quota than other phytoplankton groups, increasing their likelihood of becoming trace-metal limited (Downs et al., 2008; Facey et al., 2019; Zhang et al., 2019). The most well-studied trace-metal is iron, and when added to water, can increase phytoplankton biomass in marine (Martin and Fitzwater, 1988) and freshwater environments (Havens et al., 2012; North et al., 2007). In phytoplankton bioassays, removal of iron with a chelator caused decreased nitrate uptake and led to increased phytoplankton carbon (C):N stoichiometry (North et al., 2007). Decreasing iron supply in *Microcystis* cultures resulted in lower N cell quotas, indicating $NO_3^-$ assimilation impairments (Nagai et al., 2007). Much less is known about nickel impairing N acquisition in freshwater cyanobacteria. However, the marine non-toxic cyanobacteria, *Synechococcus* grown in nickel-limited conditions became N-limited in the presence of abundant urea (Dupont et al., 2008). Hence, the concentration of trace-metals can affect N acquisition in cyanobacteria.

Here, we examined the interactive effects of N concentration, N form, and micronutrient (MN) concentration on biomass, stoichiometry, and toxin production in *Microcystis* laboratory cultures and a natural HAB community. The first experiment examined how N concentration, N form, and MN concentration affects the growth, stoichiometry, and toxin production in a laboratory culture of *Microcystis aeruginosa*. A second experiment examined how $NO_3^-$ concentration and MN addition affected biomass production, C:N stoichiometry, and microcystin concentrations in a HAB community that was collected from a

cyanobacteria-dominated hypereutrophic reservoir. We hypothesized in our laboratory culture experiment that N form and MN concentration would interact to affect biomass and microcystin production in *Microcystis* populations. For our community HAB experiment, we hypothesized that N and MN concentration interacted to affect biomass, particulate N, and microcystin production. Therefore, we predicted for both the laboratory and community HAB experiments that greater N and MN availability would correlate with greater biomass and toxin production.

## 2. Methods

### 2.1 Laboratory Microcystis culture experiment

*Microcystis aeruginosa* (strain; LE3) that produces microcystin-LR was acquired from C.J. Gobler in April 2019 and has been maintained since then in 0.5x diluted BG-11. To acclimate *M. aeruginosa* to low MN conditions, we created a 1 L stock culture grown in 10x diluted with deionized water N- and MN-free BG-11 media. To this basal media, we added low MN concentrations (Table 1) that resulted in a decrease of the MN by 90% compared to full strength BG-11 media. We then added 16.1 mg $L^{-1}$ $NO_3$-N to generate a N:P of 50 (by mol; P-0.75 mg $L^{-}$) ensuring N replete conditions. The *Microcystis* culture was grown in a 4 L Erlenmeyer flask at 26 ºC with a light intensity of 140 μmol $m^{-2}$ $s^{-1}$ on a 14h:10h light:dark cycle. The stock culture was grown for three weeks before starting the N form and MN concentration experiment.

To test the effects of MN on N form and concentration, we set up a fully factorial design consisting of two forms of N (urea and $NO_3^{-}$), two N levels (low, 0.644 mg $L^{-1}$ N; high 16.1 mg $L^{-1}$ N), and two MN concentrations (low and high; Table 1) with each treatment combination containing five replicates resulting in 40 experimental units. The N and MN stocks used to amend the experiment were made from ACS reagent grade chemicals that could contain a maximum concentration of 4 and 10 μg $L^{-1}$ of iron in the high $NO_3^{-}$ and urea treatments, respectively. These treatment combinations were added to 20x diluted with deionized water N and MN-free (including iron) BG-11 media with 1.35 μg $L^{-1}$ of vitamin $B_{12}$ resulting in a total volume of 0.5 L grown in 1 L media bottles. We then added 5 mL of cells that corresponded to 0.25 mg $L^{-1}$ of *Microcystis* as C (C measured as described below) from our low MN acclimated stock culture to initiate the experiment. All treatments were placed in an incubator at 26 ºC with a light intensity of 140 μmol $m^{-2}$ $s^{-1}$ on a 14h:10h light:dark cycle. All experimental units were shaken every other day and a 2 mL aliquot was removed to monitor growth via *in-vivo* fluorescence twice-weekly (Turner Designs Laboratory Fluorometer). After 14 days of growth, we saved a 2 mL aliquot from each experimental unit for cell counts that were preserved in Lugol's iodine. We filtered cells onto precombusted 0.7 μm 25 mm glass fiber filters (GF/F Whatman) for particulate C and N, chlorophyll a, and particulate microcystin-LR concentration. Filters for CN and chlorophyll a were stored at −20 ºC until analyzed. Microcystin-LR filters were lyophilized on a Virtis SP Scientific benchtop pro freeze dryer (SP Scientific) for 48 h and stored at −80 ºC until analyzed.

### 2.2 Lake Fayetteville monitoring

Lake Fayetteville (36º08'11.5"N, 94º07'7.46"W), is a hypereutrophic shallow warm monomictic (average depth 3 m) flood control reservoir (Grantz et al., 2014, 2012) in Fayetteville, Arkansas, USA. Urban and agricultural land use accounts for over 75% of Lake Fayetteville's watershed (Grantz et al., 2014, 2012). During the growing season (May to September), the mean chlorophyll a concentration is 39 μg $L^{-1}$ (Grantz et al., 2014) increasing the likelihood of HABs and microcystin production (Yuan et al., 2014).

Weekly surface grab samples from Lake Fayetteville marina dock (GPS coordinates 36.137117, −94.139826) were collected in acid wash bottles to measure $NO_3^-$, nitrite ($NO_2^-$), dissolved trace-metals, and microcystin concentrations, as well as raw fluorescence of chlorophyll and phycocyanin. Nitrate and $NO_2^-$ samples were filtered using 25 mm 0.45 μm membrane filter (Pall Life Sciences) then were measured spectrophotometrically after cadmium reduction (APHA, 1992). Dissolved trace-metal samples were filtered using trace-metal clean procedures through a 0.45 μm membrane filter (Pall Life Sciences) and acidified to pH < 2 with trace-metal free HCl and $HNO_3$ and measured on an inductively coupled plasma optical emission spectrometry (ICP-OES) following the EPA method 200.7 (U.S. EPA, 1994). Whole water collected for total microcystin went through three repeated freeze-thaw cycles to lyse cells and was analyzed using an enzyme-linked immunosorbent assay according to the manufacture's protocol (Abraxis; Loftin et al., 2007). We measured the phycocyanin:chlorophyll ratios (raw fluorescence units, RFUs) using a handheld CyanoFlour (Turner Designs) from May 2019 to September 2019; phycocyanin:chlorophyll ratios above 1 suggest the community is dominated by cyanobacteria (Ogashawara et al., 2013).

