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Cyanobacterial and Microcystin Response to Nutrient Additions at Lake Fayetteville throughout the 2021 Growing Season

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Abstract

Harmful Algal Blooms (HABs) are becoming a global concern due to their increasing distribution, frequency, intensity, and the occurrence of toxins. While it is known that eutrophication influences algal blooms, there is less known about what triggers these HABs to produce toxins, especially microcystin. In this study, we conducted 21 community bioassays at Lake Fayetteville, a hypereutrophic reservoir in Fayetteville, Arkansas, from April-November 2021 to examine how the addition of phosphorous and nitrogen influence cyanobacteria concentrations, microcystin concentrations, and microcystin toxin production. These experiments included a control, nitrogen (1.0 mg/L as KNO_3), low phosphorus (0.025 mg/L as K_2HPO_4), high phosphorus (0.100 mg/L) and nitrogen plus low and high phosphorus treatments with four replicates, where the treatments were incubated in a chamber at temperature representing lake conditions below the surface and a light intensity of 140 µmol/L with 14 hours light and 10 dark. These bioassays took place throughout the nitrate decline and well into the growing season when dissolved nutrients are not readily available in the surface water. We found that cyanobacterial and algal growth were limited by nutrients, but the relative importance of N and/or P varied over time. Additionally, we found that seasonal variations in cyanobacteria and nutrient supply influenced microcystin concentration and production. Finally, while we did show that it was possible for nutrients to stimulate toxin production, for the majority of the bioassays we only stimulated algal growth and did not increase toxin production on a cellular basis.

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1. Introduction

Harmful algal blooms (HABs) are becoming a global concern due to their increasing distribution, frequency, and the intensity of toxins. In freshwaters, blue-green algae, or cyanobacteria, can produce HABs and algal toxins. These organisms are known to be highly adaptable to geochemical, climatic, and anthropogenic changes (Paerl and Otten, 2013). For instance, cyanobacteria are often more dominant than other phytoplankton when competing for resources due to their ability to regulate their buoyancy in water, store nutrients, and fix atmospheric nitrogen (Ibelings et al., 2021). Cyanobacterial HABs are a nationwide concern, having been documented in every state (NRDC, 2019). In the USA, HABs affect drinking water, tourism, aquatic life, food web dynamics, and human and animal health (EPA, 2015). Cyanobacterial blooms create issues of water clarity and quality (Brooks et al., 2016), can cause oxygen depletion which negatively impacts aquatic life (Jacoby et al., 2000; Paerl, 1988), and can produce toxins which are harmful to human and animal health (Paerl and Otten, 2013).

The major genera of cyanobacterial HABs that produce toxins are *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Microcystis*, and *Planktothrix* (Paerl and Otten, 2013). Toxins are generally released into water bodies following a bloom's death and senescence (Jacoby et al., 2000). Cyanotoxins (e.g., hepatotoxins, neurotoxins, cytotoxins, and dermatotoxins) can be both acutely and chronically toxic, which pose serious risks for human and animal health (Bláha et al., 2009). Cyanotoxin concentrations and production can vary in frequency between bloom events and spatially within a single bloom (van Apeldoorn et al., 2007). Microcystin, which is produced by many cyanobacteria, is the most frequently observed cyanotoxin across the USA and globally (Wood et al., 2015).

Cyanobacterial HABs threaten large water bodies in the United States such as Lakes Erie and Michigan in the northern U.S., the Chesapeake Bay on the mid-Atlantic coast, and Lake Okeechobee in southern Florida (Paerl et al., 2011). Small inland lakes and ponds, while often not as commonly studied as larger reservoirs (Downing, 2010), are also impacted by cyanobacterial HABs. For example, in a 2015 study, 10 of 24 routinely monitored small (< 0.5 km^2) water bodies in central Ohio showed measurable concentrations of microcystin (Mrdjen et al., 2018). Cyanobacterial HABs in small and large reservoirs may increase in occurrences with climate change, as climate change creates the ideal conditions for cyanobacterial blooms (e.g., increased water temperatures and nutrient inputs from episodic but extreme rainfall runoff events) (Wood et al., 2015).

Toxin production in cyanobacterial HABs is often triggered by the interactive effects of hydrologic, climatic, and temperature conditions such as warmer air and surface water temperature, light availability, residence time, and stratification (Paerl and Paul, 2012). For instance, cyanobacterial HABs often occur and have their maximum growth rates with warmer water temperatures (> 25 ºC; Paerl and Otten, 2013). Blooms consume CO_2 during photosynthesis which in turn increases the water column pH to alkaline conditions (i.e., pH > 9) which may play a role in cyanobacterial succession and a completive disadvantage for diatoms (Zepernick et al., 2021). Toxin production is influenced by light availability, where maximum production has been recorded at high light intensities (between 31-68 µmol of photons m^{-2} s^{-1}; Kaebernick et al., 2000), which generally occur about 1m below the surface during an algal bloom (Walsh et al., 1997). Hydrologic effects such as lake residence times and intense vertical stratification can create ideal growth conditions for cyanobacterial HABs, because of their ability to control buoyancy and overcome the mixing forces (Ibelings et al., 2021).

Nutrients are the main drivers in algal blooms, including cyanobacterial HABs. In general, eutrophication is accelerated by increasing nitrogen (N) and phosphorous (P) inputs from both point sources and non-point sources, such as effluent, atmospheric deposition, and runoff from the landscape. In nutrient enriched lakes, cyanobacteria can often make up a large share of total phytoplankton biomass throughout the growing season (Ibelings et al., 2021). Cyanobacterial HABs often dominate water bodies with an N:P ratio <15 (molar), whereas N:P ratios of > 20 are likely eukaryotic algal taxa (Paerl et al., 2001). Moreover, greater microcystin concentrations are often observed when TN:TP is within 15 and 20 (Scott et al., 2013). The prominent taxa in cyanobacterial HABs, for instance *Microcystis*, and toxin production may also be influenced by N form (e.g., nitrate, ammonium, and urea) to support bloom and toxin growth (Andersen et al., 2020; Paerl et al., 2016; Scott et al., 2013), however some studies suggest that only N supply, not N form increased microcystin biomass and toxin concentration (Wagner et al., 2021).

Nutrient limitation patterns of algal blooms and even cyanobacterial HABs in hypereutrophic reservoirs vary seasonally with the availability of nutrients and the timing and quantity of nutrient inputs (Andersen et al., 2020; Maberly et al., 2020). In the winter and early spring, the supply of dissolved N is greatest which typically results in algal growth in the reservoir to be P limited. Then, during the summer months, algal growth in eutrophic systems tends to be co-limited by both N and P (Paerl et al., 2011). When the lakes start to mix in the fall, algal growth in reservoirs returns to P limitation. However, how this seasonal nutrient limitation relates to cyanobacterial HAB concentration and algal toxin production should be researched as it is still not well understood.

While studies have examined the effects of nutrient loads on HABs (Andersen et al., 2020; Barnard et al., 2021; Guildford and Hecky, 2000; Li et al., 2014; Wagner et al., 2021), there is still uncertainty of how nutrient dynamics affect cyanobacterial biomass, toxin concentrations, and toxin production. To gain further insights into the relationships between nutrient dynamics and microcystin in cyanobacterial HABs, we conducted 21 bioassays throughout the 2021 growing season at a small reservoir in Fayetteville, Arkansas. The specific objectives of the bioassays were to: 1) determine the effect of nutrient addition on measured chlorophyll-a pigment (CHL-a), 2) to evaluate the effect of nutrient addition on total microcystin concentration in cyanobacteria, and 3) identify the role that nutrient addition plays in toxin production by answering the question: did we increase microcystin toxin production or just increase cyanobacteria growth? This study provides further investigation of nutrient dynamics as a driver of HABs and toxin production in hypereutrophic freshwater reservoirs.

