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Cyanobacterial Harmful Algal Bloom Ecology and Cyanotoxins in the Eutrophic Lake Winnebago-Green Bay Water System

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CYANOBACTERIAL HARMFUL ALGAL BLOOM ECOLOGY AND CYANOTOXINS IN THE EUTROPHIC LAKE WINNEBAGO-GREEN BAY WATER SYSTEM

by

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ABSTRACT

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Cyanobacterial harmful algal blooms (cyanoHABs) are frequently observed in water bodies used for recreation and drinking water production and can be detrimental to humans, animals, and general water quality. CyanoHABs are natural occurrences, but human activities such as agriculture, land use change, and runoff from urban and rural landscapes can promote and accelerate their expansion. The blooms are aesthetically unpleasing scums and can be laden with toxins (cyanotoxins) and toxic or otherwise bioactive peptides (TBPs) that can be harmful to humans and animals. Despite the vast research on cyanoHABs, cyanotoxin and TBP diversity and dynamics within a water column are not well studied. Furthermore, the variability in lake cyanotoxin and TBP concentrations is not fully understood at time-scales relevant to drinking water production. There is a great need for information about cyanoHABs and their toxins that may pose recreational risk to swimmers, particularly children. To begin to assess the temporal variability of cyanotoxins and TBPs, **Chapter 2** sought to use a proven technology, an automated water sampler, deployed to a water quality-monitoring buoy, to achieve a high temporal resolution sampling strategy for cyanotoxins and their associated pigments in a eutrophic lake. **Chapter 3** sought to analyze environmental variables that may be associated with cyanotoxin and TBP blooms from multiple depths (surface water to bottom waters).

Cyanobacteria have the ability to move throughout the water column in response to light or nutrient availability, however many sampling strategies focus on a singular depth. **Chapter 4** describes the first spatial assessment of cyanotoxins and TBPs in Green Bay over a two-year period and sought to characterize a cyanotoxin gradient that follows the spatial trophic gradient. There are many accounts of toxin-producing blooms in the Laurentian Great Lakes. Surprisingly, there is a lack of information on cyanotoxins in Green Bay, a highly productive region in Lake Michigan. This dissertation seeks to describe the temporal and spatial variability of cyanotoxins and TBPs in two connected water bodies that are extremely important as drinking water and recreation resources in Wisconsin. The resulting work provides important insights into less studied, but frequently TBPs in drinking water and recreational waters. Cyanotoxin and TBP sampling was paired with *in situ* fluorometers, a common tool used for monitoring cyanoHABs, to assess the variability of pigments and cyanotoxins.

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^{13}C-Phe - $^{13}C_6$-phenylalanine

ADDA -- 3-Amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid

AOC – area of concern

AP– anabaenopeptin

ATX-A – anatoxin-a

BUI – beneficial use impairment

Chl – chlorophyll-a

CP – cyanopeptolins

cyanoHABs – cyanobacterial harmful algal blooms

CYN – cylindrospermopsin

DIN – dissolved inorganic nitrogen

DO – dissolved oxygen

EPA – Environmental Protection Agency

GB – Green Bay

hATX – homoanatoxin-a

HILIC – hydrophilic interaction chromotography

LC – liquid chromotography

LOD – limit of detection

m – meter

MC – microcystin

MCLR – microcystin-leucine (L) arginine (R)

MCRR – microcystin-arginine (R) arginine (R)

MG – microginin

MS – mass spectrometry

MS/MS – tandem mass spectrometry

N – nitrogen

NH3 – ammonia

NO2 – nitrite

NO3 – nitrate

NOD – nodularin

P – phosphorus

Phy – phycocyanin

TBP – toxic or otherwise bioactive peptides

TDP – total dissolved phosphorus

TOC – total organic carbon

TP – total phosphorus

TSS – total suspended solids

WHO – World Health Organization

WN – Lake Winnebago

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Chapter 1. Introduction

Cyanobacterial harmful algal blooms (cyanoHABs) are a threat to lakes worldwide (1, 2). CyanoHABs are natural occurrences, but human activities such as agriculture, land use change, and runoff from urban and rural landscapes can promote and accelerate their expansion (3-5). Global cautionary tales warn freshwater lakes are facing increased eutrophication (6), and many studies are predicting or observing a rise in cyanoHABs (7-10), which threatens the drinking and recreational resources of freshwater lakes (11, 12).

Climate change is expected to exacerbate the threat of eutrophication and cyanoHABs in freshwater lakes (as reviewed in (13)). Climate change has already been documented by a global increase in earth surface temperature by 0.5°C and an increase in summer surface water temperature by 0.34°C decade^{-1} from 1985 - 2009 (14, 15). The effects of climate change can promote the expansion and dominance of cyanobacteria over other phytoplankton assemblages (7, 16) due to their higher temperature growth optima (17). In the event of increased lake stratification, bloom forming genera such as *Microcystsis*, *Anabaena*, and *Dolichospermum* generally prefer the thermal stability of a stratified water column (18, 19). Weak mixing and warm weather favor buoyant species such cyanobacteria. They have the ability to adjust their vertical position in the water column in response to the conditions in a stratified environment and form surface blooms, outcompeting other phytoplankton (20, 21). These tactics are employed already, as the bacteria tend to dominate eutrophic, freshwaters during the warmest times of the year.

One measured trend that can be assessed is the loss of oligotrophic lakes in the United States. Total nitrogen (TN) and total phosphorus (TP) data collected for the National Nutrient

Survey revealed lakes and rivers in all U.S. Environmental Protection Agency (EPA) nutrient ecoregions exceeded median values (22). Phosphorus data from National Lake Assessment (NLA) were collected in 2007 and 2012 and notable continental scale increases were observed in TP, while oligotrophic lakes decreased by 18.2% (23). An additional study of lake nutrient and chlorophyll trends from 1990 to 2013 using the Lake Multi-Scaled Geospatial and Temporal Database of the Northeast U.S. (LAGOS-NE) determined water quality of these midwestern and northeastern US lakes have not degraded over that timeframe, but lakes also hadn't improved (24). The shift in lake trophic status to some lakes in the US could lead to more lakes that can support cyanoHABs. There is a great need for synthesis of long-term datasets to assess the severity of increased eutrophication and cyanoHABs, however these types of data may exist only regionally or globally not at all. Local knowledge, derived from first-hand experience (25, 26) may become an important resource as scientists, lake managers and stakeholders grapple with the many ecological threats facing their lakes. A recent survey of two different lake-organizations assessed the risk of global cyanoHABs and respondents indicated eutrophication is a threat to 70% of the lakes in the dataset, reporting cyanoHABs occur in 52% of lakes studied (n = 249; data unpublished, Figure 1.1). Interestingly, an environmental nonprofit in the United States had a 40% increase in cyanoHAB outbreak reports from 2017 to 2018 (169 outbreaks to 239), a marked jump from the seven outbreaks reported in 2010 at the start of the program (27). Whether the increase in reporting was due to increased awareness of cyanoHABs or more cyanoHAB occurrences, the resulting perception is that cyanoHABs are increasing. Additionally, the observed rise in cyanoHAB reporting in the studies above could be due to increased monitoring efforts (28).

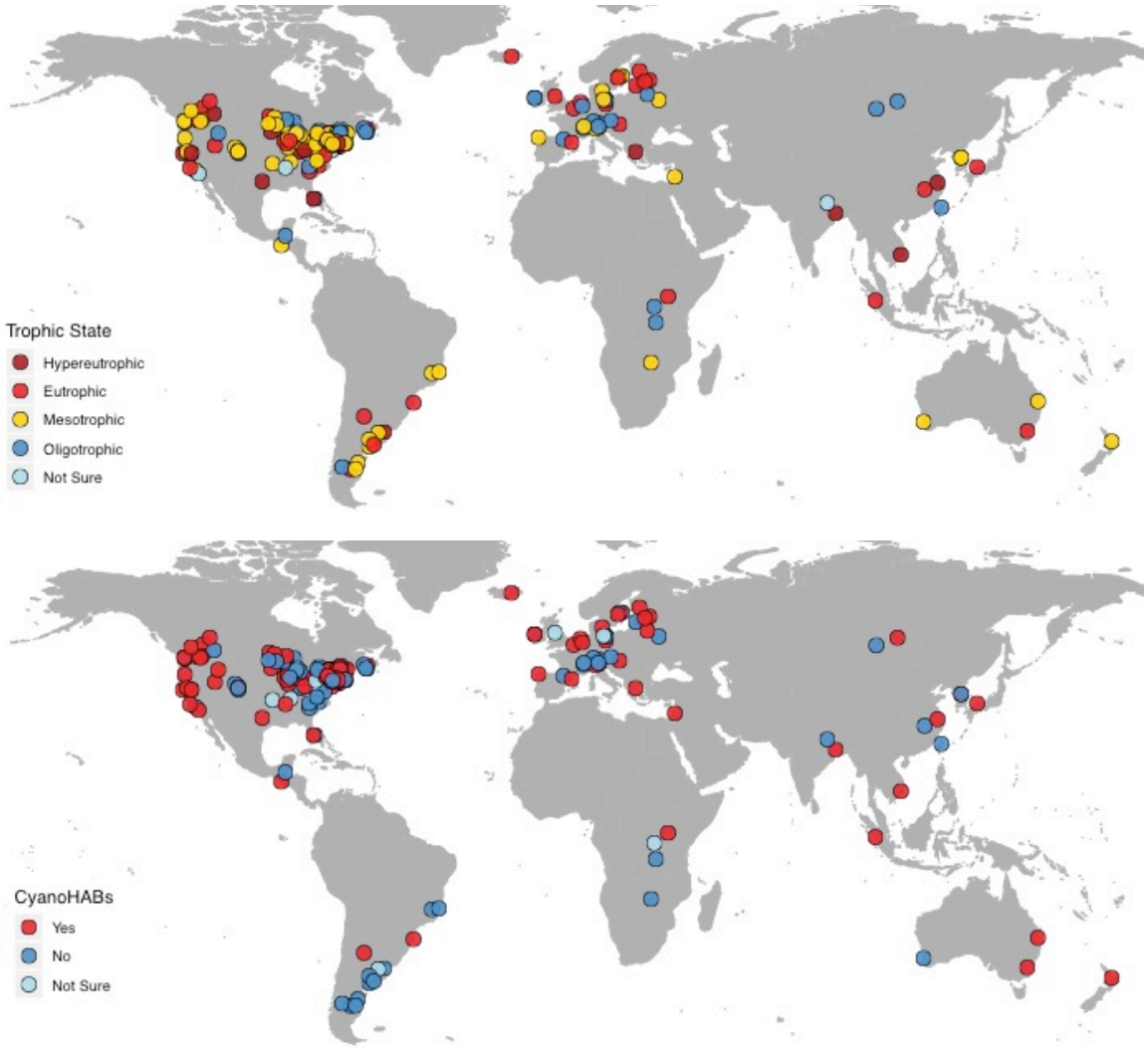

Figure 1.1: A survey of two different lake-organizations assessed the risk of cyanoHABs and respondents indicated (Top) eutrophication is a threat to 70% of the lakes in the dataset and (Bottom) cyanoHABs occur in 52% of lakes (n = 249; data unpublished).

Cyanobacteria in lakes have evolved a myriad of physiological, morphological and behavioral adaptations, which allow them to proliferate in diverse environments around the world. While often noted for dominance in eutrophic water bodies, cyanobacteria are global organisms, and species have been observed in lakes of many sizes and types including oligotrophic lakes (29), salty water bodies (30, 31) and tropical waters (32, 33). Mechanisms that aid in large-scale proliferation include buoyancy control (34), nutrient sequestration and

storage (35), and salt and temperature tolerance (17, 36-38). Additionally, cyanobacteria have the ability to photosynthesize and produce chlorophyll, a green pigment, and phycocyanin, a blue accessory pigment produced by cyanobacteria (39). These pigments are useful for monitoring cyanobacteria.

Buoyancy control allows cyanobacteria to move within the water column due to gas vacuoles. The protein gas vacuole is made up of stacked gas vesicles, shaped liked rods that repel water and diffuse gas (40). Buoyancy can be altered in response to light and nutrients, giving cyanobacteria a competitive advantage against other organisms to access nutrients from bottom waters, as well as move to the surface for photosynthesis (21). As photosynthetic organisms, cyanobacteria use sunlight to create carbohydrates, and the accumulation of carbohydrates provides short-term density control. If there is too much pressure from too many carbohydrates, gas vesicles can burst causing cyanobacteria to sink (41). Cyanobacteria will also sink in response to a lack of nutrients or if they have been exposed to too much light (42).