### 2.3 Lake Fayetteville community HAB bioassay

Coupled with this monitoring regime, we performed a bioassay experiment by collecting 25 L of surface whole lake water in an acid washed container from the end of the marina dock (GPS coordinates 36.137117, −94.139826) on July 1st, 2019. Additional samples were collected in acid washed bottles to determine the initial $NO_2^-$ + $NO_3^-$ and trace-metal concentrations. This time point was chosen because large surface scums were present, and the phycocyanin:chlorophyll ratio was around 2, indicating the phytoplankton community was dominated by cyanobacteria. The collected water was transported back to Baylor University and was screened through 80 μm mesh to remove zooplankton and debris. While no large colonies were noticed, this screening procedure would remove colonies of *Microcystis* that were larger than 80 μm. We also filtered a subsample of water through a 0.45 μm membrane filter to measure soluble reactive phosphorus using the ascorbic acid molybdate blue method (APHA, 1992). To this screened whole lake water, we conducted a fully factorial experiment to investigate the independent and interactive effects of N (as $NO_3^{-1}$) and MN concentration on biomass, C:N stoichiometry, and microcystin production. The bioassay had four levels of $NO_3^-$-N, control (0.003 mg $L^{-1}$), low (0.1 mg $L^{-1}$), medium (0.5 mg $L^{-1}$), and, high (1 mg $L^{-1}$) and two levels of MN (background, amended; Table 2). The MN stocks used for the amendments were all ACS reagent grade and could be influenced by slight impurities, therefore the concentrations in Table 2 are a conservative estimate. The bioassay had a total volume of 700 mL and four replicates, resulting in 32

experimental units grown in 1 L acid washed media bottles. The soluble reactive phosphorus sample collected determined there was under 5 μg $L^{-1}$ of soluble reactive P, thus to ensure only N or MN could become limiting we added 100 μg $L^{-1}$ of P to all experimental units. We then placed the experimental units in an incubator at 26 ºC with a light intensity of 140 μmol $m^{-2}$ $s^{-1}$ on a 14h:10h light:dark cycle for 7 days. Each day experimental units were gently shaken, and a 2 mL aliquot was removed to monitor growth via *in-vivo* fluorescence (Turner Designs Laboratory Fluorometer). On the 7$^{th}$ day, we filtered each experimental unit on 0.7 μm precombusted 25 mm GF/F filters for particulate C and N, chlorophyll a, and microcystin concentrations. Filters for CN and chlorophyll a were stored at −20 ºC until analyzed. Microcystin filters were lyophilized on a Virtis SP Scientific benchtop pro freeze dryer (SP Scientific) for 48 h and stored at −80 ºC until analyzed.

### 2.4 Carbon, nitrogen, chlorophyll a, cell counts and microcystin analysis

Particulate C and N were dried at 60 ºC for 24 h and were measured on an elemental analyzer (Thermo-Fisher NC Soil). The amount of C and N were determined by comparing the peak area to known aspartic acid standards. Filters for chlorophyll a were extracted in 100 % acetone for 24 h in the dark at −20 ºC. After extraction, chlorophyll a concentration was determined by reading the absorbance at 630 nm, 645 nm, 665 nm, and 750 nm on a spectrophotometer and converted to concentration using the trichromatic equations (Lind, 1985).

Cell counts were performed on the laboratory culture experiment that examined the interactive effects of N form, N, and MN concentration as previously described (Wagner et al., 2019). Briefly, preserved *Microcystis* LE3 cultures were counted on a flow cytometer using a side scatter forward scatter method (BD Diagnostic Systems, FACSVerse). For each experimental unit, we counted 50,000 cells and obtained the volume to calculate the cell density.

Microcystins were quantified with a liquid chromatography mass spectrometry (LC-MS) as previously described (Wagner et al., 2019). Briefly, 10 μL of a 1 mg $L^{-1}$ mixture containing four isotopically-labeled microcystins (MC- LA, LR, RR, and YR) was added to the lyophilized filters and extracted using 1 mL of 75:25 ($v$ $v^{-1}$) acetonitrile:water containing 0.1% formic acid. Filters were then sonicated for 5 min and centrifuged before the supernatant was collected. This extraction procedure was repeated twice. The pooled supernatants were blown down under a stream of $N_2$, resuspended in 1 mL of 90:10 ($v$ $v^{-1}$) water:acetonitrile buffered with 5 mM $NH_4OOCH_3$ and 3.6 mM HCOOH (pH 3.7), filtered through a 0.2 μm syringe filter, and stored in an amber HPLC vial until LC-MS analysis. Analysis was performed using an Agilent 1260 Infinity LC coupled to an Agilent 6420 mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) following a previously published isotope dilution method (Haddad et al., 2019). For both the laboratory culture and community HAB experiments, we report microcystin concentrations as the concentration within an experimental unit on a volume basis. Additionally, for the laboratory *Microcystis* culture experiment, we also standardize the microcystin concentration by cell density to calculate microcystin cell quotas.

### 2.5 Statistical analysis

All data were checked for normality and homoscedasticity prior to analysis in JMP (version 15). To assess how N form, and N and MN concentration affected biomass, C:N stoichiometry, chlorophyll a, and microcystin production in the laboratory *Microcystis* cultures, we performed a 3-way analysis of variance (ANOVA) with a Tukey's posthoc multiple comparison test in JMP. For all response variables that did not have a significant 3-way interaction, we subsequently performed a 2-way ANOVA with a Tukey's posthoc multiple comparison test. Finally, for response variables that did not have a 2 or 3-way interaction, we performed an ANOVA with a Tukey's posthoc test for multiple comparisons. We examined if N or MN addition affected biomass, C:N stoichiometry, chlorophyll a, and microcystin concentrations in our community HAB bioassay using a 2-way ANOVA. However, all response variables had non-significant interaction between N and MN addition, therefore we performed an ANOVA and Tukey's post hoc test to examine the effects of N or MN.

## 3. Results

### 3.1 Laboratory Microcystis culture experiment

We found significant 3-way ANOVA interactions between N concentration, N form, and MN concentration for *Microcystis* biomass (as particulate C), particulate N, C:N stoichiometry, and microcystin-LR concentrations (Table 3, Fig. 1). The only significant difference between N forms was that *Microcystis* cultures grown in high urea high MN conditions produced more biomass compared to cultures grown in high $NO_3^-$ high MN conditions (Fig. 1a). Cultures grown in low N conditions produced less biomass compared to cultures grown in high N conditions regardless of N form (Fig 1a). Micronutrient concentrations did not affect particulate C in low N conditions or high $NO_3^-$ conditions; however, more biomass was produced in high urea and MN conditions compared to high urea low MN conditions (Fig. 1a).

High urea conditions produced more particulate N compared to high $NO_3^-$ conditions, with no other differences noticed between N forms (Fig. 1b). Low N conditions produced less particulate N compared to high N conditions (Fig. 1b). Micronutrient concentration did not affect particulate N in low N conditions. However, in high N and high MN treatments resulted in more particulate N (Fig. 1b).

Nitrogen form did not affect the C:N stoichiometry (Fig. 1c). Low N resulted in elevated C:N stoichiometry regardless of N form when compared to high N treatments (Fig. 1c). Micronutrient concentrations did not affect the C:N stoichiometry in high N conditions (Fig. 1c). In low $NO_3^-$ conditions, high micronutrient concentrations decreased the C:N stoichiometry (Fig. 1c). Whereas, in low urea conditions, increasing the MN concentrations increased the C:N stoichiometry (Fig. 1c).

*Microcystis* cultures grown in high $NO_3^-$ conditions produced higher concentrations of microcystin-LR compared to high urea conditions (Fig. 1d). Regardless of N form, low N conditions produced less microcystin-LR compared to high N conditions (Fig. 1d).

Micronutrient concentrations did not affect microcystin-LR in either N concentration or N form conditions (Fig. 1d).

Chlorophyll a concentration resulted in a two-way interaction between N and MN concentration (Table 3, Fig 2a). In low N conditions, chlorophyll a concentrations were the same; whereas, in high N conditions, high MN concentrations resulted in more chlorophyll a compared to low MN conditions (Fig 2a).

Microcystin-LR cell quotas interacted with N form and N concentration (Table 3, Fig. 2b). In low N conditions, microcystin-LR concentrations were similar; however, in high N conditions, *Microcystis* cultures grown in $NO_3^-$ had higher microcystin-LR cell quotas compared to high urea conditions (Fig. 2b).