1.1 Study Site

Lake Fayetteville is a hypereutrophic reservoir in northwest Arkansas, U.S.A (36.137092, -94.139794) with a surface area of < 1 km^2 and a max depth of 15 m (Figure 1). Lake Fayetteville was constructed in 1949 as a drinking water source, but since 1959, the lake and its surrounding parks and trails have been used for recreational purposes such as boating, fishing, hiking, and biking. Clear Creek is the only perennial input into Lake Fayetteville and it drains 24.2 km^2 with a watershed land use of 3% wetlands and water, 8.2% forest, 38.5% urban, and 50.3% agriculture, estimated by Model My Watershed (Stroud Water Research Center, 2021). Lake Fayetteville discharges from the reservoir surface, where Clear Creek continues downstream.

The dissolved N supply at Lake Fayetteville varies seasonally (Grantz et al., 2014). Dissolved nitrogen peaks during winter (e.g., November and December) and is diminished during summer months. However, particulate N (PN) and total phosphorous (TP) concentrations peaked in the late summer months then declined in the winter months (Grantz et al., 2014). Historically, this pattern has occurred at least for the past 50 years (Meyer, 1971). Historic dissolved nitrate-N (NO_3-N) ranged from 1.30-1.60 mg/L in the winter to 0.11 mg/L in the summer (Meyer, 1971). Lake Fayetteville retains ~90% of P inputs from the watershed, but N accumulation is negligible when denitrification is considered (Grantz et al., 2014). This suggests that a lot of the P is stored in bottom sediments, which can be released via equilibrium exchange (Austin et al., 2020) and during reductive dissolution when the hypolimnion is anoxic (Haggard et al., 2021).

The phytoplankton community has included cyanobacteria, which can produce microcystin, since 1968 (Meyer, 1971). In recent years, monitoring studies have been conducted at Lake Fayetteville to understand lake nutrient and HAB dynamics (Ferri et al., 2022; Haggard et al., 2022) . In 2019, microcystin concentrations in the lake increased during late spring, reached a maximum of 16 µg/L in early June, then decreased rapidly to between 0.5 and 1 µg/L for the rest of the growing season (Wagner et al., 2021).

Figure 1: Aerial Map of Lake Fayetteville, in Fayetteville, Arkansas with sampling location marked. Map imagery from Google Maps.

2. Methods

2.1 Water Collection, Sampling, and Analysis

We performed 21 nutrient bioassays throughout the growing season (April to November 2021) by collecting lake water from the edge of the marina boat dock at Lake Fayetteville (Figure 1). Lake water was collected in a 20 L plastic carboy to be used for the bioassays and three additional 1 L samples were collected in acid washed containers to measure initial physico-chemical, nutrients, and various algal properties. The lake water samples were analyzed for chlorophyll-a raw fluorescence units (CHL RFUs), phycocyanin raw fluorescence units (PC RFUs), the phycocyanin to chlorophyll-a raw fluorescence ratio (PC:CHL), chlorophyll-a (CHL-a) pigments, total microcystin concentration, total phosphorous (TP), soluble reactive phosphorous (SRP), ammonia (NH_4-N), nitrate plus nitrite (hereafter, NO_3-N), and total nitrogen (TN). Samples were also measured for pH (Oakton pH Testr 30+ Waterproof Pocket Tester), water temperature, conductivity, and dissolved oxygen (DO; YSI ProSolo Digital Water Quality Meter) on site.

All water samples were analyzed at the certified water quality lab with the Arkansas Water Resources Center (https://awrc.uada.edu/). We measured CHL RFUs, PC RFUs, and the PC:CHL ratio using a Turner Designs CyanoFluor Handheld HAB Indicator. CHL-a pigment samples were filtered through a 0.7 µm membrane filter, stored at -18 °C in aqueous acetone, and analyzed using a Turner Designs Fluorometer (APHA Method 10200 H3). Unfiltered water samples (20 mL) for total microcystin underwent three freeze-thaw cycles to lyse cells and were analyzed using Enzyme-Linked Immunosorbent Assays (ELISA) kits on an Abraxis Plate Reader (EPA Method 546). SRP (EPA Method 365.1), NO_4-N (EPA Method 351.2), $NO_3N + NO_2$-N (EPA Method 353.2), TN (APHA Method 4500-P J; EPA Method 353.2), and TP (APHA Method 4500-P J; EPA Method 365.1) were measured using a Skalar San++ System Wet Chemistry Autoanalyzer.

2.2 Lake Fayetteville community HAB bioassays

The carboy full of lake water was brought back to the lab and was continuously mixed while 700 mL of homogenized lake water was distributed into 1 L acid washed glass media bottles. Each bioassay had six nutrient addition treatments, 1) control (no nutrient addition), 2) NO_3-N, N (1.0 mg/L), 3) low phosphate (PO_4-P), LP (0.025 mg/L), 4) low PO_4-P plus NO_3-N, LPN, 5) high PO_4-P, HP (0.1 mg/L), and 6) high PO_4-P plus NO_3-N, HPN. There were four replicates per treatment, resulting in 24 experimental units per bioassay.

We placed the bottles in the incubator (VWR, model VRI6P) in a randomized block design, which was set at the average surface water temperature (0.05 m below surface) the day the water was collected. The incubator had a minimum temperature of 15 ºC, so if surface water temperature was less than 15 ºC, then incubations were at the minimum temperature. The incubator had an LED panel lamp (Werker Lamps, model FIX12539) with a set light intensity of

140 µmol m^2/s on a 14h:10h light:dark cycle. The incubation period was between 5 to 7 days, depending on the community biomass growth rate measured as CHL and PC RFUs using the CyanoFluor. The bottles were gently mixed on various days throughout the experiment. On the last day of the incubation period, the water in the bottles were processed to measure physico-chemical and algal properties as previously described. We focused on the variables CHL-a and total microcystin in this study.

2.3 Statistical Analysis

Correlations between initial lake sample parameters were determined through linear regression with a significance level of 0.05 ($\alpha = 0.05$) in Excel. The effect of nutrient treatments was analyzed using analysis of variance (ANOVA), and treatment means were separated using least significant difference (LSD) with a significance level of 0.05 ($\alpha = 0.05$) in R (version 1.4.1717). The data from the bioassays were log-transformed prior to ANOVA since these variables are bound by zero and typically showed log-normal distributions. The graphical data are shown as the actual values, whereas the letters separating means were from the ANOVA LSD with log transformed data.

3. Results

3.1 Lake Fayetteville Water Quality

The surface water temperature at Lake Fayetteville steadily increased from 17.8 °C at the beginning of the study (April 15, 2021) into the growing season (Figure 2a). The maximum water temperature just below the surface peaked at 33.0 °C during mid-summer (July 26, 2021), and then decreased into the fall. There were two slight increases in temperature in mid-

September and then late October. Then, the last sampling date had the least surface water temperature of 14.1 °C (November 3, 2021).