Cyanobacteria have other mechanisms that allow them to compete for nutrients. Some species of cyanobacteria can fix atmospheric nitrogen (43) and in general, cyanobacteria have storage mechanisms for carbon, nitrogen and phosphorus (44). Storage capabilities play an important role for dominance as is the case with *Microcystis*, which has been shown to be less immediately dependent by nutrient availability (45, 46). Examples of storage products can be polyphosphate for phosphorus storage and cyanophycin or phycobilin for nitrogen (35, 47).

CyanoHABs are frequently observed in water bodies used for recreation and drinking water production and can be detrimental to humans, animals, and general water quality. The

blooms are aesthetically unpleasing scums accompanied with strong odors as the bloom decays. The decomposition of blooms can lead to decreased dissolved oxygen and potentially cause hypoxic conditions. Additional cyanoHAB attributes are the production of toxins and toxic or otherwise bioactive peptides (TBPs) that can be harmful to humans and animals (48-50).

Cyanotoxins and TBPs are secondary metabolites, not necessary for normal cell functioning. These secondary metabolites are acutely acting toxins and can be tumor promoters, potent liver, brain or neurological toxins, as well as being toxic or otherwise bioactive peptides. All cyanotoxins and TBPs should be of interest for public health monitoring although current health advisories and guidelines from the US EPA include microcystins (MCs) and cylindrospermopsin (CYN) (51, 52). There are many more classes of cyanotoxins and TBPs that can be produced and the classes themselves can be quite complex.

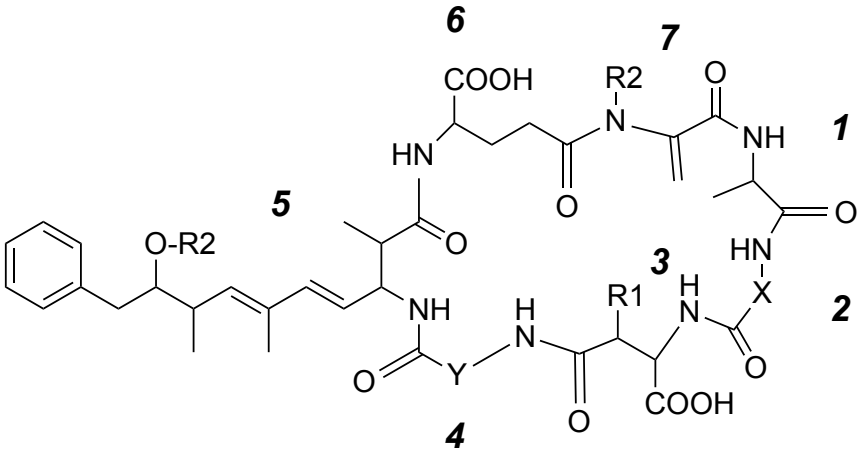

Figure 1.2: General structure of microcystin (MC). MCs contain seven amino acids (labeled 1-7) – the adda side chain (5), four non-protein amino acids (1, 3, 6, 7) and two variable amino acids (2, 4). R1 and R2 can be hydrogen or methyl groups and X and Y are variable amino acids. For MCLR, the X and Y are replaced with leucine and arginine. N = nitrogen, O = oxygen, H = hydrogen

MCs are cyclic heptapeptides and contain seven amino acids - the unique Adda side chain, four non-protein amino acids and two variable amino acids (Figure 1.2) (53). MCs are structurally diverse with high molecular weights and more than 200 congeners are possible (54). MCs are ubiquitously observed in freshwater systems. One of the most frequently studied and detected MC congener is microcystin-LR (MCLR), which has leucine (L) and arginine (R) as variable amino acids, along with microcystin-RR (arginine and arginine; MCRR). Several cyanobacterial species are known to produce MCs including *Microcystsis*, *Dolichospermum*, *Planktothrix* and *Oscillitoria* (48).

Inhibition of protein phosphatase 1/2A (PP1/PP2A) is a well-studied mechanism of MC toxicity (55). These phosphatases play critical roles in cellular processes and are major regulators of protein dephosphorylation. On the cellular level, when MCs bind and inhibit PP1/PP2A, they disrupt the cytoskeleton and cause cell death (56). As a cyclic peptide, the MC structure blocks access to other substrates at the active site (57).

MC exposure can occur via ingestion (50) and can be a common exposure route through recreation. Once ingested, MCs are not broken down in the stomach and instead are absorbed into the bloodstream (58). Given the high molecular weight and structure, MCs cannot diffuse across the cell membrane and instead require active transportation with organic anion transporting polypeptides (OATPs) (59, 60). The OATPs that have been shown to transport MCs are found in the liver and also in the brain and kidney (61). Therefore, MCs are potent liver, kidney and brain toxins (62, 63) and act by inhibiting PP1/PP2A after uptake (64, 65).

Other liver toxins include nodularin (NOD), and CYN. NOD is similar in structure to MC with the Adda structure but does not have amino acids at positions 1 and 2 (66). NOD also

inhibits protein phosphatases, but mainly occurs in brackish waters (67, 68) produced by *Nodularia* and *Aphanizomenom* (69). CYN is regarded as a liver toxin and also is capable of causing damage to kidneys (70). It is an inhibitor of protein synthesis and reduced glutathione synthesis and can act by inducing genotoxicity (71, 72). CYN is produced by *Cylindrospermopsis* and while normally associated with tropical and sub-tropical waters, CYN has been detected in temperate regions (73-75).

Among neurotoxins produced by cyanobacteria, anatoxin-a (ATX-A) and homoanatoxin-a (hATX) are some of the most frequently encountered or measured. ATX can be produced by *Aphanizomenom* and *Dolichospermum*, among others (76, 77). Both ATX-A and hATX are bicyclic alkaloids that mimic acetylcholine and bind irreversibly to the nicotinic acetylcholine receptors at the neuromuscular junction causing uncontrolled activation of the nicotinic acetylcholine receptors and overstimulation of muscles, leading to respiratory paralysis (78-81). In animal studies, ATX-A and hATX have caused staggering, muscle twitching, gasping and eventually death by respiratory arrest (76, 82). Anatoxin-a(s) is another neurotoxin, the (s) designation referring to the salvation factor, identified in the original observation (83). It is a cholinesterase inhibitor and noted for being very toxic (84).

Paralytic shellfish poisoning toxins (PSTs) are another general class of neurotoxins produced by cyanobacteria, which includes saxitoxins. PSTs were mainly thought to occur only in marine environments, but studies have shown PSTs can be produced by freshwater organisms as well such as *Aphanizomenom*, *Cylindrospermopsis* and *Lyngba* (85-87). Saxitoxins, noted for their high toxicity, act by inhibiting sodium gated channels and can lead to nerve paralysis and death by respiratory arrest (88).

An additional neurotoxin to mention is Beta-methylamino-L-alanine (BMAA). BMAA has been shown to have neurodegenerative effects and has been linked to amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (89). Research is ongoing to assess its natural production by cyanobacteria and possible human exposure to this neurotoxin.

In addition to the aforementioned toxins, cyanobacteria produce hundreds of other TBPs. Anabaenopeptins (APs), cyanopeptolins (CPs), and microginins (MGs) are among various classes of TBPs that can be produced in tandem with MCs (90-92). These TBPs have a range of bioactivity on cellular enzymes including phosphatases, chymotrypsin, thrombin, some of which may be beneficial for commercial or medicinal uses, such as antifungals, antimicrobials or antivirals (93-95). Recent studies have shown some TBPs may also be toxic to aquatic organisms like the crustacean *Thamnocephalus platyurus* and a neurotoxin in zebrafish (96, 97). Another study showed APs and CPs were toxic to the model organism *C. elegans* (98). Thus, the toxicity of cyanoHABs extends beyond hepatotoxins and neurotoxins.

Anabaenopeptins (APs) are cyclic oligopeptides that possesses a ureido linkage (99) and can also inhibit PP1 (100) as well as carboxypeptidases (101, 102). APs can be produced by *Dolichospermum*, *Aphanizomenom*, *Microcystis* and *Planktothrix*. At least 96 variants of APs have been reported and as such, the pharmacological effects of these peptides is an emerging area of study (103, 104).

Cyanopeptolins (CPs) are cyclic peptides made up of seven to nine amino acids. CPs can be produced by *Dolichospermum*, *Microcystis Planktothrix*, *Lyngbya*, and *Nostoc* and more than 68 variants have been detected (105). CPs act as serine protease inhibitors and may have pharmaceutical value as they may be applied in treatment of asthma or viral infections (106).

Alternatively, a CP variant, CP-1020, has been shown in recent studies to be toxic to the crustacean *Thamnocephalus platyurus* and a neurotoxin in zebrafish (96, 97).

Microginins (MGs) are linear peptides and can vary in length from four to six amino acids (107, 108). *Microcystis* and *Planktothrix* are both known producers of MGs and at least 38 variants are known (109). MGs are inhibitors of proteases including an angiotensin converting enzyme and may be useful in treating high blood pressure (110).

Studies have investigated environmental variables as potential attributes for cyanotoxin production, including light, temperature, nutrients, and trace metals. Toxin production has been shown to be suppressed in low light conditions and increased light intensity has been associated with increased toxin production (111, 112).Temperature has generally been accepted as a driver for cyanobacteria dominance, and increases in water temperature have been associated with increased growth rates of toxic *Microcystis* and also non-toxic *Microcystis (*113*)*. When increases in temperature occurred with increases in phosphorus, toxic *Microcystis* had the highest growth rate over non-toxic strains (113). In culture experiments, strains of toxic and non-toxic *Microcystis* were grown under different nutrient conditions and higher nutrient concentrations favored the growth of toxic strains (114). Additionally, higher phosphorus and nitrogen levels have been associated with higher MC content per cyanobacterial cell (38). Toxin production has also been shown to increase when cyanobacteria are starved of iron (115). On the biochemical level, cyanobacteria use phosphorus and nitrogen for cell and toxin construction. Cyanobacteria species can vary in size, which will drive part of the nutrient demand, which can be furthered driven by cyanotoxin production. Nitrogen is essential for the production of cyanotoxins, and it has been hypothesized that nitrogen availability can

determine bloom and toxin production (43, 116). Microcystins have shown to have an affinity for iron and bind it, thus microcystin content is an inverse relationship with the concentration of the trace metal (115).

CyanoHAB toxicity is indiscernible based on visual observation. Within a cyanobacterial bloom, there can be a diverse cyanobacterial community with different species that may be known producers of cyanotoxins or not. Even if a species e.g. *Microcystis* is a known producer of MCs, the strains present in the *Microcystis* bloom need to have the genes to encode for the toxin (117, 118). Strains can be non-producers, lacking the ability to produces MCs (119). If the strains have the genes to encode of MCs, the genes may not always be expressed (120). Therefore, even if the cyanobacterial community composition is known, the presence of cyanotoxins requires an additional biological, immunological or analytical method.

There are several factors that may regulate toxin production including light, stress, and nutrients. In one study, microcystin transcription was increased under high light and red light conditions and decreased with blue light (121). This same study found stress had a negative impact on transcription. Nitrogen, or transcription of nitrogen-regulated genes can bind to the microcystin gene cluster and act as an up or down regulator of its synthesis (122) and under nitrogen limitation conditions, the nitrogen-regulated genes were expressed more (123). Thus nitrogen starvation has been shown to increase microcystin production on a biosynthetic level as the toxic strains of a species were more tolerant to nutrient stress (124).

Commercial technology for real-time monitoring of cyanotoxins is not an option yet, and current analyses yield results within hours to days, depending on the method. A universal method to detect all cyanotoxin classes and their congeners is also not available. Commonly

employed methods include enzyme-linked immunosorbent assay (ELISA), Abraxis strip test, protein phosphatase inhibition assay, and liquid chromatography tandem mass spectrometry (LC-MS/MS). Each method comes with its own specifications for cyanotoxins classes that can be measured and length of time it takes, as well as cost and the ease or ability to use the method.