Chlorophyll a cell quotas resulted in three two-way interactions (Table 3, Fig. 2c). In low N conditions, chlorophyll a cell quotas were the same; however, in high N conditions, high MN concentrations resulted in higher chlorophyll a cell quotas (Fig 2c). Additionally, chlorophyll a cell quotas had a significant interaction between N form and N concentration (Table 3, Fig. 2c). Similar cell quotas were observed in low N conditions. Whereas, in high N conditions, $NO_3^-$ treatments produced higher chlorophyll a cell quotas compared to urea treatments. Lastly, we found chlorophyll a cell quotas interacted with N form and MN concentration (Fig. 2c). Within MN concentration or N form there were no significant differences observed in chlorophyll a cell quotas. *Microcystis* cultures grown in $NO_3^-$ with high MN had significantly higher chlorophyll a cell quotas compared to low urea low MN conditions (Fig. 2c).

Cell densities and N cell quotas were affected by N concentration, with increased densities and N cell quotas found in high N compared to low N conditions (Table 3, Fig. 3a&3b). Additionally, cell densities were also impacted by MN concentration, with increased cell densities in high MN conditions compared to low MN (Table 3, Fig. 3c).

### 3.2. Lake Fayetteville Monitoring

The *in vivo* phycocyanin:chlorophyll approached 6 in early-May when measurements were first recorded (Fig. 4a). From late June to October the *in vivo* phycocyanin:chlorophyll varied from 0.5 to 2, with most data above 1 (Fig. 4a). Lake Fayetteville's mean $NO_2^-$ and $NO_3^-$ was approximately 0.8 mg $L^{-1}$ in early spring (Fig. 4b). From May to mid-June $NO_2^-$ and $NO_3^-$ concentrations rapidly decreased and then fluctuated between 0.003 and 0.01 mg $L^{-1}$ for the remainder of the growing season (Fig. 4b). Microcystin concentrations were undetectable by ELISA in March and then began to increase by mid-spring (Fig 4b). Throughout May, microcystin concentrations increased, reaching a maximum of approximately 16 μg $L^{-1}$ in early June before rapidly decreasing to between 0.5 and 1 μg $L^{-1}$ for the rest of the growing season (Fig. 4b). Dissolved iron ranged from 11 to 75 μg $L^{-1}$ from the end of April to October (Fig. 4c). Concentrations of dissolved iron varied during the spring, but then generally showed a declining trend during the summer months until October (Fig. 4c). Dissolved molybdenum ranged between below detection limits to 7 μg $L^{-1}$ (Fig. 4c). Similar to dissolved iron, dissolved molybdenum concentrations were more

variable in late April until mid-May, then remained between below detection limits to 2 μg $L^{-1}$ with most data around 1 μg $L^{-1}$ (Fig. 4c).

### 3.3 Lake Fayetteville community HAB bioassay

The Lake Fayetteville phytoplankton community HAB bioassay resulted in no two-way interactions between N ($NO_3^-$) concentration and MN amended for particulate C, particulate N, C:N stoichiometry, chlorophyll a, and microcystin concentrations (Table 4). N concentration was a significant main effect for all the response variables (Table 4). The phytoplankton biomass significantly increased in the medium and high N treatments compared to the control and low N treatments (Fig. 5a). Particulate N increased significantly with all N amended treatments and was further increased in the high N treatment (Fig. 5b). The phytoplankton grown in the control and low N conditions had a lower C:N stoichiometry compared to the phytoplankton grown in medium and high N treatments (Fig. 5c). Chlorophyll a significantly increased in all N treatments; however, there was not a dose-dependent response with N addition (Fig. 5d). Total microcystin concentration and the two most abundant congeners (microcystin-LR, and microcystin-LA) displayed the same pattern with significant increases in microcystin concentration in the high N addition (Fig. 5e-5g). Whereas microcystin-RR and microcystin-YR concentration significantly increased in the medium N treatment compared to the control and low N treatments (Fig. 5h&5i). Further significant increases in microcystin-RR and microcystin-YR occurred in the high N addition (Fig. 5h&5i). Microcystin-LA concentrations were also sensitive to MN amendment, with phytoplankton populations grown under amended MN having significantly increased microcystin-LA concentrations compared to background MN conditions (Table 4, Fig. 6)

## 4. Discussion

Our hypothesis that N form, N and MN concentrations would interact to affect biomass, N acquisition, and microcystin concentration was supported in our laboratory *Microcystis* experiment. The high N conditions and MN concentration affected *Microcystis* N acquisition; however, in low N conditions, MN concentration had less of an effect on biomass, or particulate N, regardless of N form. *Microcystis* cultured in high urea resulted in more biomass and particulate N, similar C:N stoichiometry, and lower microcystin-LR concentrations and cell quotas than those cultured in high $NO_3^-$. Our hypothesis that MN and N addition would interactively affect biomass and microcystin concentration was not supported in the community HAB bioassay. While no interactions between N concentration and MN amendment, N addition did result in increased biomass, particulate N, and microcystin concentrations, whereas MN amendment resulted in increased concentrations of microcystin-LA. Our results highlight the complex interactions between N form, N and MN concentration on microcystin concentration, and cell quotas in both *Microcystis* laboratory cultures and phytoplankton community HABs.

### 4.1 Interactions between N form, N and MN concentration in Microcystis cultures

Increased biomass in *Microcystis* cultures grown in high urea may be caused by the decreased energetic demands of N assimilation (Finlay et al., 2010; Herrero et al., 2001). Also, the breakdown of urea results in a carbon dioxide molecule that can be further used to

support growth (Krausfeldt et al., 2019). This increased growth in urea compared to $NO_3^-$ might be experiment/strain-specific with some *Microcystis* strains demonstrating similar growth on both N forms (Chen et al., 2019; Erratt et al., 2018), while others have displayed increased growth with urea (Li et al., 2016). Regardless of whether urea was able to increase biomass or not in previous experiments, all these examples and our study were completed in N-free BG-11 media that lacks nickel. Despite the fact nickel is a cofactor required for urea assimilation using urease, we found high MN concentrations increased biomass and particulate N. While only identified *in vitro*, urease may be able to substitute the nickel cofactor with cobalt or manganese resulting in the reduction of urease activity (Carter et al., 2009) or use an iron co-factor as identified in pathogenic bacteria (Carter et al., 2011). Alternatively, some of the trace-metal components in BG-11 contain nickel as a contaminant and this trace amount of nickel might have met the urease synthesis demands, resulting in higher biomass and particulate N compared to $NO_3^-$ in high urea and MN conditions. Similar results were found in a bioassay that had the same chlorophyll concentrations with or without nickel additions (Chaffin and Bridgeman, 2014). With the potential of increased urea inputs from fertilizer, we suggest examining how individual trace-metal components, including nickel, affect the growth and cyanotoxin production of *Microcystis* cultures grown with urea.

Cyanobacteria have a higher MN demand compared to other phytoplankton, especially in diazotrophic cyanobacteria species (Facey et al., 2019; Glass et al., 2009). In MN limited environments, the addition of MN (e.g., molybdenum and cobalt) can increase cyanobacteria-dominated phytoplankton biomass upwards of 40% (Downs et al., 2008). Additionally, Lake Taihu, known for significant *Microcystis* blooms, can be limited/co-limited by iron, cobalt, or copper within different regions of the lake (Zhang et al., 2019). Micronutrient concentration affected the C and N acquisition and/or assimilation in our *Microcystis* laboratory experiment. Nitrogen transport and assimilation enzymes that contain iron and molybdenum cofactors are highly sensitive to environmental N conditions (Herrero et al., 2001), with higher expression in low N environments (Harke and Gobler, 2015). This would result in higher MN demands in the low N treatments; however, MN concentration did not influence biomass or particulate N. Although, we found the MN concentration affected biomass and particulate N in high N conditions regardless of N form. This result is likely caused by MN limitation in the higher N treatments caused by the decreased MN concentration per cell. Once the MN concentration decreased, the N acquisition/assimilation likely decreased which resulted in less biomass and particulate N produced. Overall, our results support that MN concentration can limit the biomass and particulate N of *Microcystis* cultures, especially in high N conditions where growth rates are expected to be high.