Lake Fayetteville's dissolved nutrient supply near the water surface varied over the growing season (Figure 2). NO_3-N concentration was greatest at the beginning of the growing season (April 15, 2021; 0.724 mg/L), then steadily decreased through June (June 23, 2021; 0.066 mg/L); there was one decrease and then increase in NO_3-N in mid-May (Figure 2b). NO_3-N concentration remained low (mean of 0.017 mg/L) from July through mid-October when concentrations started to increase. When the study ended, NO_3-N had a concentration of 0.274 mg/L (November 3, 2021). NH_4-N was variable at the beginning of the study, peaking at 0.36 mg/L on May 27, 2021 (Figure 2c). Then, NH_4-N sharply decreased following this sampling date and, after July 12, 2021, maintained concentrations less than the method detection limits (MDL; $\leq$ 0.014 mg/L) until early October. On this sampling date, NH_4-N began to drastically increase (0.47 mg/L; October 8, 2021) until November. When the study ended, NH_4-N had a concentration of 0.71 mg/L (November 3, 2021). SRP concentrations were low at the beginning of the sampling period (April 15, 2021; 0.001 mg/L) and then increased to 0.04 mg/L on May 6, 2021 (Figure 2d). However, the SRP concentration decreased in late May, and after June 4, 2021, remained below MDL ($\leq$ 0.005 mg/L) for the rest of the study.

TN concentration at the beginning of the study was 1.26 mg/L (April 15, 2021) but quickly decreased to 0.63 mg/L on April 27, 2021 (Figure 2e). Then, concentrations increased to the greatest TN value over the growing season of 2.6 mg/L (May 18, 2021), sharply decreased, then increased again back to 2.5 mg/L (June 4, 2021). After this spike in TN, the concentrations fell to an average of 0.89 mg/L until fall, where the TN concentration began to increase once again until the end of the sampling period (1.54 mg/L; November 3, 2021).

TP concentration at the beginning of the study was 0.029 mg/L (April 15, 2021), but two weeks later it oddly decreased to 0.002 mg/L (April 27, 2021; Figure 2f). Then, TP increased to approximately 0.078 mg/L in early May, decreased again, then sharply increased to its greatest concentration of the growing season of 0.185 mg/L on June 4, 2021. Following this sampling date, TP concentrations decreased and stayed around 0.032 mg/L until late October. The TP concentration at the end of the study at Lake Fayetteville increased slightly to 0.043 mg/L. During this study, TP was strongly correlated with TN ($r = 0.78$, $p < 0.0001$).

PC RFUs were variable throughout the growing season, with the greatest values observed during the spring and early summer months (Figure 2g). PC RFUs increased from the first sampling date (334 RFUs; April 15, 2021) to the greatest value of 20,323 RFUs on May 18, 2021. There is missing RFU (PC, CHL, and PC:CHL ratio) data from the May 6, 2021, sampling date due to routine instrument maintenance. PC RFUs sharply decreased, increased again to 9919 RFUs (June 4, 2021), then decreased to 2076 RFUs on June 14, 2021. PC RFUs increased from this sampling date until September 23, 2021 (7758 PC RFUs), then decreased through the end of the study (2428 PC RFUs; November 3, 2021).

Throughout the study, Lake Fayetteville had CHL RFUs with a generally positive trend ($r = 0.65$), whereas there were fluctuations between sampling dates (Figure 2h). CHL RFUs at the beginning of the study were 1042 RFUs (April 15, 2021). The RFUs fluctuated between approximately 700 RFUs and 2600 RFUs from the beginning of the study until June 30, 2021. Then, CHL RFUs increased to 6716 RFUs (July 12, 2021), decreased on the next sampling date, and then increased back up to 6599 RFUs (August 18, 2021). Between September 9, 2021, and October 8, 2021, CHL RFUs averaged 3957 RFUs with fluctuations. October 15, 2021, had the greatest CHL RFU value of 9253 RFUs.

The PC:CHL ratio was 0.37 at the beginning of the study (April 15, 2021), but then had increased to the greatest ratio value of 7.8 on May 15, 2021 (Figure 2i). After this sampling date, the PC:CHL ratio decreased to 1.3 (May 27, 2021), but then increased to 4.2 the next week (June 4, 2021). The PC:CHL ratio then steadily decreased to 0.59 (July 12, 2021) and following this sampling date remained around a mean of 1.4 from late July to early October. Then, the PC:CHL ratio began to decrease; at the end of the study period, the ratio was 0.32 (November 3, 2021).

Lake Fayetteville was considered hypereutrophic during the 2021 growing season, with the majority of sampling dates having CHL-a concentrations greater than 20 mg/L (Figure 2j). CHL-a was least at the beginning of the study (April 15, 2021, 3.36 µg/L), then sharply increased to over 140 µg/L on May 5, 2021. After this spike in CHL-a, the system crashed (May 27, 2021; 13.8 µg/L) then increased again to 69.7 µg/L on June 4, 2021. Lake Fayetteville maintained a relatively high CHL-a concentration for the rest of the sampling period (mean of 33.6 µg/L). CHL-a pigment had a strong, positive correlation with PC RFUs across the sampling period at Lake Fayetteville ($r = 0.99$, $p = 0.003$). Mean CHL-a pigment concentrations across sampling dates were also correlated to mean TN ($r = 0.67$, $p < 0.001$) and mean TP ($r = 0.48$, $p = 0.026$) during this study.

Lake Fayetteville's total microcystin concentration was least at the beginning of the study (0.055 µg/L; April 15, 2021) then increased to 0.304 µg/L on April 27, 2021 (Figure 2k). There was a decrease in microcystin concentration after this sampling date, but then a gradual increase to the peak microcystin concentration of the growing season (1.047 µg/L, June 4, 2021). After this peak, concentrations decreased, then increased again up to 0.716 µg/L on June 23, 2021, then steadily decreased through July (0.187 µg/L; July 12, 2021). Microcystin concentration remained low (mean of 0.109 µg/L) from late-July through mid-October when concentrations

began to increase once again. At the end of the study period, total microcystin had a concentration of 0.355 µg/L (November 3, 2021). Mean total microcystin concentrations were correlated with TN ($r = 0.45$, $p = 0.039$) and TP ($r = 0.58$, $p = 0.006$) across this study.

Microcystin production at Lake Fayetteville was widely variable during the first two months of the study (Figure 2l). The initial microcystin production was 0.016 µg/µg (April 15, 2021), but by the next sampling date it increased to the greatest microcystin production of the study (0.041 µg/µg; April 27, 2021). Then, production decreased drastically to 0.002 µg/µg (May 18, 2021), but the next week increased back up to 0.038 µg/µg (May 27, 2021). On June 4, 2021, microcystin production decreased to 0.015 µg/µg, then increased throughout the month of June up to 0.025 µg/µg (June 30, 2021). Microcystin production decreased and remained low from mid-July to mid-October (mean of 0.003 µg/µg), but then increased to 0.029 µg/µg on October 22, 2021. Microcystin production decreased to 0.012 µg/µg at the end of the study period (November 3, 2021). Microcystin production was correlated with NO_3-N ($r = 0.65$, $p = 0.0016$) and PC RFUs ($r = -0.46$, $p = 0.039$) during this study.