A test strip can be used for a rapid, qualitative assessment of cyanotoxins in water. The principal of a test strip is a toxin conjugate competing against possible toxins in a water sample for binding spots with antibodies. The test strips have a test line and a control line; the intensity of the test line is compared to the intensity of the control. When there are toxins present in a sample, they fill the binding sites and prevent the formation of the colored test line. If the test line is very light or doesn't appear, the sample is said to be greater than the maximum detection range. Abraxis test strips are available for MCs and the detection range is 0 – 10 ug/L (125). Abraxis has test strips for ATX-A and CYN, as well (126, 127).

ELISA is an immunoassay that can provide quantitative and/or qualitative results. ELISA kits are commercially availability and often considered a cost-effective method for cyanotoxin detection for the following: MC, AP, CYN, ATX, SAX, BMAA. Briefly, this method works by binding the cyanotoxin and its congeners in a sample with antibodies and results in a colorimetric response that is proportional to the amount of cyanotoxin present (128, 129). When considering ELISA as a screening tool for MCs, for example, ELISAs measure any and all MC congeners present in a sample (130) which is beneficial if there are more rare congeners present and a reference standard is not yet available. However, this method does not consider the toxicity of a sample as it does not differentiate between the congeners. MCLR and MCRR have much different toxicities or LD50 (as reviewed in (131)). Using ELISA can provide a more

rapid (1-8 hours) assessment of cyanotoxins in a water sample but should be verified with an additional technique if sensitivity and specificity is desired.

Protein phosphatase inhibition assay is a rapid, quantitation method to detect MCs via phosphatase inhibition activity (132). Samples containing MCs will inhibit enzymes in this test kit. The resulting sample concentration is determined from a standard curve after absorbances are measured. A sample that does not inhibit protein phosphatase will produce a substrate with an absorbance at 405 nm that can be measured. It is important to note other cyanotoxin congeners, such as anabaenopeptins, are also phosphatase inhibitors and studies have shown the two classes, MCs and APs, co-occur in samples (92, 133). Therefore, results of this assay may over quantify MCs, however it still could be a valuable method for the sake of public health as APs are considered a toxin of emerging concern.

Analytical instrument techniques, such as LC-MS/MS can provide the most direct quantitative result for the available reference standards on hand. LC-MS/MS is often considered the 'gold standard' for quantitative measurement of specific cyanotoxins but comes at the highest cost/sample and often requires more training to operate the analytical equipment. Sample results can be provided the same day or up to several days, as there are several steps for sample preparation and analysis including sample lyophilization and freeze/thaw cycles (90). Also, depending on the target analyte, the extraction process will differ as more polar compounds such as CYN, SAX, and ATX will need to be extracted differently from MCs, APs, CPs, MGs, to achieve optimal detection. Therefore, LC-MS/MS may not be best suited as a screening tool for a public health monitoring program but could be used to verify a positive result of the test strip or ELISA.

Monitoring

Many studies report the co-occurrence of MCs, ATXs and other bioactive peptides (134-136), but examining the diversity of cyanotoxins and TBPs and changes in the cyanotoxin profile of a lake at high resolution has not yet been done. This is especially important because the variability in lake cyanotoxin concentrations is not fully understood at time-scales relevant to drinking water production, which occurs 24 hours a day, seven days a week. Furthermore, there is a great need for information about cyanoHABs and their toxins that may pose recreational risk to swimmers, particularly children (137, 138)**.** Currently, the Environmental Protection Agency (EPA) has published guidelines for recreational cyanotoxin limits as well as limits for drinking water. However, it is unclear what it means to have limits if it still has not been determined how to monitor for cyanotoxins on a scale that is appropriate for both recreational and drinking waters to determine if a water body is under said limits.

Achieving real-time monitoring of drinking water for cyanotoxins can be costly and is not feasible. Many studies have examined cyanotoxin concentrations on a weekly scale or greater (139, 140) or rely on other monitoring mechanisms (visual inspection, cell-counts, pigment analysis) before obtaining a sample for cyanotoxin analysis (141). Traditional sampling strategies often occur during the day, when conditions are sunny or favorable and, except for a few studies, cyanotoxin concentrations have not been measured at night or over a 24-hour period (142-144). When there is no cyanotoxin sampling, measurements of pigment fluoresence using *in situ* fluorometers for chlorophyll and phycocyanin have been one method for monitoring cyanobacteria at drinking water treatment plants (145-147). Previous studies have examined variability in cell density, chlorophyll or phycocyanin fluorescence in comparison

to MC concentrations, but these measures of cyanobacterial abundance fail to consistently correlate with cyanotoxin levels (148). To begin to assess temporal variability of cyanotoxins, **Chapter 2** sought to use a proven technology, a Teledyne ISCO water sampler, deployed to a water quality-monitoring buoy, to achieve a high-resolution sampling strategy for cyanotoxins and TBPs. This study took place in Lake Winnebago, Wisconsin, at the site of a drinking water intake pipe. This high-resolution cyanotoxin and TBP sampling was paired with *in situ* fluorometers to assess the variability of pigments and cyanotoxins.

CyanoHABs can form surface scums that can be magnitudes higher in toxin concentrations than the water beneath it. In a shallow lake, cyanotoxin concentrations could differ throughout the water column but few studies have considered cyanotoxins and environmental drivers throughout the water column. In a shallow, well-mixed lake like Lake Winnebago, this study assessed differences in cyanotoxin and TBP diversity from surface water samples to bottom water samples. Addressing this question could have implications for drinking water monitoring which pulls from the bottom of the water column versus recreation exposure which focuses on cyanotoxins concentrations form the surface. **Chapter 3** addresses the differences in cyanotoxin and TBP concentration and diversity and assessing environmental variables that may be associated with cyanobacterial secondary metabolites by depth, in a multi-year analysis.

There are many accounts of cyanotoxins in prominent, eutrophic lakes in Wisconsin (92, 149, 150). One of the first recorded measurements of cyanotoxins was in the late 1960's in Lake Winnebago, followed by a statewide survey in 1967-1969, 1986 and 1993, which also found cyanotoxins in Lake Winnebago, as well as in other lakes around Wisconsin such as Lake Delton,

Lake Menomin and Wapogasset Lake (151-153). Toxin-producing cyanoHABs have been described in the Great Lakes, although most studies have focused on the lower Lakes. Surprisingly, there is a lack of information on cyanotoxins in Green Bay, Lake Michigan, a highly productive region in the Laurentian Great Lakes (154). **Chapter 4** describes the first spatial assessment of cyanotoxins and TBPs in Green Bay over a two-year period, 2014-2015, from samples collected at 0 meters (m) and 1 m. This study also assessed the gradient, if any, of cyanotoxin classes in relationship to the known trophic gradient in the bay.

This dissertation seeks to describe the temporal and spatial variability of cyanotoxins and TBPs and environmental drivers in two connected water bodies. The relationship between cyanotoxins and in-situ fluorometers will be investigated, as fluorometers are often used as a monitoring tool for cyanoHABs. **Chapter 5** will begin to draw some conclusions between the two systems that are extremely important as drinking water and recreational resources in Wisconsin.

CHAPTER 2

High resolution monitoring of toxic or otherwise bioactive peptides produced by cyanobacteria

ABSTRACT

Occurrence of cyanotoxins in lakes at high temporal resolution is not well known, particularly near drinking water intakes. Here we characterized sub-daily variability of cyanotoxins in a eutrophic lake over a drinking water intake. A surface buoy was equipped with an autosampler to collect samples every six hours and was deployed for one cyanobacterial growing season. Eleven microcystins, (homo)anatoxin-a, nodularin, cylindrospermopsin, anabaenopeptins A, B and F, cyanopeptolins 1007, 1041, and 1020, and microginin 690 were targeted by liquid chromatography tandem mass spectrometry. Of the twenty-two cyanotoxins targeted, all but seven were detected in the lake on at least one date. Microcystins (MCLR/MCRR) plus Anabaenopeptin B were detected in 100% of samples and MCLR and MCRR had the highest mean and max concentrations. The max microcystin concentration (18.4 µg/L) was recorded in a midnight sample during the October bloom and the highest cyanotoxin concentrations occurred during non-bloom periods. Cyanotoxin profile variability followed temporal patterns, increasing in complexity over time. A lower sampling frequency is shown to underestimate maximum microcystin levels by >3 fold. Maximum changes in toxin levels occurred during non-bloom periods when microcystin levels increased from 5.4 µg/L to 15.1 µg/L (179% change) over 6 hours. Overall these data show that cyanotoxin levels are highly variable at point source sampling points, including drinking water intakes. Furthermore, maximum levels are not necessarily associated with bloom conditions.

INTRODUCTION

An increasing number of freshwater lakes and rivers in the United States are becoming eutrophic, supporting large accumulations of cyanobacteria known as cyanobacterial harmful algal blooms (cyanoHABs). CyanoHABs are natural occurrences, but human activities such as agriculture, land use change, and runoff from urban and rural landscapes can promote and accelerate their expansion (3-5). Excessive proliferation of cyanoHABs leads to a decrease in dissolved oxygen, creating hypoxic or even anoxic conditions as the bloom decays which can be harmful to fish and other aquatic life (155). Cyanobacteria can produce toxins (cyanotoxins) and toxic or otherwise bioactive compounds (TBPs) (48) which can affect invertebrate and vertebrate animals including humans (49, 156). These cyanotoxins and TBPs can be particularly concerning in lakes used not only for recreation, but also for drinking water production.

Cyanotoxins and TBPs include different classes of linear and circular peptides that cause varying degrees of toxicity to humans and animals. Microcystins (MCs) are commonly observed cyanotoxins in freshwater systems with more than 200 structural variations reported due to substitutions, methylations and modifications of its amino acids. One of the more frequently studied and detected MC congeners is microcystin-LR (MCLR), which has leucine (L) and arginine (R) as variable amino acids. The general structure of MC is a cyclic heptapeptide containing the Adda side chain, plus four non-protein amino acids and two variable amino acids (53). MCs are potent liver toxins (62, 63), and act by inhibiting protein phosphatases (PP) 1 and 2A (64, 65). Other liver toxins include nodularin (NOD), and cylindrospermopsin (CYN). NOD is similar in structure to MC and also inhibits protein phosphatases, but mainly occurs in brackish waters (66-68). CYN affects both the liver and kidneys and is an inhibitor of protein synthesis

(71). While CYN is normally associated with tropical and sub-tropical waters, it has been detected in temperate regions (73, 74).

Among neurotoxins produced by cyanobacteria, anatoxin-a (ATX-A) and homoanatoxin-a (hATX) are some of the most frequently encountered or measured. Both ATX-A and hATX are bicyclic alkaloids that mimic acetylcholine and bind irreversibly to the nicotinic acetylcholine receptors at the neuromuscular junction causing uncontrolled activation of the nicotinic acetylcholine receptors and overstimulation of muscles, leading to respiratory paralysis (78-81). The neurotoxin has been shown to have a half-life on the order of hours under certain pH and light conditions (157), compared to MCLR which could have a half-life of 3-9 weeks under similar conditions (158, 159).

In addition to the aforementioned toxins, cyanobacteria produce hundreds of peptides that can be toxic or otherwise bioactive (TBPs) by inhibiting various proteases and may be beneficial for commercial or medicinal uses (93, 94). Microginins (MGs), are linear peptides and inhibitors of proteases including angiotension converting enzyme (110). Anabaenopeptins (APs) are cyclic peptides that possess a ureido linkage (99) and are inhibitors of phosphatase 1 (100, 160) but also inhibitors of carboxypeptidases (101, 102). Cyanopeptolins (CPs) are cyclic serine protease inhibitors and in a recent study a CP variant, CP-1020, has been shown to be a neurotoxin in zebrafish (96, 97). Additionally, a recent study assessed the toxicological effects of several congeners of APs and CPs to the model organism *C. elegans* and found APs to have the greatest toxicity, resulting in reduced reproduction, shortened lifespan and severe aging-related vulval defects (98).

Many studies report the co-occurrence of MCs, ATXs and other TBPs (134-136), but changes in cyanotoxin diversity at high temporal resolution (i.e. sub- daily) is not well known. This is especially important for drinking water production, which occurs continuously 24 hours a day, seven days a week. Furthermore, there is a great need for information about cyanoHABs and their toxins that may pose recreational risk to swimmers, particularly children (137, 138). Currently, the Environmental Protection Agency (EPA) has published guidelines for recreational cyanotoxin limits as well as limits for drinking water. However, it is unclear what it means to have limits if it still has not been determined how to monitor for cyanotoxins on a scale that is appropriate for both recreational and drinking waters.