Our results support the ecological stoichiometric hypothesis that states increasing N supply relative to demand would result in increased microcystin production in the *Microcystis* experiment. This higher N supply compared to growth demand also results in a lower C:N, thus microcystin cell quotas and concentrations are negatively correlated to *Microcystis* C:N ratios (van de Waal et al., 2009; Wagner et al., 2019). Our results support this finding, with lower C:N *Microcystis* cultures having greater microcystin-LR concentrations than higher C:N cultures irrespective of N form. Another prediction is that microcystin is produced proportionally to growth (Downing et al., 2005; Long et al., 2001). However, we found that

*Microcystis* grown in high urea conditions produced more biomass, had higher growth rates (0.29 $day^{-1}$; determined from initial and final biomass C measurements), and less microcystin-LR concentration and cell quota, compared to *Microcystis* grown in high $NO_3^-$ conditions produced less biomass, had decreased growth rates (0.26 $day^{-1}$), and higher microcystin-LR cell quota and concentration. Consequently, our results do not support that microcystin production is regulated by growth, but do support that N form can affect microcystin production (Chen et al., 2019; Peng et al., 2018). Both urea and $NO_3^-$ are enzymatically cleaved and reduced to $NH_4^+$ that becomes aminated by the glutamine synthetase-glutamate synthase cycle (Flores and Herrero, 2005; Herrero et al., 2001) before being incorporated into proteins or secondary metabolites. *Microcystis* cultures grown with different N forms have resulted in dissimilar amino acid composition, with populations grown in $NO_3^-$ having higher concentrations of amino acids involved in microcystin production compared to cultures grown in urea (Chen et al., 2019). Similar results have been reported in phytoplankton communities' response to different N forms with $NO_3^-$ additions causing higher cyanotoxin concentrations compared to urea additions (Davis et al., 2010; Donald et al., 2011). Overall, our results suggest that both the form of N and the N:P supply does influence microcystin-LR in laboratory *Microcystis* cultures that are independent of growth.

### 4.2 Effects of MN and N in a community HAB bioassay

Given the extreme magnitude of biomass reached during phytoplankton blooms, we expected the phytoplankton community nutrient addition to have high MN demands. In addition to the high biomass, ambient $NO_2^-$ and $NO_3^-$ were low (ranged between 0.003 to 0.1 mg $L^{-1}$; Fig. 4b) relative to commonly observed values in eutrophic lakes and reservoirs (0.5 to 6 mg $L^{-1}$; Harris et al., 2016), and Lake Fayetteville tends to become N-limited annually in the summer months (Grantz et al., 2014). This should result in increased production of N assimilation enzymes containing iron and molybdenum cofactors (Herrero et al., 2001; Herrero and Flores, 2019) or boron needed for heterocyst formation in N fixing cyanobacteria (Bonilla et al., 1990). Even with the increased demand for MN to acquire N in low N conditions, MNs were likely not limiting growth in Lake Fayetteville given there was no MN effect on growth or particulate N during our community HAB bioassay. The concentrations of dissolved iron remained well above detection from May until October but did show a general decline over the growing season. Whereas, the concentrations of molybdenum did decrease below detection limits periodically in June and September but were detectable during our community HAB bioassay. As cyanobacteria are better competitors of some MNs such as iron (Sorichetti et al., 2016), it may suggest there was enough MN present to not elicit a MN effect in our community HAB bioassay. Additionally, given that there was background dissolved trace metals present in the Lake Fayetteville community HAB bioassay and amending with additional trace metals did not result in increased biomass or particulate N, we suspect the HAB community was not trace metal limited in early July 2019.

While MN limitation, particularly iron limitation, is becoming more apparent in freshwater ecosystems (Downs et al., 2008; Havens et al., 2012; North et al., 2007), many *in situ* lake assessments examining MN limitation do not include microcystin or other cyanotoxin

concentrations. In a eutrophic cyanobacterial dominated system, microcystin production was suppressed by amending mesocosms with low levels of iron, and it was further decreased in the presence of higher iron loading (Orihel et al., 2016). This was caused by an unknown mechanism in low iron additions and decreased water column P in high iron additions causing community dynamic shifts (Orihel et al., 2016). While it may be due to non-MN limiting conditions, we found that MN additions did not influence total microcystin concentration; however, the concentration of microcystin-LA was higher in MN amended conditions. Regardless of the microcystin congener, the enzymes responsible for microcystin synthesis are the same (Dittmann et al., 2013). Different microcystin congeners can occur through horizontal gene transfer and point mutations that allow for different selectivity in amino acids (Dittmann et al., 2013). Harmful algal blooms are composed of many species and strains of cyanobacteria, (Davis et al., 2010; Monchamp et al., 2014) with the resulting increase in microcystin-LA under MN addition possibly caused by a change in community structure as seen in other HABs (Monchamp et al., 2014).

Nitrogen supply also affected the cyanotoxin concentrations in Lake Fayetteville. The highest microcystin levels occurred when $NO_2^-$ and $NO_3^-$ were above 0.2 mg $L^{-1}$ and remained low when $NO_2^-$ and $NO_3^-$ were below 0.1 mg $L^{-1}$. This observation is supported in the literature, with toxin producing strains dominating in waters that have higher inorganic N and P concentrations and non-toxin producing strains dominating in low nutrient waters (Davis et al., 2010). While our N addition bioassay occurred when N supply was low, we were able to stimulate microcystin production, either by selecting for more toxic species/strains as found in Davis et al. (2010) or increasing cyanotoxin concentrations in the dominant species/strains present (van de Waal et al., 2014). In phytoplankton communities, microcystin congeners have been correlated with different cyanobacteria species instead of environmental variables including nutrient concentrations (Monchamp et al., 2014). Differences in toxic cyanobacterial species/strains may explain our results that microcystin-RR and microcystin-YR concentrations were significantly increased at lower N inputs than microcystin-LA and microcystin-LR, where increases only occurred at the highest N supply.

In general, microcystin is a N-rich molecule, and depending on the two different amino acids in the microcystin congener, the N content can increase further. For example, microcystin-RR contains 17.5 % N, whereas microcystin-LR has 14% N, and relatively N-poor microcystin-LA only contains 10.6% N. Cyanobacteria strains that can produce more than one microcystin congener have been found to prioritize the production of microcystin-RR over microcystin-LR with increasing N supply (van de Waal et al., 2009). Our results support this finding with microcystin-RR concentrations increasing in the 0.5 mg $L^{-1}$ $NO_3^-$ treatment compared to less N rich congeners not increasing until the 1 mg $L^{-1}$ $NO_3^-$ treatment. Whether our results are from changes in cyanobacteria dynamics or increased microcystin production, our results reveal that N addition can alter the microcystin concentration and that individual microcystin congeners are affected differently by N supplies. Such differential congener production dynamics across N conditions represent important considerations for water quality assessment and management efforts because specific microcystin congeners vary in toxicological mechanisms of action, potencies (e.g., microcystin-LA is more toxic than microcystin-LR; Chernoff et al., 2021), and adverse

outcomes, which are highly relevant to public health and the environment (Wang et al., 2020).