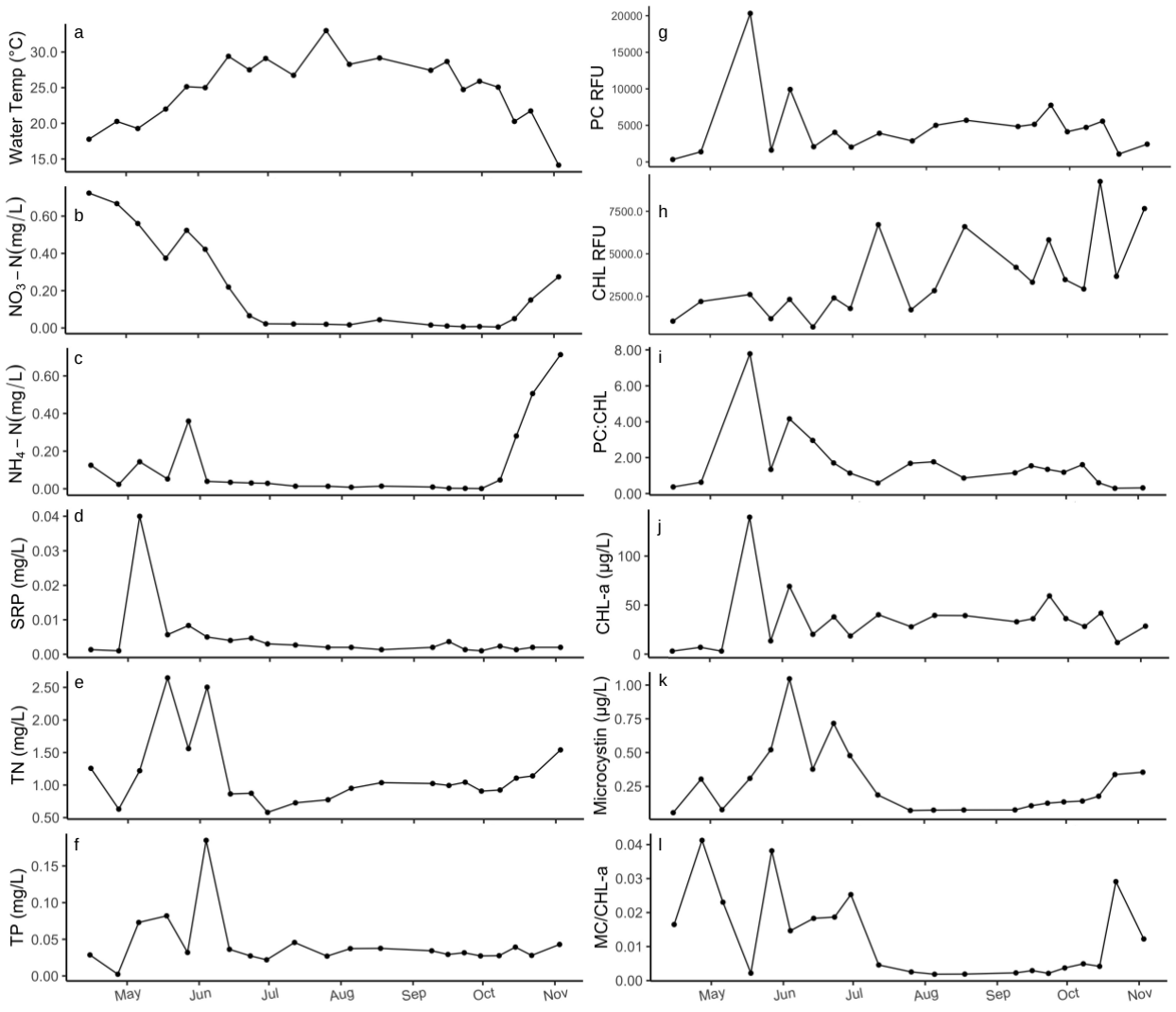

Figure 2. Initial mean conditions just below the water surface from 2021 Lake Fayetteville Bioassay experiment sampling dates; parameters include water temperature, NO_3-N concentration, NH_4-N concentration, SRP concentration, TN concentration, TP concentration, PC RFUs, CHL RFUs, PC:CHL ratio, chlorophyll-a concentration (CHL-a), total microcystin concentration, and microcystin toxin production (MC/CHL-a).

3.2 Lake Fayetteville community HAB bioassays

3.2.1 Effect of Nutrients on Chlorophyll-a Concentrations

Chlorophyll-a concentrations in each bioassay over the study showed three distinct patterns in response to nutrient additions (Figure 3). The first type of response was that CHL-a was P-limited. This response was observed from bioassay 1 (April 15, 2021) through bioassay 7 (June 14, 2021). In these bioassays, the CHL-a in P treatments generally increased relative to the control, while the N treatments did not differ from the control. However, when examining each bioassay individually, the details vary a little bit. For instance, in bioassay 1, CHL-a concentrations in the LP, LPN, HP, and HPN treatments were significantly greater than the control, but not significantly different from each other (mean of 36.1 µg/L). Whereas CHL-a concentrations in bioassays 2 and 4 (mean of 88.4 µg/L and 104.7 µg/L, respectively) were greatest in both HP and HPN treatments; these two treatments were not different. Oddly, in bioassay 7, the greatest CHL-a concentration (129.2 µg/L) was observed in the LPN treatment. The exception to this first type of response was bioassay 3 (May 6, 2021), where the measured CHL-a was not different across any treatments.

The most consistent pattern of CHL-a concentration response to nutrients was N and P co-limitation. This pattern was observed from bioassay 8 (June 23, 2021) through 18 (October 8, 2021). During this period, we observed that CHL-a increased in treatments receiving both N and P relative to the control, suggesting nutrient co-limitation. However, the N and P treatment that had the greatest CHL-a concentration differed by experiment. In bioassays 12, 14, 16, and 17, we observed that the HPN treatment had the greatest CHL-a with respect to the other treatments. In bioassays 8, 9, 10, 13, 15, and 18, the greatest CHL-a response occurred with both LPN and HPN treatments. The exception was bioassay 11 (July 26, 2021) where the HP treatment had the

greatest CHL-a concentration (129.9 µg/L) relative to the other nutrient treatments and the control (33.0 µg/L).

The third type of CHL-a response pattern was where CHL-a concentration response to nutrients was again P-limited. This response shift occurred with bioassays 19 (October 15, 2021) through 21 (November 3, 2021), where we observed that CHL-a concentrations were significantly greater than the control in all P treatments. In bioassay 19, we observed that CHL-a concentration was greatest in the HPN treatment (72.3 µg/L) relative to the control (26.6 µg/L). However, in bioassays 20 and 21, we observed that CHL-a concentrations were greatest in both the HP and HPN treatments, but these treatments did not differ from each other (mean of 73.5 µg/L and 97.8 µg/L for bioassay 20 and 21, respectively).