Achieving real-time monitoring of drinking water for cyanotoxins can be costly and not feasible. Few studies report cyanotoxin concentrations at a drinking water site at high resolutions (i.e. several times a day, daily or even several times per week) (142, 147, 161). Many studies have examined cyanotoxin concentrations on a weekly scale or greater (139, 140) or rely on other monitoring mechanisms (visual inspection, cell-counts, pigment analysis) before obtaining a sample for toxin analysis (141). Traditional sampling strategies often occur during the day, when conditions are sunny or favorable and, except for a few studies, cyanotoxin concentrations have not been measured at night or over a 24-hour period (142-144). When there is no cyanotoxin sampling, measurements of pigment fluoresence using *in situ* fluorometers for chlorophyll (Chl) and phycocyanin (Phy), an accessory pigment specific to cyanobacteria (39), have been one method for monitoring cyanobacteria at drinking water treatment plants (145-147). Previous studies have examined variability in cell density, Chl or Phy fluorescence, or genes involved in toxin production in comparison to MC concentrations,

but these measures of toxic cyanobacterial abundance fail to consistently correlate with cyanotoxin levels (148, 162). Finding a reliable monitoring mechanism associated with cyanotoxins is an area for further research.

In this study, we sought to capture the temporal patterns and sub-daily variability of twenty-two cyanotoxins using an automated water sampler that could be deployed and scheduled to collect at a 6-hour (hr) frequency. A preservation method for a suite of cyanotoxins and TBPs was tested, as samples would be sitting for several days in the auto sampler before retrieval. Additionally, we determined if pigments measured at high resolution by the water quality-monitoring buoy, are associated with cyanotoxins. Focusing on a suite of cyanotoxins produced in Lake Winnebago, a eutrophic lake in Northeastern Wisconsin, we measured eleven microcystins – MCLR, MCRR, MCYR, MCLA, Dha^{7}MCLR (dmLR), MCLF, MCLY, MCLW, MCWR, MCHtyR, MCHilR, three anabaenopeptins – AP-B, AP-F, and AP-A, three cyanopeptolins – CP-1007, CP-1041, CP-1020, one microginin analog – MG-690, two anatoxin analogs – ATX-A and hATX, and two other toxins – CYN and NOD, at high resolution at a fixed monitoring station, located at the site of a drinking water treatment plant intake pipe.

EXPERIMENTAL

Study Site.

Lake Winnebago is part of the Lake Michigan watershed and the largest inland lake in Wisconsin, USA with a surface area of 557.3 km^2 and maximum depth of 6.4 m. The lake is

primarily fed by the Fox River through Lake Winneconne and Lake Butte des Morts to the west known as the Lake Winnebago pool (Figure 2.1). The Fox River exits Lake Winnebago to the north and empties into Green Bay. Together, the Fox River and Lake Winnebago provide an estimated one-third of all phosphorus to Lake Michigan (163). In addition to serving as a recreational resource, the lake is a drinking water source to four major cities –Appleton, Oshkosh, Neenah and Menasha, with a total population of approximately 200,000 people.

Figure 2.1: Lake Winnebago, Wisconsin supplies drinking water to four major cities – Oshkosh (study site), Appleton, Neenah and Menasha.

Lake Winnebago experiences large accumulations of cyanobacteria in late summer and fall due to nonpoint-source nutrient inputs from the Fox River Basin (164, 165). The presence of toxic cyanobacteria in Lake Winnebago was first documented in the late 1960's, followed by a statewide survey in 1986 and 1993 which detected cyanotoxins in Lake Winnebago, as well as in other lakes around Wisconsin (151, 152). Recently, the presence and concentration of cyanotoxins were measured from not only lake water but also raw intake drinking water in Lake Winnebago (91).

Water Quality Monitoring Buoy

The buoy (Mooring Systems, Inc) provided 500 pounds of buoyancy and was moored with three anchors in 4 meters (m) of water, near the drinking water intake pipe. Power was supplied by a 12 V, 50 amp-hour marine battery (Optima 34M), which was charged by three 45 watt, 2.52 amp max solar panels (Solartech). Charging was controlled by a 10 amp charge controller (Morningstar Sunsaver 10) (Figure 2.1B). The buoy was equipped with both Phy and Chl *in situ* fluorometers (Turner Cyclops 7), as well as optical dissolved oxygen (DO) and temperature probes (InSitu RDO Pro) deployed at 0.5 m. Data was collected using a CR1000 datalogger (Campbell Scientific) and telemetered to the Verizon network using a cellular modem (Raven XT). Loggernet software (Campbell Scientific) was used to retrieve and store the data on a laboratory computer. Sensors were programmed to take measurements every minute and data were retrieved every 5 minutes.

Deploying an automated water sampler in a buoy comes with challenges unique to high-resolution sampling. A certain amount of ballast on the buoy is required to combat a counterbalance problem that is created from the constant flux of partially filled water sample bottles that sit above the water. Maintaining a buoy and a water sampler on Lake Winnebago can further be challenging when weather conditions create unsafe conditions to travel by boat to the buoy and further, can tip the buoy over if there is not enough ballast. Samples were lost as a result of the challenges described during September 15 – 17 and October 14 (Figure 2.2).

Sampling

Whole (unfiltered water) samples (200 ml) were obtained autonomously with a portable programmable water sampler (Teledyne ISCO model 6712) every six hours (00:00, 06:00, 12:00, 18:00) from a depth of 0.8 meter (m). The sampler was deployed on a moored buoy

approximately 0.5 miles off the western shore of Lake Winnebago (N 44º01.329' W 88º30.319'). Sample bottles were preloaded with 10 ml of glacial acetic acid as a preservative. Samples were retrieved every six days, transported on ice to the laboratory, and immediately frozen at -80 °C. Samples (*n* = 259) were collected between August and October 2013, which encompassed late September and early October cyanobacterial toxin blooms.

Acetic Acid as a Preservative

Acetic acid (5% final concentration) was used as a preservative of the water sample to prohibit degradation of the cyanotoxins. Preservation was tested in the laboratory by adding a known amount of a mixed cyanotoxin standard to replicate lake water. Samples were stored in a dark environment at 25 °C for 6 days to mimic the length of time samples would be left in the water sampler on the lake. After six days, samples were extracted for cyanotoxins using the methods described below. To our knowledge, this is the first study to use 5% acetic acid for cyanotoxin preservation. Acetic acid was chosen because it worked as an acidifying agent to the sample as the first step of the cyanotoxin extraction, and secondly it was an approved acid that could be lyophilized per the parameters of the freeze dryer (Labconco FreeZone).

Extraction and Analysis of Microcystins, Anabaenopeptins. Cyanopeptolins, Microginin, Cylindrospermopsin and Nodularin

Whole frozen water samples were lyophilized, non-selectively concentrating the target analytes in a sample, and the dried mass was resuspended in 1 mL of 0.1% formic acid and subjected to three freeze-thaw cycles at -80 °C and 55 °C, respectively. After adding 2 mL of 100% methanol, samples were placed in a sonicating water bath at 45 °C for 10 minutes and

then centrifuged at 10,000 x g for 15 minutes. One mL portions of the supernatant were transferred to liquid chromatography (LC) vials and stored at -20 °C until analysis.

Cyanotoxins were measured in 20 µg/L injections using liquid chromatography tandem mass spectrometry (LC-MS/MS) with electrospray ionization. Method specific details have been previously referenced (90), but briefly, an ABSciex 4000 QTRAP equipped with a Shimadzu Prominence HPLC was used with a reverse phase C18 column to elute the cyanotoxins. Cyanotoxins eluted from the column were detected on the mass spectrometer using a scheduled multiple reaction monitoring method. Optimized mass spectrometer settings as well as retention times and estimated detection limits are shown in Supporting Information (SI) Table S2.1.

Extraction and Analysis of Anatoxins

Using the whole water sample obtained from the automated water sampler as described above, 1 mL of sample was aliquotted into a 1.5mL tube and spiked with 5 µg/L $^{13}C_6$-Phenylalanine (^{13}C-Phe) (>99%, Cambridge Isotopes, Tewksbury, MA) in 0.1% formic acid, and acidified with 1 µL of formic acid. Samples were subjected to three freeze-thaw cycles at -80 °C and 55 °C, respectively. Samples were placed in a sonicating water bath at 45 °C for 10 minutes and then centrifuged at 12,000 x g for 15 minutes. The top 500 mL portions of the supernatant were transferred to LC vials and stored at -20 °C until analysis.

Anatoxin congeners were measured in 15 µg/L injections using LC-MS/MS with electrospray ionization as described in the method above. Toxins were separated using an isocratic gradient elution on a hydrophilic interaction liquid chromatography (HILIC) column (SeQuant ZIC-HILIC, 150 x 2.1 mm, EMD Millipore, Darmstadt, Germany) where the mobile

phase consisted of buffer A (60mM formic acid in HPLC grade water) and buffer B (60 mM formic acid in 100% acetonitrile). The isocratic gradient was 60% buffer B for 15 min with a 1 min equilibration between each sample run. Anatoxins eluted from the column were detected on the mass spectrometer using a non-scheduled multiple reaction monitoring method. Optimized mass spectrometer settings as well as retention times and estimated detection limits are shown in Table S1.

Isotopically labeled ^{13}C-Phe was used as a surrogate standard with the HILIC extraction method to differentiate between ATX-A and phenylalanine, given their identical product ion spectrum and same molecular weight, and to monitor percent recovery of target analytes. Adding a known amount of ^{13}C-Phe to lake water samples (n = 99), we recovered 99% of the compound.

Cyanotoxin Standards

Whenever possible, certified reference standards were used. Nodularin, MCLR and dmLR (Dha^{7}-MCLR) were certified reference materials from the National Research Council of Canada Biotoxins program (Halifax, Nova Scotia). MCLA (> 95%), MCRR (> 90%), and MCYR (> 90%) were purchased from Sigma-Aldrich (Milwaukee, WI) and MCLF (> 95%), MCLY (> 95%), MCWR (> 95%), MCLW (>95%), MCHtyR (> 95%), (> 95%), and MCHilR (> 95%) were purchased from Enzo Life Sciences (Farmington, NY, USA). AP-A (> 95%), B (> 95%) and F (> 95%), CP-1007 (> 95%), 1020 (> 95%), and 1041 (> 95%), and MG-690 (> 95%) were purchased from MARBIONC (Wilmington, NC, USA). ATX-A (> 96%) was purchased from Tocris Bioscience (Minneapolis, MN) as a racemic mixture. hATX (> 95%) and CYN (> 95%) was purchased from Abraxis (Warminster, PA).

Data Analysis

Cyanotoxin concentrations were calculated by comparing the peak area of transition ions in unknown samples to a standard curve of calibration standards for the C18 column and HILIC using a linear regression. All statistics were performed using R statistical software (166). A matrix of calculated cyanotoxin concentrations was imported into the R-statistical package to perform all descriptive statistics and Wilcoxon Ranked Sum tests were used to test for significant differences in mean concentrations of cyanotoxins. Spearman Rank correlations were used to compare the cyanotoxin classes to pigments and nutrients. A principal component analysis was performed on log transformed cyanotoxin concentrations, and individual points represent toxin profiles at every 6 hours, colored by month. The sampling map was created using 'ggmap' (167)

RESULTS

Preservation Efficiency

Testing the preservation of cyanotoxins with 5% acetic acid in a laboratory analysis revealed the recovery of cyanotoxins ranged from 100% to 46% with the majority of cyanotoxins having greater spike recovery with the acetic acid than without after 6 days (Table 2.1). Notably, APs and CPs would have unlikely been detected in this study if not preserved with acetic acid. The average percent recovery for all targeted cyanotoxins is 117 +/- 31%. In the absence of acetic acid as a preservative, the average percent recovery for all targeted cyanotoxins is 62 +/- 41%

Cyanotoxin Detection Frequency and Average Concentrations

Of the twenty-two cyanotoxins targeted, all but seven (MCLF, MCLY, MCHtyR, MCLW, CYN, NOD and hATX) were detected in the lake on at least one date. MCLR, MCRR and AP-B were detected in every sample, although with varying mean concentrations. Measured concentrations of cyanotoxins produced throughout the sampling season revealed MCLR (mean = 2.1 μg/L +/- 1.9 μg/L) and MCRR (1.2 μg/L +/- 1.5) had the two largest means of all the cyanotoxin congeners. Although present in every sample, AP-B's mean concentration was approximately 75% less than that of MCLR (AP-B = 0.6 μg/L +/- 0.5 μg/L) while CP-1007 had the third greatest mean concentration (1.07 μg/L +/- 1.5 μg/L) despite being largely not-detected in the August samples (Table 2.2, Figure 2.2).