## 5. Conclusion

Overall, our results support that increasing N supply irrespective of N form increases microcystin cell quotas and concentration in both laboratory *Microcystis* populations and community HABs. Thus, these results support the need to manage N inputs into aquatic systems to decrease the bloom size, toxin production, and water quality risks from cyanobacteria HABs (Gobler et al., 2016; Paerl et al., 2016). High urea high MN concentrations caused *Microcystis* populations to increase biomass and particulate N; however, this combination had lower microcystin-LR concentration and cell quotas compared to high $NO_3^-$ high MN conditions. Extending this result to lakes and reservoirs that receive high urea inputs, may result in larger cyanobacteria blooms, but less microcystin production depending on the species and strain composition (Monchamp et al., 2014). Micronutrient concentration did not affect microcystin-LR concentration in our *Microcystis* experiment, but the MN amendment did increase microcystin-LA concentration in our phytoplankton community HAB bioassay. How MN concentration affects microcystin production should be further explored, especially in freshwater ecosystems where there is little research on how MN individually and collectively influences cyanobacteria community structure and cyanotoxin concentrations (Facey et al., 2019). Our results highlight the complexity of how abiotic variables control biomass and cyanotoxin production in both laboratory populations of *Microcystis* and HABs in the field.

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## Highlights

- Manipulated nitrogen (N) form, N and micronutrient (MN) concentrations in *Microcystis* culture and community HAB bioassay
- *Microcystis* grown in high urea and MN conditions produced the most biomass and particulate N
- Highest microcystin-LR concentrations occurred in high nitrate and MN conditions
- MN concentration did not affect microcystin-LR concentration in *Microcystis* cultures
- N addition stimulated biomass and microcystin concentrations in the community HAB bioassay
- MN amendment increased microcystin-LA concentrations in the community HAB bioassay

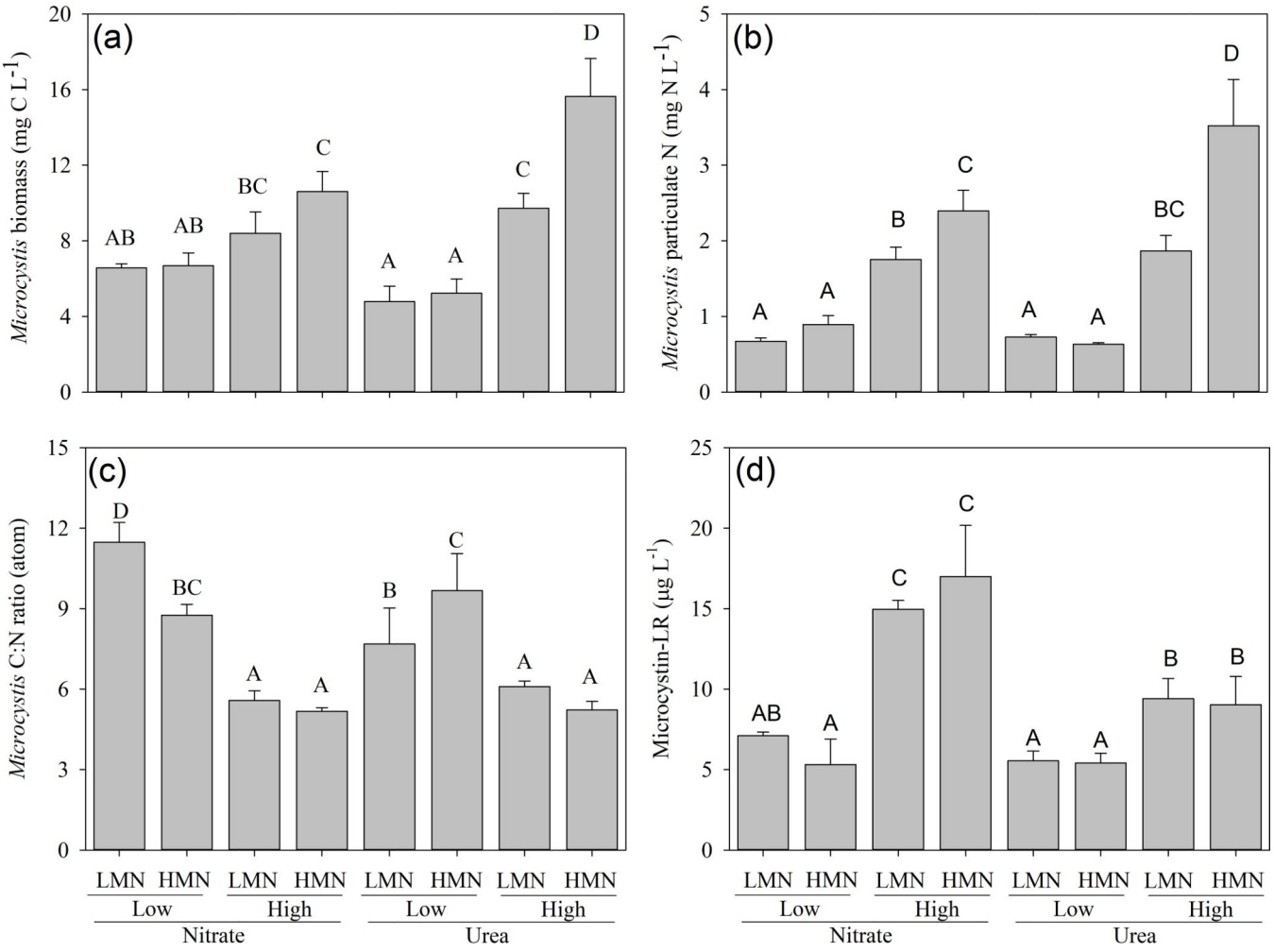


**Figure 1.**

Three way ANOVA results from the *Microcystis* population experiment describing mean differences (plus standard deviation) in nitrogen form (nitrate or urea) and in low (LMN) and high (HNM) micronutrient concentration for (a) particulate carbon (mg C $L^{-1}$) (b) particulate nitrogen (mg N $L^{-1}$), (c) carbon to nitrogen ratio (C:N by atom) (d) microcystin-LR (µg $L^{-1}$). Tukey's post-hoc significant differences indicated by different letters.