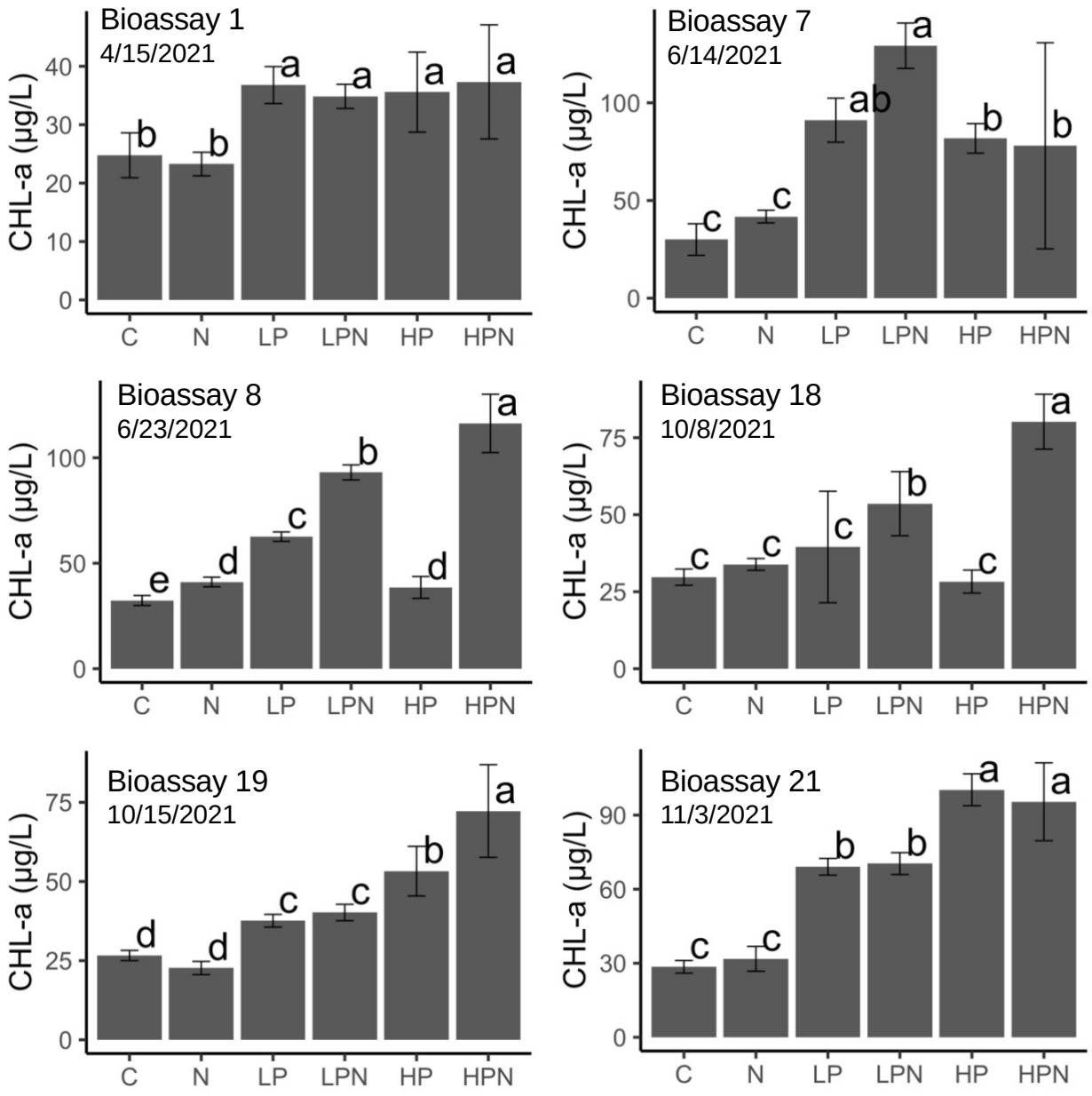

Figure 3. Select mean (± standard deviation) chlorophyll-a (CHL-a) concentrations (µg/L) from 2021 Lake Fayetteville Bioassay experiments; Letters above error bars show means that are significantly different from each other. Treatments include the control (no nutrient addition), nitrogen (N), low P (LP), high P (HP), LPN, and HPN.

3.2.2 Effect of Nutrients on Total Microcystin Concentrations

Total microcystin concentrations were measurable across all bioassays, and the magnitude of the concentration was dependent on that of the lake at the beginning of the incubations. Across all bioassays, we observed four distinct patterns in the response of microcystin to nutrient additions (Figure 4). The first type of response pattern was that there was no significant nutrient affect. This response was observed from bioassay one (April 15, 2021) and ended with bioassay six (June 4, 2021), where these bioassays marked the least (0.055 µg/L) and greatest (1.05 µg/L) initial microcystin concentrations in the lake water. In these bioassays, the general pattern was that while microcystin did increase during the incubation, there was not a significant difference between the control and the nutrient treatments. The exception to this pattern was bioassay 4 (May 18, 2021) where the microcystin concentration in the HP treatment (1.8 µg/L) was significantly greater than the control (1.0 µg/L).

In bioassay 7 (June 14, 2021), we started to see a microcystin response to nutrient additions. The second pattern was a high P stimulation of microcystin. The observed microcystin concentration in the N (0.95 µg/L) and LP (1.2 µg/L) treatments did not differ from the control (1.03 µg/L). However, the rest of the treatments had significantly greater microcystin concentrations relative to the control. The microcystin concentration in the LPN treatment (1.6 µg/L) was greater than the control but not different from the HP treatment (2.0 µg/L). The greatest microcystin concentration was observed in the HPN treatment (3.5 µg/L).

Bioassay 8 (June 23, 2021) showed an unexpected microcystin response to nutrient addition. The control had a mean total microcystin concentration of 1.3 µg/L, but microcystin was highly variable across replicates and it was not different than any nutrient treatments. The total microcystin concentrations in the P treatments (LP and HP) were not significantly different

than the control. However, the addition of N to the P treatments (LPN and HPN) had 36% less microcystin than that in the P treatments alone.

The most consistent microcystin concentration response to nutrients was observed from bioassay 9 (June 30, 2021) through 18 (October 8, 2021). The third pattern was that microcystin response was stimulated by N. During this period, we observed that microcystin increased in any treatments receiving N additions relative to control or P treatments. In select bioassays, the greatest microcystin response occurred with the HPN treatment.

The fourth type of response was P stimulation. Microcystin concentration response to nutrients shifted with bioassays 19 (October 15, 2021) and 20 (October 22, 2021). In bioassay 19, total microcystin concentration was greater in the P treatments than the control (0.58 µg/L); the greatest microcystin concentration was observed in the HPN treatment (1.1 µg/L). However, in bioassay 20, any addition of P increased microcystin concentration relative to the control (0.76 µg/L), but these P treatments (LP, LPN, HP, and HPN) did not differ from each other (mean of 1.1 µg/L).

The last bioassay (21, November 3, 2021) repeated the no nutrient response pattern from the first six bioassays, where nutrient addition did not increase microcystin concentration relative to the control (0.58 µg/L). There were no differences between treatments.