Focusing on the MCs, the mean of MCLR was significantly higher than that of MCRR ($p < 0.001$) and highest means following MCLR and MCRR were MCYR > dmLR > MCHilR > MCLA > MCWR (Table 2). Looking at the MC toxin profile for the sampling season, the concentration and distribution of MCs was markedly different from August to October. Distribution of rare or less abundant MC congeners were dissimilar; MCLA predominately occurred in August through mid-September while MCHilR and dmLR were present sporadically for the duration of the sampling season, and MCWR wasn't detected until the end of September and then persisted through the end of October (Figure 2.2). MCLR and MCRR appeared to have a similar pattern of high and low toxin concentrations throughout the season, but interestingly their individual max concentrations were not measured from the same sample, or even the same day. The maximum MCLR concentration was measured September 22 at 18:00, during a non-bloom event (an event in which cyanotoxins were present but Chl and Phy fluorescence were not at

elevated levels that would indicate a cyanoHAB; Figure 2.2). The max MCRR concentration was measured more than two weeks later during the October toxin bloom (October 9 at 00:00).

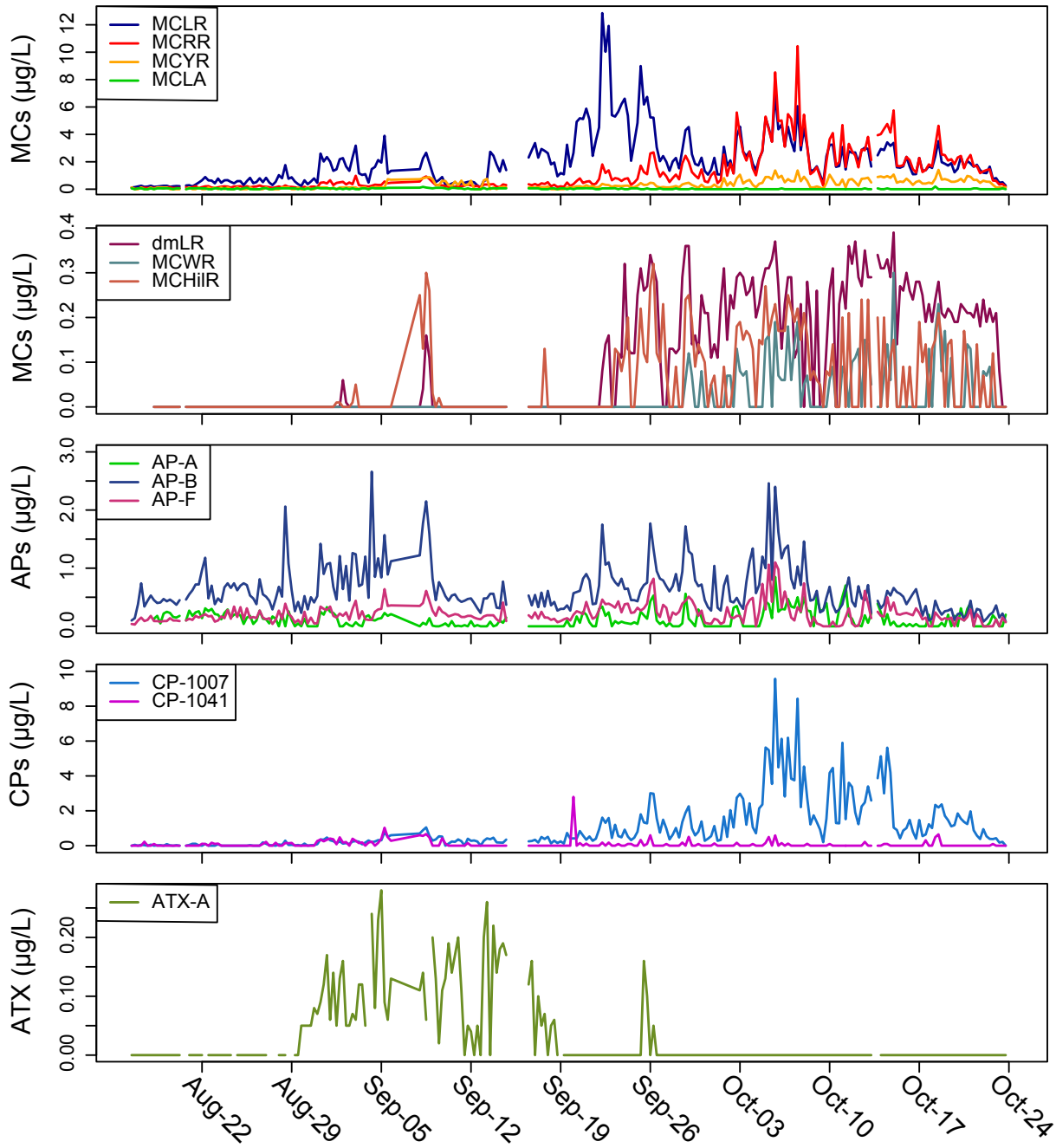

Figure 2.2: Time series of the individual cyanotoxin congeners (n = 22) measured at 6-hr intervals. MCs = microcystins, APs = anabaenopeptins, CPs = cyanopeptolins, and ATX = anatoxin-a. All but seven congeners (MCLF, MCLY, MCHtyR, MCLW, CYN, NOD and hATX) were detected in the lake on at least one date. CP-1020 and MG-690 were present sporadically and omitted from the line plot.

All three CPs targeted in this study were detected during the sampling season. CP-1007 was the dominant CP ($p < 0.001$) with a mean concentration of 1.03 µg/L (max = 9.57 µg/L, SD = 1.46 µg/L) followed by CP-1041 (mean = 0.08 µg/L, max = 2.8, SD = 0.22) and CP-1020 (mean = 0.01 µg/L, max = 0.14, SD = 0.03). There were two CP-1007 blooms, during early October and then again in mid-October. The max CP-1007 concentration was measured from the same sample as the max MCRR, midnight on October 9. The max CP-1041 was recorded during the September non-bloom event (Sep 20 at 12:00) (Figure 2.2). CP-1020 was present in 5% of samples with a max concentration 0.14 µg/L and as such, not included in Figure 2.2.

Anabaenopeptins were present throughout the sampling season at concentrations less than 3 µg/L. Specifically, AP-B was the dominant AP ($p < 0.001$) and present in every sample with a mean concentration of 0.64 µg/L (max = 2.65, SD = 0.42). The max AP-B was recorded in early September, September 4 at 18:00, whereas the max AP-F (max = 1.16 µg/L, mean = 0.21, SD = 0.17) and AP-A (max = 0.85 µg/L, mean = 0.12, SD = 0.14) were recorded during the October bloom from the same sample (October 6 at 6:00). AP-F and AP-A were present throughout the sampling season and mirrored the temporal pattern of AP-B but were detected at lower concentrations (Figure 2.2). Microginin was present in 5% of samples at less than 0.11 µg/L and is not shown in Figure 2.2. This cyanotoxin mainly occurred August to early September.

Anatoxin-a was detected at concentrations less than 0.3 µg/L (mean = 0.03 µg/L and SD = 0.06 µg/L) beginning August 30th and fluctuated at these low levels through September 19th, until it was detected again on September 26th. ATX-A was not detected in the October bloom (Figure 2.2) and hATX was not detected at all.

Cyanotoxin diversity of the water column followed a temporal trajectory, with timing within the season explaining 46% and 15% diversity of cyanotoxin congeners from August to October (Figure 2.3). Although ATX-A, MG-690 and MCLA were present in August and September, CP-1007, MCRR, dmLR, MCYR, MCHilR and MCWR were in greater abundance and concentration beginning mid-September and into October. The cluster of arrows extending from the origin of the PCA indicates the variability of analytes that can be described together. Positively correlated analytes point towards one side of the graph and negatively corelated analytes point to the opposite side.

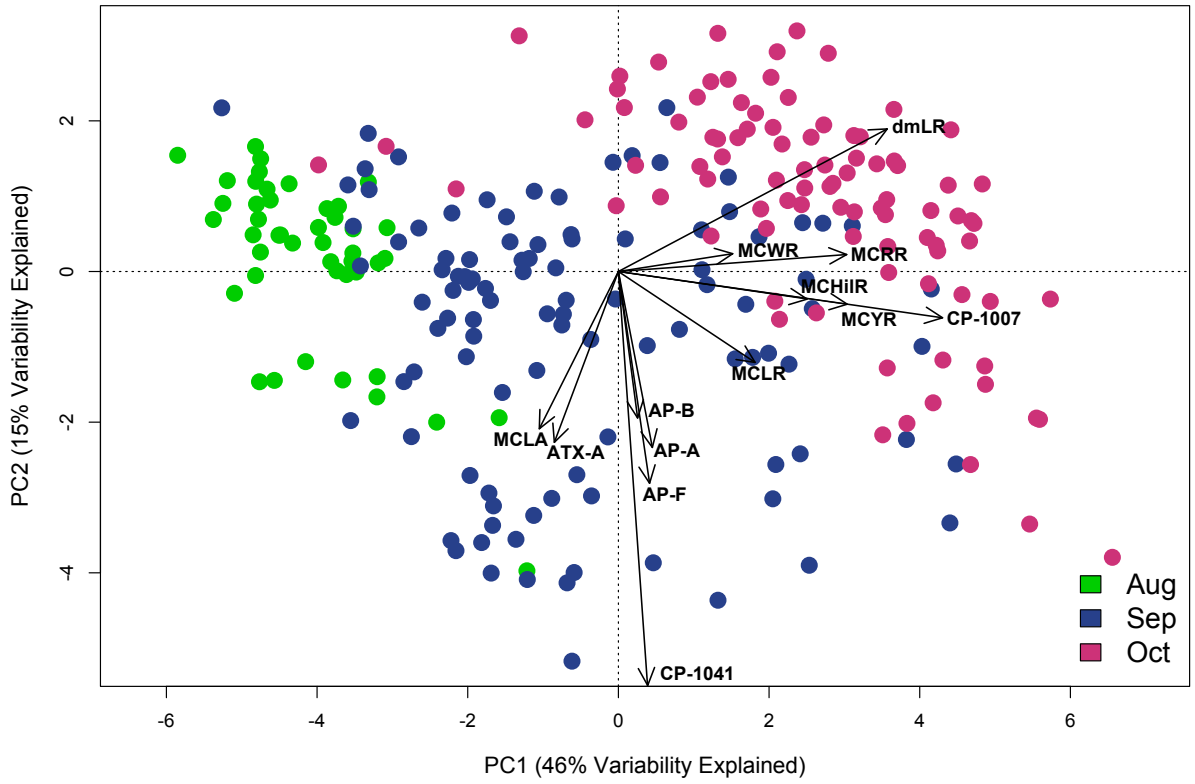

Figure 2.3: A principal component analysis was performed on log transformed cyanotoxin concentrations to explain cyanotoxin sample diversity, which followed a temporal trend. Samples are colored by sample collection month and arrows pointed towards one side of the graph indicate analytes that are positively correlated.

Phycocyanin, Chlorophyll, and Microcystin Relationship.

In situ fluorometers showed elevated levels of Phy greater than 500 millivolts (mV) on the first day the sensors were deployed, August 22nd. Phycocyanin remained elevated between 500-1500 mV through September 8th and decreased to 100 millivolts on September 9th demarcating the first bloom event, as recorded by fluorometers (Figure 2.4). A second bloom began October 7th and persisted through the remainder of the sampling season until October 24th when the sensors were retrieved from Lake Winnebago.

Chlorophyll fluorometers recorded fluctuating fluorescence levels of the pigment around 500 mV for the first two weeks of deployment (August 22nd – 31st), and a bloom was recorded September 29th and then again October 4th. This bloom continued until the sensors were removed October 24th, marking the end of the sampling season (Figure 2.4).