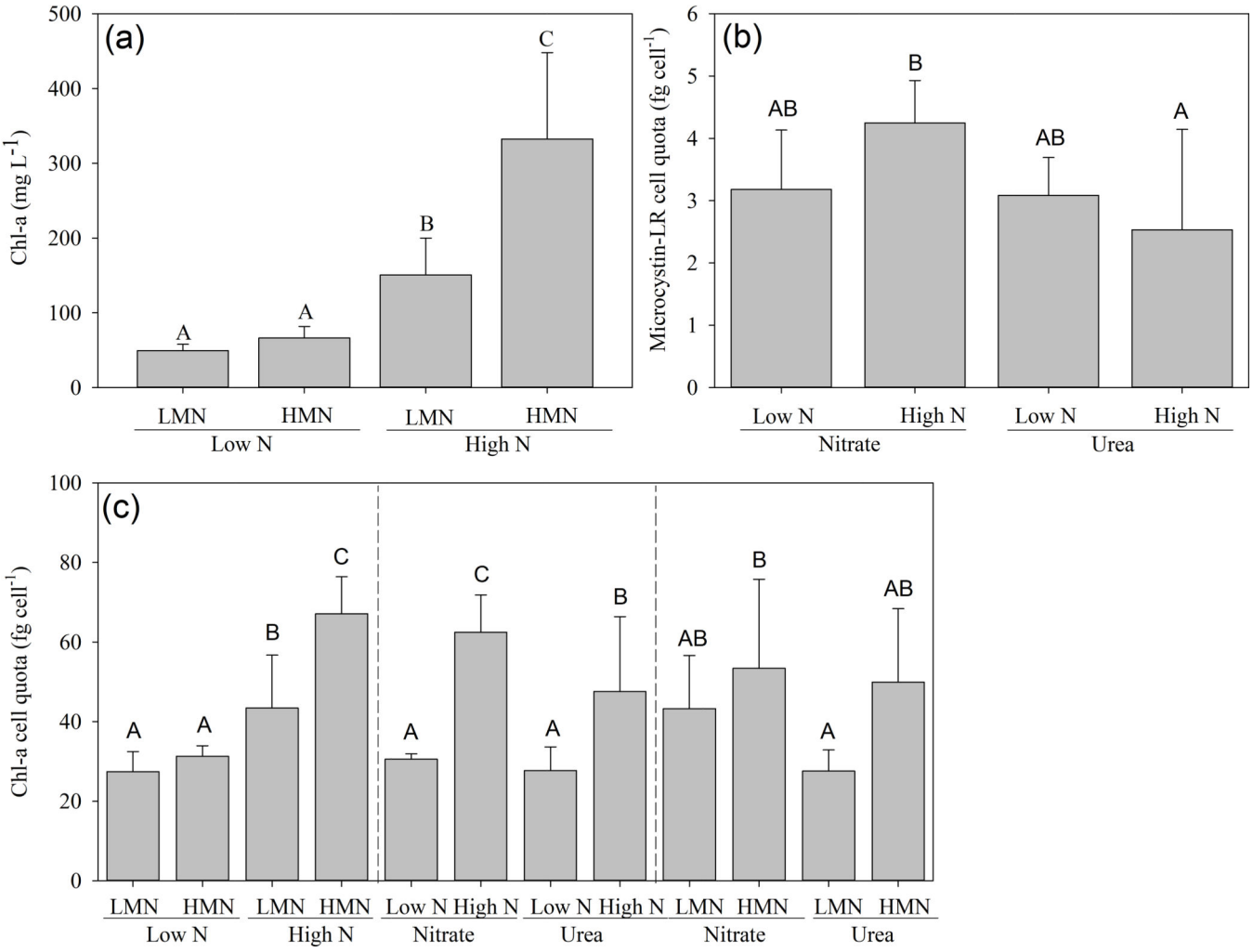


**Figure 2.**

Two way ANOVA results from *Microcystis* population experiment for (a) nitrogen (N) concentration and low (LMN) and high (HMN) micronutrient concentration effects on chlorophyll a concentration (μg $L^{-1}$) (b) form of N and N concentration effects microcystin-LR cell quotas (fg $cell^{-1}$) (c) Two-way interactions separated by vertical dashed lines of N concentration and low (LMN) and high (HMN) micronutrient concentration, N form and N concentration, and N form and low (LMN) and high (HMN) micronutrient concentration effects on chlorophyll-a cell quotas (fg $cell^{-1}$). All data are means plus standard deviation. Tukey's post-hoc significant differences indicated by different letters.

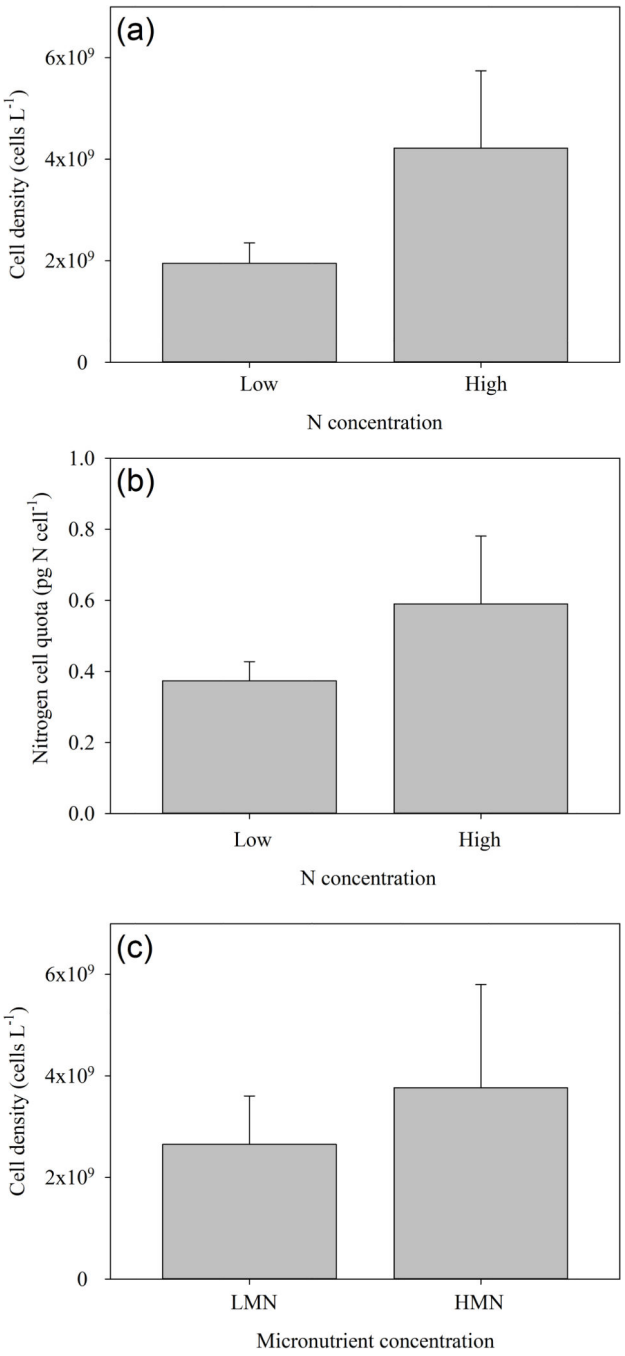


**Figure 3.**

One way ANOVA results from *Microcystis* population experiment for effect of nitrogen (N) concentration on (a) cell density (cells $L^{-1}$) (b) nitrogen cell quota (pg N $cell^{-1}$) (c) effect of low (LMN) and high (HMN) micronutrient concentration on cell density (cells $L^{-1}$). All data are means plus standard deviation.