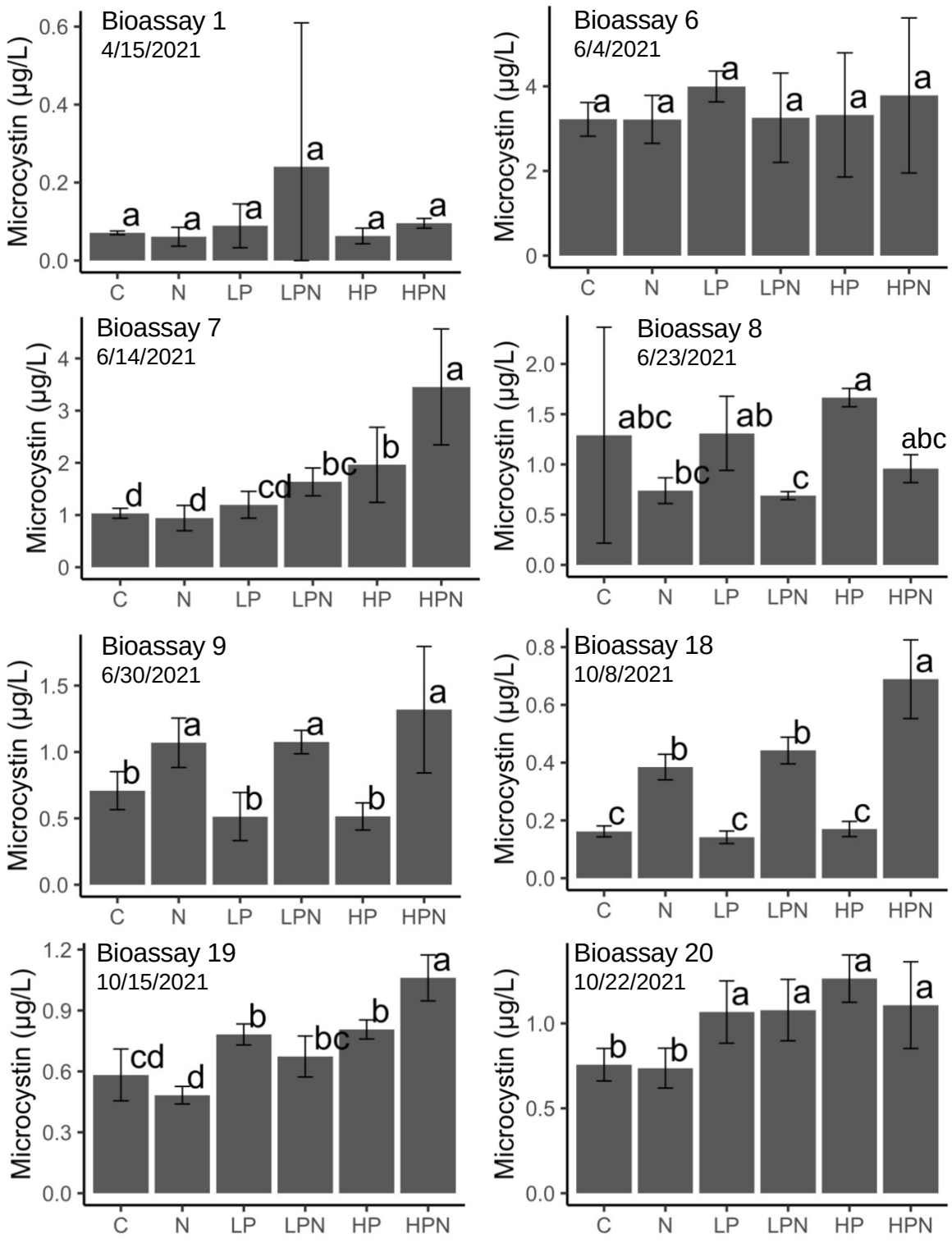

Figure 4. Select mean (± standard deviation) total microcystin concentrations (µg/L) from 2021 Lake Fayetteville Bioassay experiments; Letters above error bars show means that are significantly different from each other. Treatments include the control (no nutrient addition), nitrogen (N), low P (LP), high P (HP), LPN, and HPN.

3.2.3 Effect of Nutrients on the Total Microcystin to Chlorophyll-a pigment Ratio

To see the role that nutrients play in toxin production, we divided the total microcystin concentration by the CHL-a concentration, where CHL-a concentration was a proxy for cyanobacterial and algal biomass. We used this ratio to determine if the amendments increased microcystin production per unit biomass or just increased cyanobacterial growth with intracellular toxins. We observed some interesting patterns in what nutrient treatments stimulated toxin production, i.e., µg microcystin per µg CHL-a or µg/µg (Figure 5).

There were no clear patterns of increases in toxin production with nutrient additions in the first eight bioassays (April 23, 2021, to June 29, 2021), however, there were some important observations. First, microcystin production was generally greatest in the control and decreased with either all nutrient treatments or the high nutrient treatments. For example, microcystin production in bioassay 4 (May 18, 2021) decreased by more than half in the HP and HPN treatments relative to the control (32.3 µg/µg). Another observation was that in some bioassays during this period, there was no difference in microcystin production between the control and nutrient treatments. For example, in bioassays 1 and 3, the microcystin production across all treatments had a mean of 3.34 µg/µg and 1.1 µg/µg, respectively. Finally, we observed that microcystin production in the controls varied by more than an order of magnitude between bioassay 3 (1.2 µg/µg) and 8 (41.4 µg/µg).

In bioassays 9 (June 30, 2021) and 10 (July 12, 2021), microcystin production on a biomass basis was increased by N, but the response varied between bioassays. The microcystin production in the N treatment (65.4 µg/µg) from bioassay 9 was significantly greater than the control (36.7 µg/µg), but it was more than 2x less than the control in all other nutrient treatments. In bioassay 10, while the microcystin production was greatest in the N treatment (7.5 µg/µg), the

microcystin production in the LPN (5.1 µg/µg) and HPN (3.5 µg/µg) treatments were also significantly greater than the control (2.3 µg/µg). The magnitude of microcystin production was much less in bioassay 10 relative to 9.

Between bioassays 11-16 (July 26, 2021- September 23, 2021), we observed that the magnitude of microcystin production was not highly variable in the controls (3.0 to 8.3 µg/µg). There were no consistent responses in either the increase or decrease of toxin production relative to the control between treatments, but microcystin production did not differ between the HPN treatments and the control in each bioassay. On occasion, the microcystin production was significantly increased in the N and LP treatments relative to the controls. For example, in bioassay 11, microcystin production in the N (7.4 µg/µg) and LP (7.6 µg/µg) treatments was significantly greater than the control (4.0 µg/µg), and in bioassay 16, it was almost doubled in the LP treatment relative to the control (9.9 µg/µg).

However, in bioassays 17 (September 30, 2021) and 18 (October 8, 2021), the N treatments clearly stimulated microcystin production on a biomass basis. Toxin production in bioassay 17 was greatest in the N (12.5 µg/µg) and LPN (11.2 µg/µg) treatments relative to the control (6.0 µg/µg); the rest of the treatments did not differ from each other. Whereas in bioassay 18, the microcystin production was greatest in the N treatments (N, LPN, and HPN), though the N treatment alone had the greatest amount of production (11.4 µg/µg) relative to the control (6.0 µg/µg). The toxin production in the P treatments did not differ from the control.

The microcystin production response to nutrients completely shifted with bioassays 19 (October 15, 2021) through 21 (November 3, 2021), where adding nutrients actually reduced toxin production. In bioassay 19, the toxin production in the HP (15.4 µg/µg) and HPN (15.1 µg/µg) treatments was less than the control (21.9 µg/µg), whereas the other treatments did not

differ from the control. In bioassays 20 and 21, the microcystin production was over 2x less in all P treatments relative to the controls (45.3 and 20.3 µg/µg, respectively).