In the time defined as the non-bloom event per the low fluorescence recorded by the sensors, the sum of all MC congeners targeted in this study (SumMC) exceeded 8 µg/L (September 22nd), 2.4 standard deviations above the average MC concentration for the entire sampling period. MC concentrations increased from 5 to 15 µg/L over a 6-hour period during this non-bloom interval (Figure 2.4), a change of 3.2 standard deviations. The beginning of another MC event began October 3rd, with SumMCs greater than 10 µg/L during which Phy and Chl fluorescence suggested a cyanobacterial bloom was occurring.

From a monitoring standpoint, the use of Chl and Phy fluorometers for monitoring would have been useful as an indicator for noting changes in cyanotoxins concentrations. However, there are limitations to these tools as observed with the SumMC concentrations that were measured in the absence of the bloom. Chl was significantly correlated to SumMCs ($R = 0.2$, $p = 0.002$), SumAPs ($R = -0.14$, $p = 0.03$), and SumCPs ($R = 0.34$, $p < 0.001$). Phy was

significantly negatively correlated to SumMCs (R = -0.34, p < 0.001) and SumCPs (R = -0.019, p = 0.004), and not correlated to SumAPs (R = -0.062, p > 0.05).

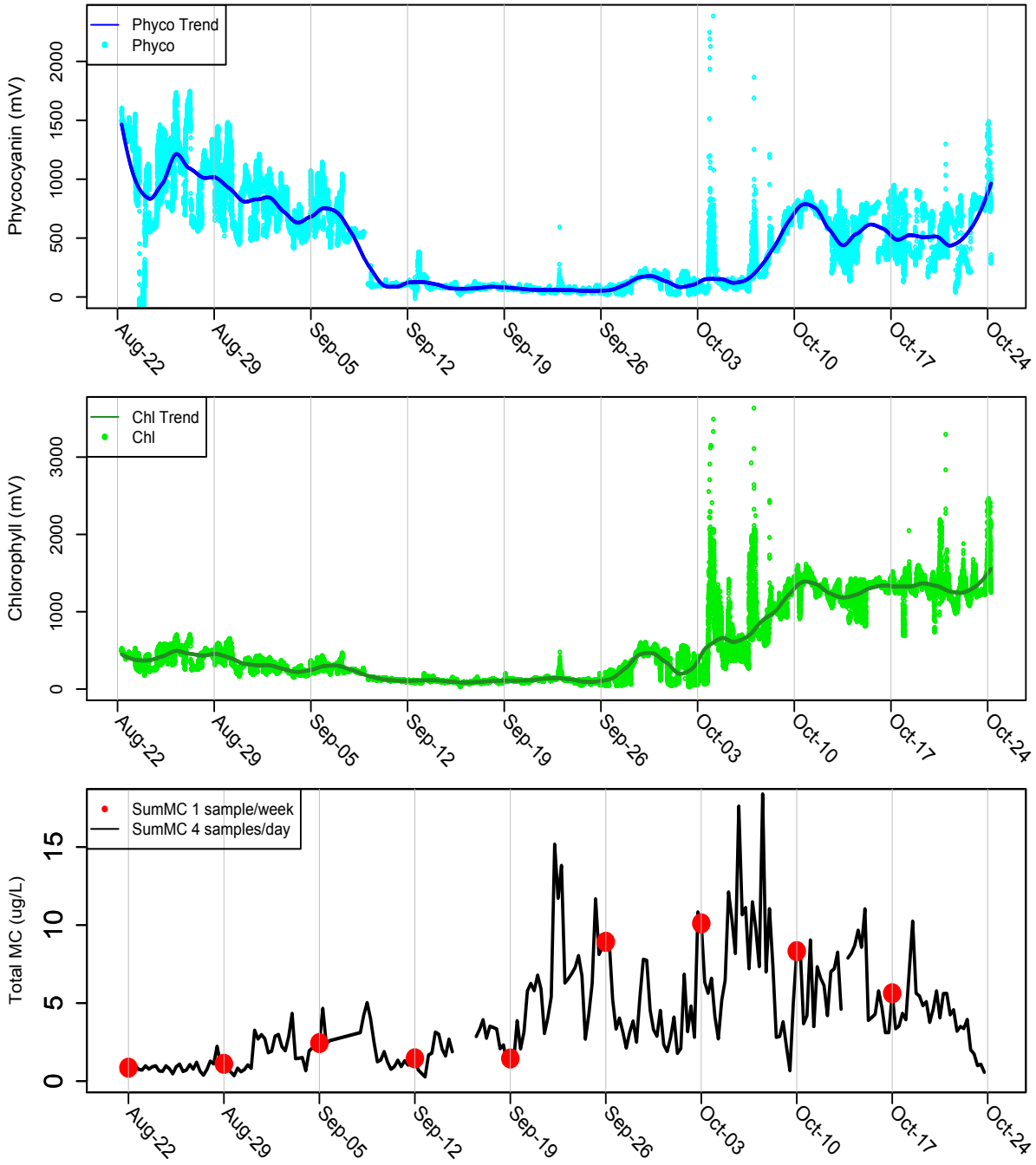

Figure 2.4: Time series plot of phycocyanin (top), chlorophyll (middle), and SumMCs (bottom). Chlorophyll and Phycocyanin were collected every 15 minutes, and SumMCs were collected every 6 hours. Red circles indicate samples collected at 12:00 on a weekly sampling strategy.

Sampling Frequency Analysis of Microcystins

Data points were removed from the original data set (sampling frequency of every 6-hours) to achieve a sampling frequency typical of sampling schedules for drinking water treatment of once or twice daily, and a sampling schedule typical of manual sampling by boat of once per week and twice per month (fortnight) (Table 2.3). The means between these sampling strategies were not significantly different ($p > 0.05$), however, the high-resolution sampling strategy provided a robust look at drinking water and recreational MC exceedances. Drinking water guidelines provided by the EPA state water containing 0.3 µg/L or greater MCs is not advisable for bottle-fed infants and pre-school children and 1 µg/L is the guideline for finished drinking water. Samples exceeded 0.3 µg/L MCs 97% of the time with a sub-daily sampling schedule and 77% of samples exceeded 1 µg/L. Guidelines for recreation are set at 8 µg/L MCs, of which 12% (n =31) of samples exceeded the guideline value whereas a daily 12:00 sample strategy would have missed 19 recreational exceedances.

Sampling every six hours versus sampling once per week (focusing on the samples taken at the noon hour) revealed an average difference in the max SumMC concentration of 9.1 µg/L (Table 2.3, Figure 2.4). More importantly, if sampling only once per week, it's possible the two MC bloom periods (as defined when SumMC sustained concentrations exceeding 8 µg/L) that were captured with the 6-hour sampling frequency would have been missed (Figure 2.4) as the duration of each MC event was less than one week. The first MC bloom occurred September 21th – 26th, and the max toxin concentration was measured from a 18:00 hour sample. High resolution sampling exceeded 8 µg/L 9 times while noon daily sampling exceeded it just twice. The second MC event that was observed due to high-resolution sampling occurred October 3rd

through October 8th. High resolution sampling exceeded 8 µg/L 11 times whereas noon sampling exceeded it 5 times. Interestingly, the max SumMC concentration, 18.4 µg/L, was observed at midnight (October 8th), whereas the daytime (i.e. noon) max was 12.1 µg/L (October 5th). However, there was no one sampling time (00:00, 06:00, 12:00, 18:00) that was most significant for SumMCs ($p > 0.1$).

DISCUSSION

In this study, a high-resolution sampling strategy for cyanotoxins was employed at the site of a drinking water treatment plant intake pipe. Sampling frequency was investigated to observe differences in cyanotoxin concentrations from a sub-daily sampling strategy with little concern for inclement weather to sampling strategies that are more attainable for a monitoring program (e.g. once per week, once per day and sampling in daylight hours). In this dataset, which encompassed one cyanobacterial growing season, we observed rapid changes in SumMC concentrations on a 6-hour basis and overall, sampling every six hours versus once per week captured the first, second and third max MC levels throughout the season (measured at 00:00, 06:00, and 06:00). Sampling once per week also would miss the detection of rarer cyanotoxin congeners, such as CP-1020, MG-690, which were measured sporadically.

Analyzing the samples collected every six hours, we observed the daytime (12:00) maximum for SumMCs was 12.1 µg/L, which was the fourth max SumMC concentration of the four sampling time points. The highest max SumMC was recorded at the midnight sample time. The max 06:00 sample was 17.6 µg/L, the 18:00 sample was 15.2 and 12:00 was 12.1 (Table 2.3). This may be explained by cyanobacteria's motility in the water column. Cyanobacteria have a unique ability to exploit high light conditions as well as maintain buoyancy during low

light conditions (168). In the case of low light conditions, cyanobacteria can start sinking after sunrise and continue in that manner throughout the day as carbohydrate stores increase (169). In early evening, when light intensity is low, cyanobacteria can then migrate to or near the water surface (143). Further complicating the ability to predict where cyanobacteria will be in the water column throughout the day, the bacteria can alter or reverse their buoyancy in response to small changes in their cell density (34). Cyanobacterial cells have been shown to be sensitive to ballast and gas-filled vacuoles so that density changes can happen on the magnitude of 0.5-5 hours (170). In fact, the greatest change in SumMC concentration was nearly 10-fold and occurred between samples collected at midnight and 6:00. However, no one time period (e.g. 00:00-6:00 or 12:00-18:00) can account for the majority of rapid changes in SumMC concentrations that we observed on a 6-hour basis. As drinking water production occurs 24 hours a day, 7 days per week, it is important to recognize the highest concentration of cyanotoxins may not occur in the hour at which sampling occurs. Furthermore, it cannot be assumed that the greatest concentration of toxins occur during the daylight, which makes monitoring for cyanotoxins a challenge.

Provisional guideline values were established by the World Health Organization (WHO) for total allowed MCLR equivalents in finished drinking water. Currently, it is recommend that drinking water should be concentrated with no more than 1 µg/L of MCLR equivalents (171). Of the 259 samples obtained in this study, there were 177 samples (68%) that exceeded the 1 µg/L MCLR guideline. If one considers a time of the day sampling is most likely to occur and focus only on noon samples, 72% of the days in this study had MCLR concentrations greater than the 1 µg/L WHO guideline value. Although noon samples were not always the most toxic sample in

a given day, the samples could contain a sufficient concentration of MCs to trigger a more intense sampling strategy if samples (raw water) are continually exceeding 1 µg/L.

Of further concern is the lack of provisional guidelines that consider the many different classes of cyanotoxins in drinking or recreational water. During peak bloom conditions, the max combined cyanotoxin and TBP concentration was 32.1 µg/L and comprised of 11 individual congeners, all with varying levels of toxicity and human health effects. Guidelines by the United States Environmental Protection Agency (EPA) recommend recreation should be avoided when waters exceed 8 µg/L MCs (51). The EPA also provided recommended recreational water quality criteria for CYN, although CYN was not detected in this dataset. These recommendations are for individual cyanotoxins classes and don't take into account the cumulative or additive effect of the many cyanotoxin congeners present in a cyanoHAB. Additionally, the mixture of cyanotoxins and TBPs in the water column changes throughout a sampling season (Figure 2.2). It's hard to tell from this dataset if the mixture of cyanotoxins and TBPs was more diverse later into the cyanobacterial season (Figure S2.2) because the max number of cyanotoxins and TBPs detected occurred on the first day of sampling (n=13) in mid-August. For some monitoring programs, August might be considered the middle or the end of the sampling season. The month of August overall had the lowest median (n=7) number of cyanotoxin and TBPs detected while September and October were n= 9. This could be attributed to the cyanobacterial species present throughout the season. It is important to note that ATX-A was measured near the beginning of the dataset in late August and September. ATX-A detects did not occur at any time when SumMCs were greater than the recreational guideline value of 8 µg/L, but they did co-occur with MCs, APs, CPs, and the sporadic MG-690.