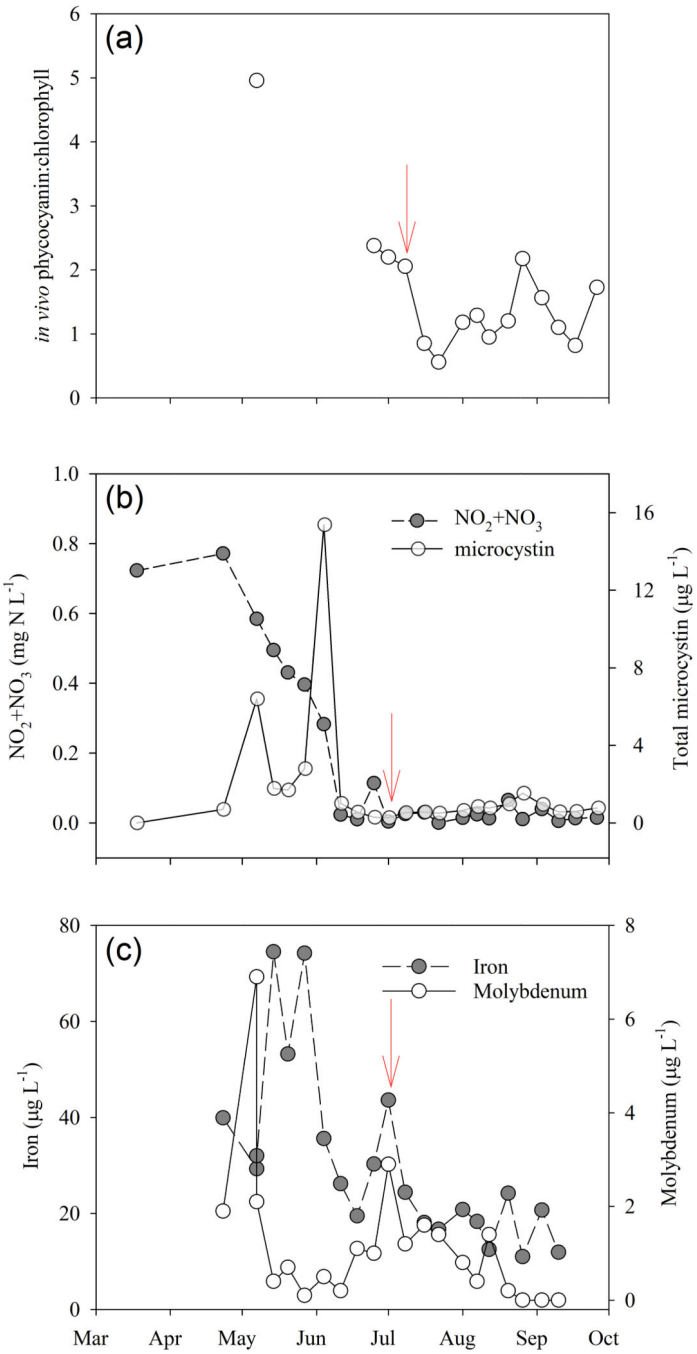


**Figure 4.**

Temporal dam sampling of (a) *in vivo* phycocyanin:chlorophyll:(b) dissolved nitrite ($NO_2$) and nitrate ($NO_3$, mg $L^{-1}$) in dark grey circles and total microcystin (µg $L^{-1}$) (c) dissolved iron (µg $L^{-1}$) in dark grey circles and dissolved molybdenum (µg $L^{-1}$) from March to October for Lake Fayetteville 2019. Arrow in each panel is when water was taken for the community HAB bioassay experiment.

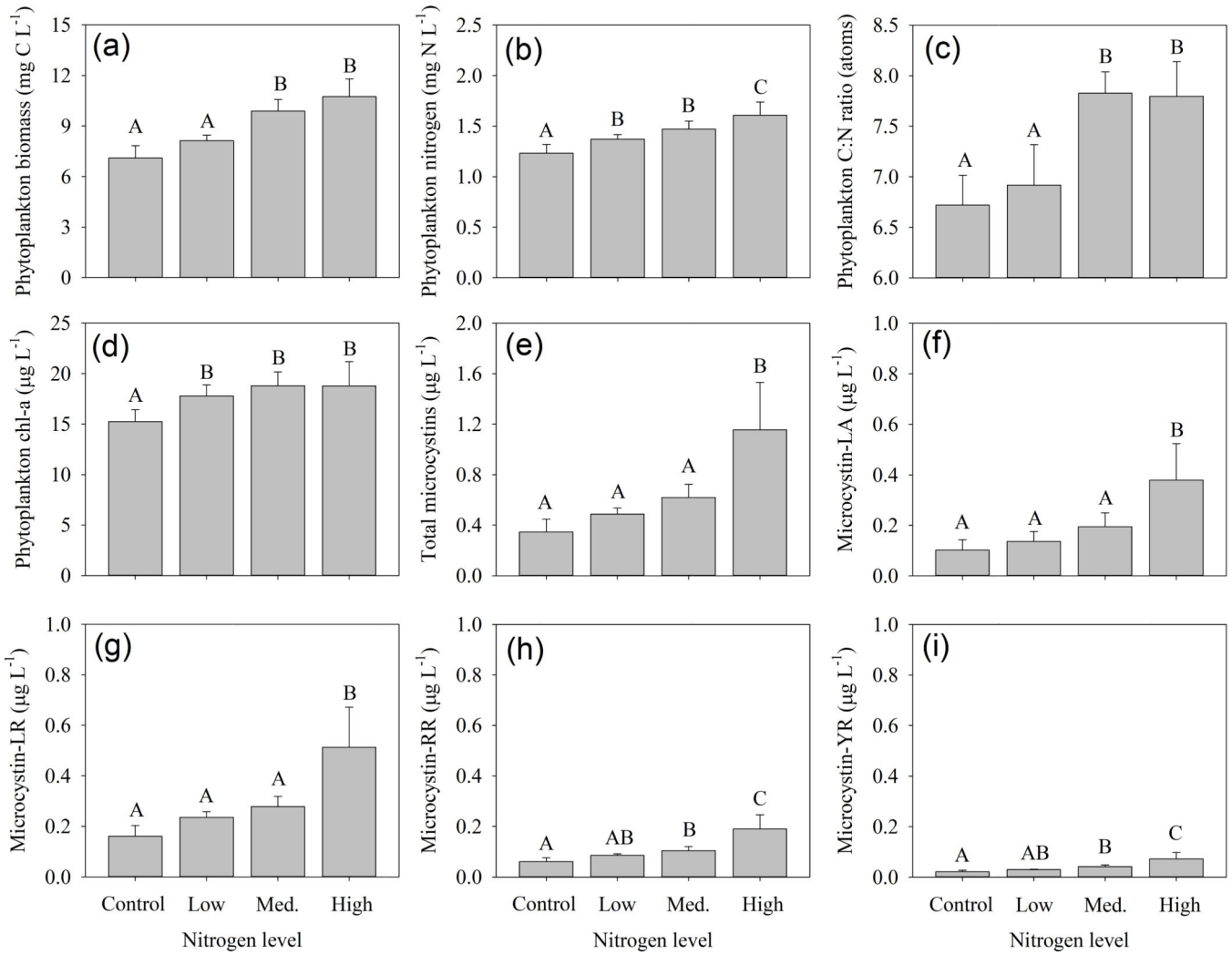


**Figure 5.**

One way ANOVA results from phytoplankton community bioassay showing the effect of N addition (Control; 0.1 mg $L^{-1}$ -N, Low; 0.5 mg $L^{-1}$- N, Med; 1 mg $L^{-1}$-N, High) on (a) particulate carbon (mg $L^{-1}$), (b) particulate nitrogen (mg $L^{-1}$), (c) carbon to nitrogen ratio (C:N by atom), (d) chlorophyll a (Chl-a µg $L^{-1}$), (e) total microcystin (µg $L^{-1}$), (f) microcystin-LA (µg $L^{-1}$), (g) microcystin-YR (µg $L^{-1}$), (h) microcystin-LR (µg $L^{-1}$), and (i) microcystin-RR (µg $L^{-1}$). All data are means plus standard deviation.