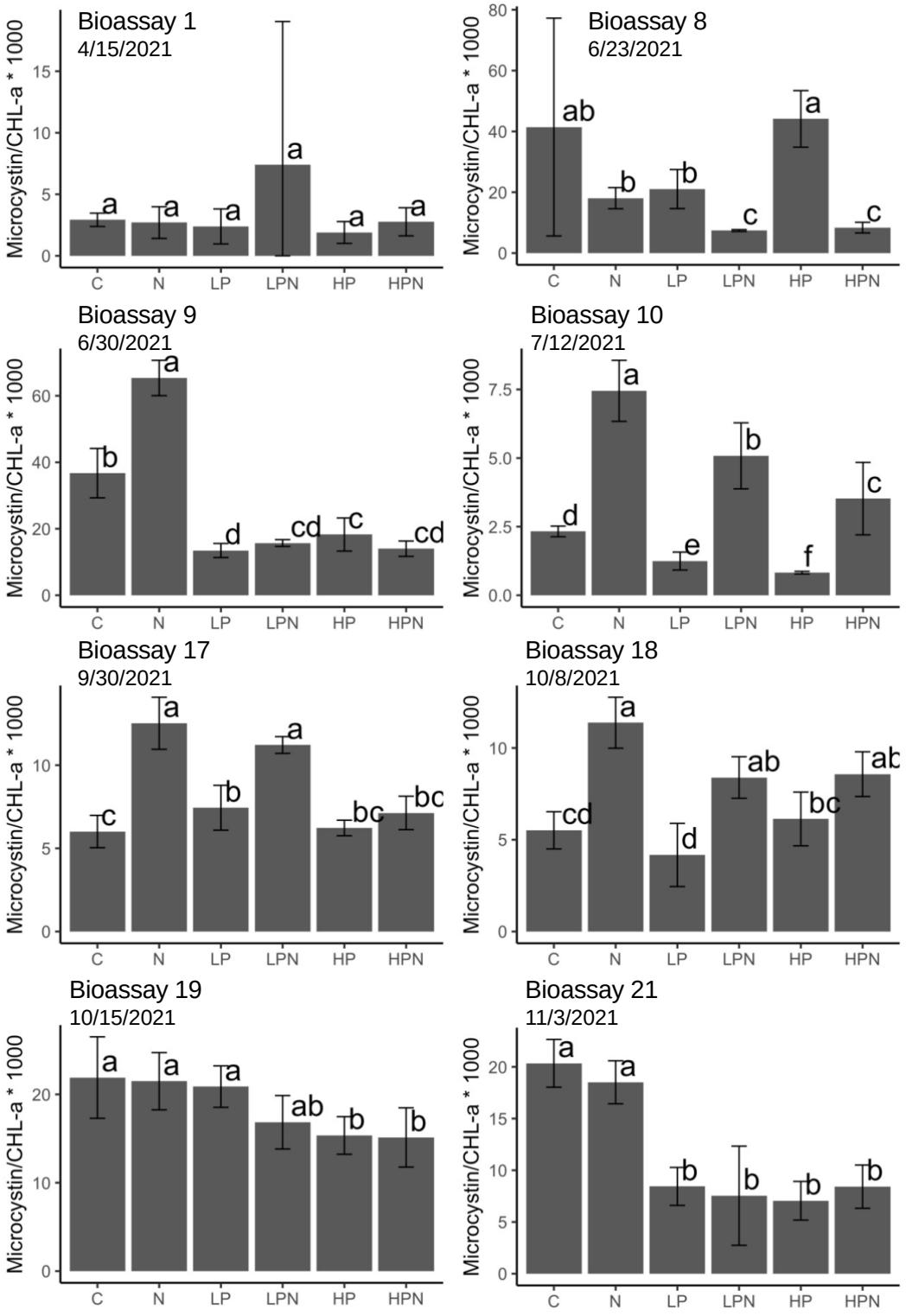

Figure 5. Select mean (± standard deviation) total microcystin to chlorophyll-a pigment ratio (µg microcystin/µg CHL-a) from 2021 Lake Fayetteville Bioassay experiments; Letters above error bars show means that are significantly different from each other. Treatments include the control (no nutrient addition), nitrogen (N), low P (LP), high P (HP), LPN, and HPN.

4. Discussion

4.1 Chlorophyll-a as a proxy for cyanobacterial biomass

Chlorophyll-a concentrations are often used as a proxy for algal biomass (Canfield et al., 2019; Desortová, 1981; Guan et al., 2022). Using our data from the 2021 growing season, we found that CHL-a and PC RFUs were highly correlated ($r = 0.99$, $p = 0.003$). Based on this strong correlation, chlorophyll-a pigment concentration is a good proxy for cyanobacterial biomass, which aided us in calculating toxin production on a cellular or per unit CHL-a basis. This observation is supported in the literature, where the relation between PC RFUs and cyanobacterial biovolume have been explored in both laboratory-cultured cyanobacterial and environmental samples across numerous studies (e.g., see citations above). PC RFUs from a CyanoFluor were shown to be an effective and cost-effective proxy for cyanobacterial biomass and biovolume (Thomson-Laing et al., 2020). Additionally, *in vivo* PC fluorescence have been correlated with cyanobacterial biovolume in cyanobacteria-dominated phytoplanktonic lakes (McQuaid et al., 2011).

While phycocyanin is strongly correlated to cyanobacterial biovolume, this relationship can be species and lake specific (Bertone et al., 2019). This relationship could be further developed for Lake Fayetteville with algal identification but is out of the scope of this project. With this study, we are limited to using the measured CHL-a pigment data from initial lake samples and the bioassay experiments to estimate cyanobacterial and algal biomass. To further investigate community composition of Lake Fayetteville at the time of sampling, lake water samples from bioassays were saved and sent to a colleague for cyanobacteria and species identification analysis using a FlowCam Cyano, but the data are not yet available.

This strong relationship between CHL-a and PC RFUs suggests that within this study period, CHL-a is produced mainly by cyanobacteria. This relationship is further supported by the observation that on a majority of the sampling dates, the PC:CHL RFU ratios were above 1.0. The PC:CHL ratio suggests the abundance of cyanobacteria in the total phytoplankton population (Turner Designs, 2017), which suggests that most if not all of the CHL-a production in Lake Fayetteville was from cyanobacteria. This observation aligns with previous research at Lake Fayetteville in summer 2019, where the PC:CHL ratio ranged from 0.5 to 2 with most measurements above 1.0 (Wagner et al., 2021). CHL-a concentrations are likely representative of cyanobacterial responses at Lake Fayetteville and in our bioassays.

4.2 Nutrient Limitation of Cyanobacteria

Throughout the study period, Lake Fayetteville had nutrient dynamics and algal growth limitations that were characteristic of a classic hypereutrophic reservoir where the importance of N and/or P varied over time (Andersen et al., 2020; Lawson, 2021; Wang et al., 2019). In the spring (April through June), NO_3 and TN concentrations were much higher than SRP and TP concentrations, suggesting that the lake was P-limited. Cyanobacteria and algal growth are often limited by supply of P in lakes in both *in vivo* and *in situ* bioassays (Andersen et al., 2020; Kolzau et al., 2014; Lawson, 2021). At Lake Fayetteville, cyanobacterial growth was P-limited, where CHL-a concentrations increased with SRP addition in the bioassays.

In late June, the NO_3 supply in the water at Lake Fayetteville declines drastically. We then saw a shift in nutrient response in the bioassays, where cyanobacterial growth shifted from only P limitation to N and P co-limitation in the summer months (late June through early October). Strong co-limitation by N and P of cyanobacterial and algal growth in hypereutrophic

lakes during the summer growing season is well supported in the literature (Andersen et al., 2020; Dzialowski et al., 2005; Hughes and Marion, 2021; Wang et al., 2019).

Following lake turnover in the fall (mid-October through early November), NO_3 and NH_4 concentrations sharply increased at Lake Fayetteville, whereas the SRP supply remained low. These observations suggest P would limit cyanobacterial growth, and in the bioassays, we saw cyanobacterial growth shift from being co-limited back to only P-limited. This pattern has been observed in the literature, where many lakes have shown P-limitation of cyanobacteria and algae after fall turnover (Andersen et al., 2020; Barnard et al., 2021; Maberly et al., 2020).