Different cyanobacterial species could be responsible for the production of different cyanotoxins. ATX-A can be produced by *Aphanizomenom* and *Dolichospermum*, among others (76, 77). Several cyanobacterial species are known to produce MCs including *Microcystsis*, *Dolichospermum*, *Planktothrix* and *Oscillitoria* (48), while APs can be produced by *Dolichospermum*, *Aphanizomenom*, and *Microcystis*, among others. The co-occurrence of MCs and APs has also been observed in several other studies (92, 172, 173). Given the 100% frequency detection of MCLR, MCRR and AP-B it is possible the same cyanobacteria species was responsible for the production of these three congeners. Both *Planktothrix* and *Anabaena* have genes for MC and AP production (136, 174). *Microcystis* is also a producer of APs and MCs but some studies have shown that specific strains from a *Microcystis* culture do not contain genes for both (74-76). It is also possible that the three APs were produced by the same cyanobacteria, given their similar temporal profile (Figure 2.2). To understand the dynamics of toxic cyanoHABs, future work should include research on the cyanobacterial community, and the percent of a cyanoHAB that is toxic. This could be achieved by analyzing the percent of toxic and non-toxic strains of *Microcystis* by quantifying MC synthetase genes (113). This analysis would further be beneficial when considering an opposite scenario to the one observed at the end of September (i.e. MCs in the absence of a visual bloom)– a bloom with the absence of cyanotoxins. CyanoHABs aren't exclusively toxic. In addition to a diverse cyanobacterial community within a cyanoHAB, different strains of an individual species may be known producers of cyanotoxins or not. An example is *Microcystis,* a known producer of MCs, may not have strains present in the *Microcystis* bloom that have the genes to encode for the toxin (117,

118). Strains can be non-producers, lacking the ability to produces MCs (119). If the strains have the genes to encode of MCs, the genes may not always be expressed (120).

From a monitoring standpoint, trying to monitor all possible cyanotoxins would be challenging, expensive and not feasible. As the means between the sampling strategies were not significantly different, a weekly sampling or even biweekly could be enough to capture the cyanotoxin dynamics. It would be advantageous to understand cyanotoxin patterns with respect to Chl and Phy fluorescence. *In situ* fluorometers are becoming increasingly prevalent in lakes worldwide. Chlorophyll and phycocyanin fluorescence can give an indication of cyanobacterial biomass and this information can be useful for supporting monthly or bi-monthly sampling strategies. There are limitations to *in situ* fluorometers. One immediate limitation is the bias that can occur when a small but dense bloom or colony is measured by the fluorometer, which may not represent the conditions of the larger surface area of water. A heterogeneous sample is preferred instead. Secondly, fluorometers can be impeded by other suspended solids. There is an additional concern for fluorescent quenching – too much light can damage the pigments or even cause cell death (175). These are several reasons why fluorometers could underestimate or overestimate the pigment fluorescence, and in turn, the estimation of algal biomass.

Some monitoring strategies may rely on chlorophyll as an indicator for algal biomass and phycocyanin as an indicator for cyanobacteria toxicity (176) however, relying on fluorescence as an indicator of toxicity is inadequate. On a temporal scale, biomass is not an indicator of bloom toxicity. Despite the significant correlation between SumMCs and pigments, we observed periods of max toxin concentrations during non-cyanobacterial bloom events as

recorded by the fluorometers, and toxin per unit biomass was elevated during the pre-bloom period in late September. MCs are stable compounds (159, 177) and can persist in the environment when there is little cyanoHAB biomass (178) which could explain the first phycocyanin bloom that preceded the non-bloom event when max toxin per unit biomass occurred. Alternatively, laboratory studies have shown increases in toxic-strain production are associated with higher growth rates (179). Thus, it's possible that microcystin concentrations were highest during exponential growth just prior to any large accumulation of cyanobacterial biomass.

Without the use of an autonomous device, observing the variability of cyanotoxins and the relationship of pigments to toxins for an entire cyanobacterial growing season would be unfeasible. Furthermore, given the unpredictability of a lake and weather system, sampling at six-hour intervals is unrealistic. There is a need to develop autonomous sensors that not only can measure cyanotoxins in real-time, but are also affordable and accessible. The Environmental Sample Processor (ESP) is a notable example of an autonomous device that can measure *in-situ*, but it is extremely expensive. The alternative to an autonomous sensor is to use predictive modeling to forecast cyanotoxins. This method relies on high resolution water quality data, usually collected with a buoy deployment, accompanied with data from weather sensors and wave sensors to make cyanotoxin predictions in real-time. Arguably, acquistion of these data can also be costly. Being able to capture the true variation of cyanotoxin concentrations might be important for a fixed monitoring station, particularly a drinking water intake. Although there is an increasing concern for MCs in drinking water (52), there is not yet a federal MC monitoring mandate for drinking water treatment plants. It is recommended that

visual inspections for cyanobacterial blooms begin early in the season and occur often (180), but as observed in this study, it is possible for cyanotoxins to be present in the water without the visual indication of a cyanobacterial bloom. Maximum cyanotoxin levels are not necessarily associated with bloom conditions. Overall these data show that cyanotoxin levels are highly variable at a single point source but the success from this monitoring strategy would help provide valuable insight to cyanotoxin and pigment dynamics in the absence of real-time toxin monitoring.

ACKNOWLEDGEMENTS

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Table 2.1: Cyanotoxin preservation and recovery for individual cyanotoxin congeners. The recovery of the congener was tested with acetic acid as a preservative versus the recovery of the congeners in the absence of the preservative. Samples were left in the dark environment for six days to mimic the environment in the autosampler.

Cyanotoxin Congener	Acetic Acid Preservation (% Recovered)	No Preservation (% Recovery)
MCLR	136	101
MCRR	133	100
MCYR	113	73
MCLA	117	113
MCLF	103	108
MCLY	125	123
MCWR	60	57
MCHilR	129	70
MCHtyR	121	79
MCLW	47	81
dmLR	120	93
NOD	129	80
CYL	46	59
AP-B	154	0
AP-F	148	2
AP-A	134	0
CP-007	95	14
CP-1041	167	39
CP-1020	122	42
MG-690	134	0
ATX-A	125	NA
hATX	105	NA

Table 2.2: Mean and maximum concentrations and standard deviation (SD) of the cyanotoxin congeners measured in this study from August to October of 2013.

	Mean (µg L^{-1})	Max (µg L^{-1})	SD (µg L^{-1})	Coefficient of Variation	Frequency of Detect
SumMCs	3.83	18.40	3.31	0.87	1.00
MCLR	2.07	12.85	1.89	0.91	1.00
MCRR	1.23	10.44	1.53	1.23	1.00
MCYR	0.32	1.41	0.31	0.97	0.80
dmLR	0.11	0.39	0.13	1.16	0.44
MCHilR	0.05	0.32	0.08	1.52	0.35
MCLA	0.03	0.20	0.04	1.25	0.43
MCWR	0.02	0.30	0.05	2.29	0.18
MCLY	0.00	0.01	0.00	7.18	0.02
SumAPs	0.97	4.34	0.63	0.65	1.00
AP-B	0.643	2.655	0.416	0.65	1.00
AP-F	0.213	1.100	0.171	0.80	0.93
AP-A	0.12	0.85	0.14	1.16	0.70
SumCPs	1.12	10.16	1.48	1.32	0.92
CP-1007	1.03	9.570	1.462	1.42	0.88
CP-1041	0.083	2.800	0.228	2.75	0.29
CP-1020	0.007	0.119	0.030	4.54	0.05
MG-690	0.003	0.106	0.013	4.66	0.05
ATX-A	0.028	0.275	0.058	2.06	0.23

Table 2.3: Mean and maximum concentration of SumMCs determined by sampling frequency. Sub-daily sampling occurred every 6-hours.

	Mean (µg/L)	Max (µg/L)
4X/day	3.8	18.4
Daily		
06:00	3.9	17.6
12:00	4.2	12.1
18:00	3.7	15.2
00:00	3.6	18.4
Weekly		
Mon	4.6	9.8
Tue	4.9	11.1
Wed	2.8	6.3
Thu	4.5	10.1
Fri	3.4	9.0
Fortnight	4.3	9.0

CHAPTER 3

A multi-year analysis of cyanotoxins and toxic or otherwise bioactive peptides over a drinking water intake and possible environmental drivers

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Abstract

The presence and co-occurrence of toxins produced by cyanobacteria is problematic in water bodies used for recreation and drinking water production. This study sought to analyze environmental variables that may be associated with cyanotoxin blooms from multiple depths (surface water to bottom waters). Samples were collected over three years from the site of a drinking water intake pipe. A total of 151 samples were analyzed for 12 congeners within 5 classes of cyanotoxins. Pigment blooms and increases in cyanotoxins concentrations occurred in August during 2014 and 2015 and during September of 2014, which would have impeded recreation. Cyanotoxins were measured from subsurface as well as from the bottom waters. Of the environmental variables assessed, chlorophyll and phycocyanin were most correlative to cyanotoxins, although this relationship was specific to microcystins (MCs), and was weak with anabaenopeptins (APs) and cyanopeptolins (CPs). Concentrations of SumMCs, SumAPs and SumCPs were not significant with depth ($p > 0.05$). The recreational MC threshold established by the US Environmental Protection Agency was exceeded in 7 consecutive samples over the course of several weeks from August-September 2014. The drinking water threshold was exceeded 22 times during the same year, and several more times in 2015 and 2016. The cyanotoxin dynamics assessed here provide important insights into less studied, but frequently occurring toxic or otherwise bioactive peptides in drinking water and recreational waters.

1. Introduction

The occurrence of cyanobacterial harmful algal blooms (cyanoHABs), otherwise known as blue green algae, is on the rise (2). CyanoHABs have been observed in water bodies used for recreation and drinking water production. Cyanobacteria are fueled by nutrients, particularly nitrogen (N) and phosphorus (P) (5, 181) and human activities such as agriculture, land use change, and runoff from urban and rural landscapes can promote and accelerate their expansion (3, 4). CyanoHABs can be detrimental to humans, animals, and to general water quality. The blooms are aesthetically unpleasing and can form surface scums accompanied with noxious odors. The decomposition of blooms can lead to decreased dissolved oxygen and potentially cause hypoxic conditions. Additionally, cyanobacteria can produce toxins (cyanotoxins) that can be harmful to humans and animals (182, 183).

Commonly measured cyanotoxins include microcystins (MCs), which are potent liver toxins (184). MCs are cyclic heptapeptides and contain seven amino acids - the unique Adda side chain, four non-protein amino acids and two variable amino acids (53). MCs are structurally diverse with high molecular weights and more than 200 congeners are possible (54, 57). MCs are ubiquitously observed in freshwater systems. One of the most frequently studied MC is microcystin-LR (MCLR), which has leucine (L) and arginine (R) as variable amino acids, along with microcystin-RR (arginine and arginine; MCRR) (92, 185). MCs can inhibit protein phosphatase 1/2A (PP1/PP2A), phosphatases that play critical roles in cellular processes (55, 58, 186). Nodularin (NOD) is another liver toxin and protein phosphatase inhibitor, the same mode of toxicity as MCs. NOD is similar in structure to MC but does not have amino acids at positions

1 and 2 (66, 68, 187). NOD mainly occurs in brackish waters, but is increasingly being detected in freshwaters (48, 92).

In addition to the aforementioned toxins, cyanobacteria produce hundreds of other toxic or otherwise bioactive peptides (TBPs) (134-136). Anabaenopeptins (APs), cyanopeptolins (CPs), and microginins (MGs) are among many classes of TBPs that can be produced in tandem with MCs (90-92). These TBPs have a range of bioactivity on cellular enzymes including phosphates, chymotrypsin and thrombin. Some TBPs may be beneficial for commercial or medicinal uses such as antifungals, antimicrobials or antivirals (93, 94). However, recent studies have shown some TBPs may also be toxic to aquatic organisms like the crustacean *Thamnocephalus platyurus* and a neurotoxin in zebrafish (96, 97). Another study showed APs and CPs were toxic to *C. elegans* (98). The toxicity of cyanoHABs extends beyond MCs and other cyanotoxins.

Cyanobacteria have evolved adaptations to survive and even thrive in a range of environmental conditions although they are often noted for their dominance in warm, eutrophic waters. Mechanisms that aid in large-scale proliferation include buoyancy control (34) and nutrient sequestration and storage (35). Buoyancy control allows cyanobacteria to move throughout the water column due to gas vacuoles (40). Buoyancy can be altered in response to light and nutrients, giving cyanobacteria a competitive advantage against other organisms. Cyanobacteria have other mechanisms that allow them to compete for nutrients. Some species of cyanobacteria can fix atmospheric nitrogen (43) and in general, cyanobacteria have storage mechanisms for carbon, nitrogen and phosphorus (35, 44, 47). Storage capabilities can play an important role for dominance as cyanobacteria may be less immediately dependent

by nutrient availability (45, 46). Despite knowing many competitive adaptations associated with cyanobacteria, cyanotoxin production and dynamics remains an important area for further research.