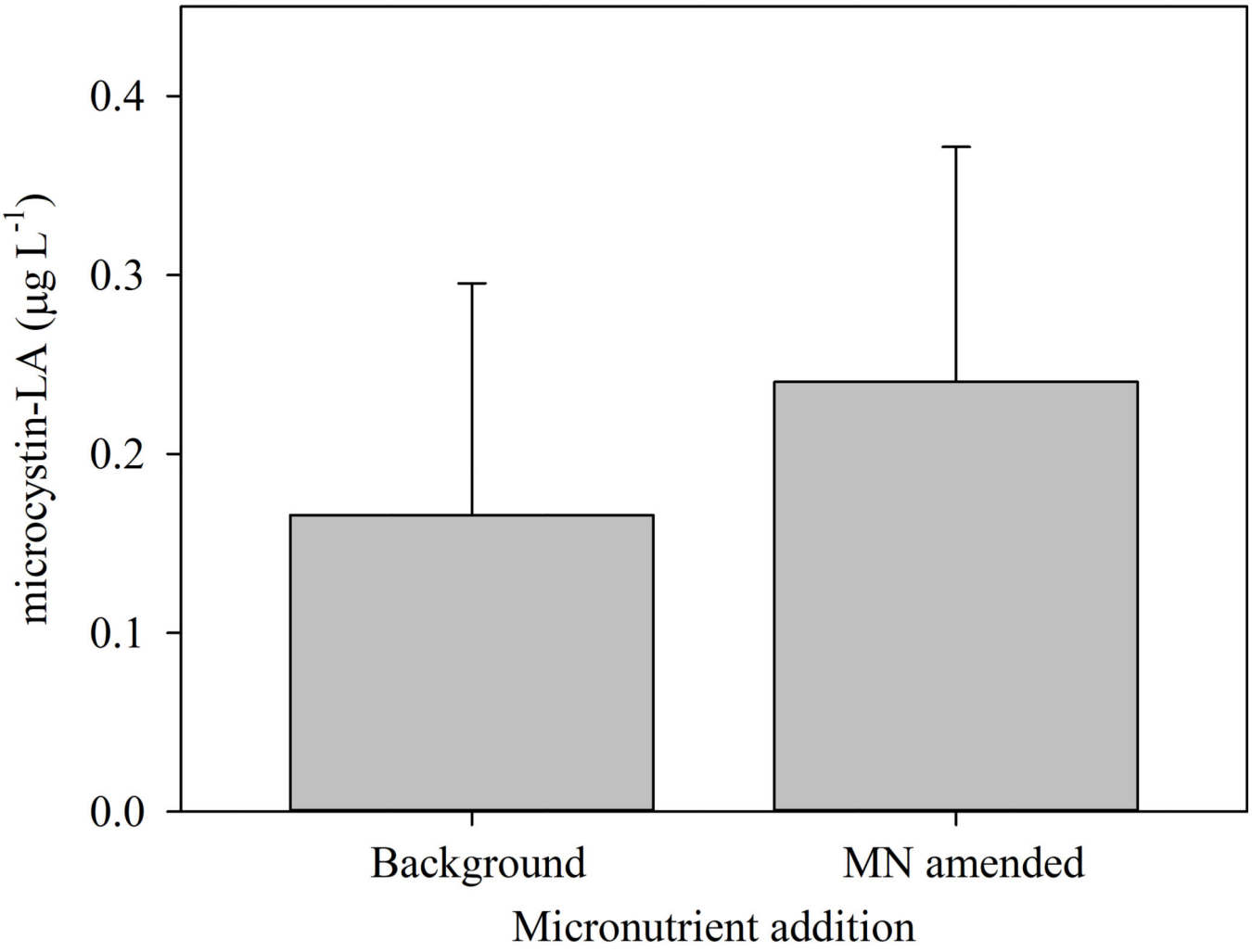


**Figure 6.**

One way ANOVA results from phytoplankton community bioassay showing the effect of background MN and MN amended micronutrient concentration on microcystin-LA (µg $L^{-1}$). All data are means plus standard deviation. See table 2 for background and amended MN concentrations.

**Table 1:**

Micronutrient (MN) concentration in the low and high MN treatments

| Micronutrient | Compound | High MN (μg $L^{-1}$) | Low MN (μg $L^{-1}$) |
|---|---|---|---|
| Fe | $(NH_4)_5[Fe(C_6H_4O_7)_2]$ | 59.6 | 3.66 |
| B | $H_3BO_3$ | 24.9 | 1.55 |
| Mn | $MnCl_2 \cdot 4H_2O$ | 27.0 | 1.69 |
| Zn | $ZnSO_4 \cdot 7H_2O$ | 2.52 | 0.157 |
| Mo | $Na_2MoO_4 \cdot 2H_2O$ | 7.68 | 0.480 |
| Cu | $CuSO_4 \cdot 5H_2O$ | 0.983 | 0.061 |
| Co | $Co(NO3)_2 \cdot 6H_2O$ | 0.501 | 0.031 |

**Table 2:**

Micronutrient (MN) concentration in Lake Fayetteville on day the community HAB bioassay was collected. Concentrations of the MN amended to the Lake Fayetteville community HAB bioassay and the resulting MN concentrations

| Micronutrient | Lake Fayetteville MN background (μg $L^{-1}$) | MN amended (μg $L^{-1}$) | Total MN in amended (μg $L^{-1}$) background + amended |
|---|---|---|---|
| Fe | 43.6 | 59.6 | 103.2 |
| B | 28.5 | 24.9 | 53.4 |
| Mn | 5.5 | 27.0 | 32.5 |
| Zn | 11.1 | 2.52 | 13.62 |
| Mo | 2.9 | 7.68 | 10.5 |
| Cu | 3.1 | 0.983 | 4.08 |
| Co | 4.8 | 0.501 | 5.30 |

**Table 3.**

P-values of the three-way Analysis of Variance (ANOVA) from the laboratory culture experiment using *Microcystis aeruginosa* (LE3).

| Variable | N form | [N] | [MN] | N form x [N] | N form x [N] | [N] x [MN] | N form x [N] x [MN] |
|---|---|---|---|---|---|---|---|
| Cell density (cells $L^{-1}$) | 0.6072 | **<0.0001** | **0.0092** | 0.0996 | 0.4588 | 0.1089 | 0.0810 |
| Particulate carbon (mg $L^{-1}$) | **0.0413** | **<0.0001** | **<0.0001** | **<0.0001** | **0.0098** | **<0.0001** | **0.0280** |
| Particulate nitrogen (mg $L^{-1}$) | **0.0087** | **<0.0001** | **<0.0001** | **0.0005** | 0.0701 | **<0.0001** | **0.0011** |
| C:N (mol) | **0.0346** | **<0.0001** | 0.0649 | **0.0026** | **0.0003** | 0.6080 | **<0.0001** |
| Chlorophyll a (μg $L^{-1}$) | 0.4890 | **<0.0001** | **<0.0001** | 0.9217 | 0.0873 | **0.0005** | 0.0774 |
| Microcystin-LR (μg $L^{-1}$) | **<0.0001** | **<0.0001** | 0.8859 | **<0.0001** | 0.6971 | 0.0746 | **0.0454** |
| C cell quota (pg $cell^{-1}$) | 0.6719 | 0.5362 | 0.9587 | 0.0707 | 0.1835 | 0.1854 | 0.3852 |
| N cell quota (pg $cell^{-1}$) | 0.2590 | **<0.0001** | 0.3399 | 0.4897 | 0.8374 | 0.0977 | 0.4484 |
| Chl-a cell quota (fg $cell^{-1}$) | **<0.0001** | **<0.0001** | **<0.0001** | **0.0010** | **0.0012** | **<0.0001** | 0.3549 |
| MC-LR cell quota (fg $cell^{-1}$) | **0.0303** | 0.2970 | 0.0554 | **0.0138** | 0.3287 | 0.3790 | 0.1897 |

**Table 4.**

P-values of the two-way Analysis of Variance from community HAB Lake Fayetteville bioassay.

| Variable | [N] | [MN] | [N] x [MN] |
|---|---|---|---|
| Particulate carbon (mg $L^{-1}$) | | 0.5341 | 0.8497 |
| Particulate nitrogen (mg $L^{-1}$) | **<0.0001** | 0.4665 | 0.7336 |
| C:N (mol) | **<0.0001** | 0.7690 | 0.3042 |
| Chlorophyll a (μg $L^{-1}$) | **0.0003** | 0.6145 | 0.1807 |
| Total Microcystin (μg $L^{-1}$) | **<0.0001** | 0.1291 | 0.6892 |
| Microcystin-LA (μg $L^{-1}$) | **<0.0001** | **0.0102** | 0.7646 |
| Microcystin-YR (μg $L^{-1}$) | **<0.0001** | 0.2830 | 0.4681 |
| Microcystin-LR (μg $L^{-1}$) | **<0.0001** | 0.5427 | 0.7122 |
| Microcystin-RR (μg $L^{-1}$) | **<0.0001** | 0.2048 | 0.3949 |