One factor to consider is that nutrient bioassays remove the water from the lake, which is an open system, and put it in a closed system for the bioassays. Sediments in the lakes release SRP through equilibrium processes and under reductive dissolution (Austin et al., 2020; Grantz et al., 2014). While SRP was low in the lake water and usually less than MDLs, cyanobacteria and algae can quickly take up SRP that is released from sediment (Austin et al., 2020; McCarty, 2019). We need to keep this in mind when considering bioassay responses to nutrient addition.

4.3 Effects of Nutrients on Microcystin

The seasonal variations in cyanobacteria and nutrient supply influenced microcystin concentration and production (i.e., µg microcystin/µg CHL-a) at Lake Fayetteville over the 2021 growing season. When the nutrient supply was greatest in spring, total microcystin concentrations in the lake were increasing, whereas toxin production (µg microcystin/µg CHL-a) was greatest but variable. This relationship corresponds to findings in similar studies where there was an abundance of *Microcystis* that can produce toxins under high nutrient supply (Davis et al., 2009, 2010). However, the bioassays during the spring months showed that microcystin concentration did not increase with nutrient addition and often toxin production decreased with

nutrient treatments. The bioassays, instead of increasing toxin production, could have promoted growth of cyanobacteria, especially non-toxin producing species.

Interestingly, there are some clear examples of the effects of nutrients, particularly SRP, on responses on microcystin concentration and production in initial lake sample measurements at Lake Fayetteville. For instance, there is a significant spike in SRP on the third sampling date. On the fourth sampling date, CHL-a and the PC:CHL ratio have spikes, and NO_3 and toxin production decline. Since these points all follow increased SRP availability, this response could suggest that the spike in SRP could have stimulated a blue-green bloom that was made up of non-microcystin or non-toxin producing cyanobacteria.

In mid-June, when NO_3 supply was steadily decreasing in the lake, we observed that increasing SRP additions in the bioassays increased total microcystin concentrations but not production. This response suggests that the addition of P and P plus N increased the growth of cyanobacteria with toxin content (Jankowiak et al., 2019), but the individual cells likely did not produce more microcystin. The next week, however, we saw an increase in microcystin concentration in the lake, but in the bioassays observed that N addition actually decreased microcystin concentration and production. This N response was unusual, suggesting that we stimulated cyanobacteria growth but not microcystin concentration or toxin production.

When the NO_3 supply was gone (late June), total microcystin concentrations from the bioassays were stimulated by N. This observation is supported in the literature, where in a past Lake Fayetteville study, the addition of N in any form increased total microcystin concentrations (Wagner et al., 2021). Additionally, studies on Lake Erie in the U.S.A, (Barnard et al., 2021; Chaffin et al., 2018; Newell et al., 2019), Lake Wascana in Canada (Bogard et al., 2020), and in

laboratory grown monocultures (Wei et al., 2022), have found similar relationships where N availability can control cyanobacterial HABs.

In mid-July, we observed the first instance of NO_3 stimulating toxin production on a cellular basis in the bioassays. We see this observation right after the nutrient supply was diminished at Lake Fayetteville. However, this response did not continue in late July through the summer. Toxin production by cyanobacteria during the bioassay varied in response to nutrients, whereas total microcystin concentrations were stimulated by NO_3. During this period (mid-July through summer), NO_3 addition more often increased growth of cyanobacteria, rather than toxin production on a cellular level. An interesting observation is that microcystin toxin production is least during the summer months, which follows the patterns of microcystin concentration. During this period, microcystin concentration and production were limited by N in the bioassays, which supports findings in the literature that non toxin producing strains of cyanobacterial blooms dominate in waters with low nutrient concentrations (Davis et al., 2010).

Nutrient concentrations in Lake Fayetteville begin to increase again in late September and early October. The microcystin production cellular basis response shifts to N stimulation in late September in the bioassays, which precedes the major increase in lake nutrient concentrations by about a week. The microcystin response to nutrients shifts with the mid-October bioassay, where microcystin was again stimulated by P (Andersen et al., 2020; Wang et al., 2019). This microcystin response continued through the end of the end of the study period. By the end of the study period, as lake nutrient concentrations continued to increase, toxin production in the bioassays returned to decreasing with nutrient addition, suggesting that cyanobacteria growth was stimulated, but not toxin production.

Surface water temperatures in lakes may also have an impact on microcystin concentration and toxin production (Hayes et al., 2020). As the lake warmed in the spring, we saw our greatest microcystin concentrations, with the microcystin peaking at 25 °C, which is comparable to other studies (Davis et al., 2009; Hayes et al., 2020; Paerl and Otten, 2013). When lake conditions were above this temperature threshold, we saw a serious decline in initial microcystin concentration. On the other hand, toxin production was N-limited during two bioassays, both of which occurred when surface water temperature increased to ~27 °C and down to ~26 °C. This may show a relationship between temperature thresholds and the nutrient levels that promote toxin production. We found that lower surface water temperatures, below the 25 °C threshold, had the greatest initial toxin production. A combination between surface water temperature and nutrient stress may influence microcystin toxin concentration and production.

The third objective of this study was to identify the role that nutrients play by answering the question: did we increase microcystin toxin production or just increase cyanobacteria growth? Over the study period, we observed only two, two-week instances of N addition stimulating microcystin toxin production above the control. For the remainder of the study period, the nutrient treatments had reduced levels of toxin production relative to the control. While we did show that it is possible for nutrients to stimulate toxin production, the majority of the study period showed that through the bioassays, we only stimulated algal growth and did not increase toxin production on a cellular basis.

Although P limits cyanobacterial growth when toxin production is greatest, it is the combination of N and P that limits cyanobacterial growth during the growing season. Developing dual N and P HABs management techniques to reduce eutrophication and improve water quality will depend on an understanding of lake-specific nutrient and temperature dynamics (Barnard et

al., 2021; Newell et al., 2019). The historic focus has been on P as the limiting nutrient in anthropogenic eutrophication and even HABs, however, growing research suggests nutrients, especially N, influence not only algal growth, but also cyanobacterial concentration and toxin production (Gobler et al., 2016). We found that the importance of nutrients for cyanobacteria and microcystin toxin concentration and production varies with season and nutrient dynamics. Eutrophic lakes, like Lake Fayetteville, with lower N:P ratios will experience N and P co-limitation (Zhou et al., 2021). As the reservoir trophic state increases, cyanobacterial co-stimulation by N and P may become more widespread (Paerl et al., 2016), which suggests that dual management of N and P will be important in mitigating and preventing HABs in Lake Fayetteville.

5. Conclusion

This study demonstrated that nutrient limitation and stimulations changed with time and available nutrients in Lake Fayetteville. We found that, given the initial lake conditions, cyanobacteria were P-limited in the spring, N and P co-limited in the summer, and then P-limited again into the fall, according to our bioassays. Lake Fayetteville's microcystin concentration was not stimulated by nutrient addition in the spring but shifted to being stimulated by N addition in the late summer through early fall months. In the fall, microcystin concentration was stimulated by P. Microcystin toxin production was generally not stimulated by nutrient addition in the bioassays, with the exception of production being stimulated by N at the beginning of the summer and beginning of fall. Generally, we found that in the majority of bioassays we did not actually increase microcystin toxin production on a cellular basis. Instead, the addition of nutrients stimulated algal growth.

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