Understanding cyanotoxin dynamics relevant to drinking water production and recreation remain an area of active research. CyanoHABs have been frequently observed in Lake Winnebago, an important recreational and drinking water resource in Wisconsin, USA (188). Lake Winnebago is a shallow, eutrophic inland lake. This multi-year study sought to analyze cyanotoxins and TBP's from a fixed monitoring station located near the site of the drinking water intake pipe. Sampling occurred from several depths within the water column – from the surface waters, which may be important for recreation, to the bottom waters, which can impact the intake of raw drinking water. This study focused on a suite of cyanotoxins and TBPs including MCLR, MCRR, MCYR, MCLA, Dha^{7}MCLR (dmLR), two anabaenopeptins – AP-B and AP-F, three cyanopeptolins – CP-1007, CP-1041, CP-1020, one microginin analog – MG-690 and nodularin (NOD). The temporal variability of these cyanotoxins was assessed along with pigments and nutrients to identify potential environmental drivers of cyanotoxins and toxic or otherwise bioactive peptides.

2. Methods

2.1 Study site

Lake Winnebago is the largest inland lake in Wisconsin with a max depth 6.4 meters (m) and surface area of 557 km^2. The lake is part of the Fox-Wolf watershed, fed by Lake Butte de Morts and Lake Winneconne and flows to the north into the Fox River (Figure 3.1). The Fox River and Lake Winnebago empty into Green Bay, Lake Michigan and provide an estimated one-

third of all phosphorus to Lake Michigan (163). In addition to being an important recreational resource, the lake serves as a drinking water source for more than 250,000 people in four municipalities. The Wisconsin Department of Natural resources sponsored a study of Wisconsin lakes in the mid 1960's that provided the first documentation of toxic algae in Lake Winnebago, followed by a statewide survey in 1986 and 1993 that found cyanotoxins (151-153). Since then, few studies have focused on cyanotoxin dynamics in Lake Winnebago although a recent study detected measurable amounts of cyanotoxins in raw intake drinking water (91).

Figure 2.1: Samples were collected from a fixed station indicated by the red circle, off the western shore of Lake Winnebago, WI.

2.2 Sampling

Samples were collected from a single station (N 44º01.329' W 88º30.319') approximately 0.5 miles off the western shore of Lake Winnebago during the cyanobacterial growing seasons from 2014 – 2016. Depth discrete samples were collected using a Van Dorn sampler that was lowered to 0, 1, and 3 meter (m) depths. The max depth at the site of this station was 3.8 m. Samples were transported to the laboratory on ice and aliquots of whole

water were immediately frozen at -80 °C for cyanotoxin analysis. Additional aliquots were collected for measurements of chlorophyll-*a*, phycocyanin (an accessory pigment for cyanobacteria), and total and dissolved nutrients, described in more detail below (Table 3.1). Samples for chlorophyll and phycocyanin were filtered through glass fiber filters (Whatman, 0.7 µm nominal pore size) and filters stored at -20 °C until analysis.

Sampling transpired May – October and a total of 151 samples were collected over three years. The majority of samples collected during August (34%) and July (24%). Sampling efforts were greatest in 2014, for a total of 29 sampling days or 58% of the total samples. Collection for 2014 spanned May – October with an increased sampling effort during July, August, and September. In 2015, sample collection spanned July – September, for a total of 7 sampling days. In 2016, sampling began June through August, and a final sample was collected in October, for a total of 14 sampling days (Figure S3.1).

2.3 Extraction and analysis of Cyanotoxins

Frozen whole water samples (10 mL) were lyophilized, non-selectively concentrating the target analytes in a sample and the dried mass was resuspended in 1 mL of 0.1% formic acid and subjected to three freeze-thaw cycles at -80 °C and 55 °C, respectively. After adding 2 mL of 100% methanol, samples were placed in a sonicating water bath at 45 °C for 10 minutes and then centrifuged at 10,000 x g for 15 minutes. One mL portions of the supernatant were transferred to liquid chromatography (LC) vials and stored at -20 °C until analysis.

Cyanotoxins were measured in 20 µg/L injections using liquid chromatography tandem mass spectrometry (LC-MS/MS) with electrospray ionization. Method specific details (e.g. optimized mass spectrometer settings, retention times and estimated detection limits) have

been previously referenced (90) but briefly, an ABSciex 4000 QTRAP equipped with a Shimadzu Prominence HPLC was used with a reverse phase C18 column to elute the cyanotoxins. Cyanotoxins eluted from the column were detected on the mass spectrometer using a scheduled multiple reaction monitoring method. Individual samples were analyzed quantitatively for 12 different cyanotoxins including 5 different MC congeners.

2.4 Analytical measurements

Total and total dissolved phosphorus (TP, TDP) were measured in non-filtered and filtered samples, respectively according to Valderrama (189). Particulate phosphorus (PP) was calculated by subtracting TDP from TP. Nitrite (NO_2) and nitrate (NO_3) was measured using Griess reagent and cadmium reduction method (190) and ammonia (NH_3) was measured according to Koroleff (191). Dissolved inorganic nitrogen (DIN) is the sum of NO_3, NO_2 and NH_3. Total organic carbon (TOC) was measured in non-filtered samples using a Teledyne TOC analyzer. Chlorophyll and phycocyanin were measured spectrophotometrically after methanolic or buffered water extraction on filters as described previously (43, 149).

2.5 Cyanotoxin Standards

Whenever possible, certified reference standards were used. MCLR, dmLR, and NOD were certified reference materials from the National Research Council of Canada Biotoxins program (Halifax, Nova Scotia). MCLA (> 95%), MCRR (> 90%), and MCYR (> 90%) were purchased from Sigma-Aldrich (Milwaukee, WI). APB (> 95%) and F (> 95%), CP1007 (> 95%), 1020 (> 95%), and 1041 (> 95%), and MG690 (> 95%) were purchased from MARBIONC (Wilmington, NC, USA).

2.6 Wind and Air Temperature

Wind and air temperature were collected from the Appleton International airport weather station (192). Data were used as 24-hour cumulative averages. Wind was reported in miles per hour (mph) and air temperature was in Fahrenheit (F).

2.7 Data Analysis

All statistics were performed using R statistical software (166). A matrix of calculated cyanotoxin concentrations was imported into the R-statistical package to perform all descriptive statistics. Kruskal Wallis was used on log transformed concentrations to test the differences in the mean of the cyanotoxin classes and individual congeners to depth. Spearman Rank correlations were used to compare the cyanotoxin classes to pigments and nutrients. A principal component analysis was performed on log transformed samples, focusing on cyanotoxin congener diversity for each sample point. Samples were grouped by trophic state, calculated from measured chlorophyll concentrations and an ANOSIM was performed to assess if samples (specifically if cyanotoxin congener profile) within each trophic state were similar to each other and dissimilar to other trophic state. Multiple linear regression was used to assess the association of environmental variables to SumMCs, SumAPs, and SumCPs from different depths. Environmental variables included in the regression analysis were significant ($p < 0.5$) to at least one of the cyanotoxin classes. Cyanotoxin concentrations were log transformed after setting concentrations of zero to 0.001.

3. Results

3.1 Cyanotoxin Congeners

<u>Cyanotoxin Class by Depth</u>

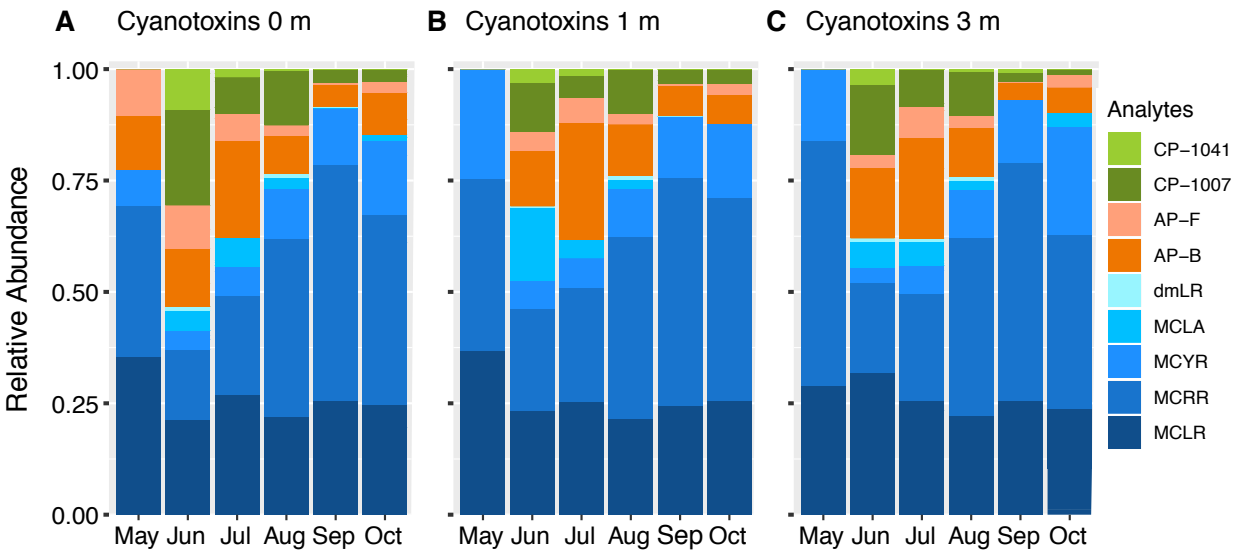

Figure 3.2: The relative abundance of each cyanotoxin profiles for cumulative years 2014 – 2016 for (A) samples from 0 m (B) Samples from 1 m and (C) Samples from 3 m. Microcystins (MCs) are represented in blue, anabeanopeptins (APs) in orange, and cyanopeptolins (CPs) in green.

Samples were collected from 0 m, 1 m, and 3 m and the max cyanotoxin concentrations were measured from 0 m samples for all cyanotoxin congeners except AP-B (Table 3.2). Among the three cyanotoxin classes, MC, AP, and CP, the MC congeners (n = 5) made up 78.5 % of the total cyanotoxin concentration from the three years of analysis. While MCs were the dominant class of the cyanotoxin pool for all three years, when CPs and APs are summed as the TBPs class, then TBPs were almost 50% of the cyanotoxin pool in 2015 (43%; Figure 3.2 B) and 47% in 2016 (Figure 3.2 C). TBPs were 14.5% of the cyanotoxin pool in 2014 (Figure 3.2 A).

The mean of the sum of all MC congeners (SumMCs) was 4.0 µg/L from 0 m, 3.3 µg/L from 1 m and 2.5 µg/L from 3 m (Table 3.2). The max SumMC, 21.4 µg/L, was measured in 2014, compared to the 2015 max SumMC, 9.0 µg/L, and 2.4 µg/L in 2014. The max SumMC for each year was measured from 0 m (Figure 3.4). The means of SumMCs were compared between the three depths and were not significantly different ($p > 0.05$; Kruskal Wallis). The mean of the sum of all AP congeners (SumAPs) was 0.7 µg/L at the surface, 0.6 µg/L from 1 m

and 0.5 µg/L from 3 m. The max SumAPs, 3.3 µg/L, was measured in 2016 from 1 m, followed by 3.2 µg/L in 2014, also from 1 m, and 2.2 µg/L (2015) from 1 m. The means of SumAPs were compared between the three depths (surface, 1 m and 3 m) and were not significantly different ($p > 0.05$; Kruskal Wallis). However, SumAPs were notable because the max concentrations were detected at 1 m. The mean of the sum of all CP congeners (SumCPs) was 0.6 µg/L from the surface, 0.3 µg/L from 1 m and 0.3 µg/L from 3 m. The max SumCPs, 3.8 µg/L, was measured in 2015, followed by 2.2 (2014), and 1.6 (2016). All three yearly max concentrations were measured from 0 m. The means of SumCPs were compared between the depths and were not significantly different ($p > 0.05$; Kruskal Wallis). However, even in this shallow system concentrations of cyanotoxin and TBP classes were stratified by depth with 0 m toxin profiles organizing differently than the 1 m and 3 m (Figure 3.3).

Individual Congeners by Depth

Within the MC class, MCRR and MCLR had the two largest means of cyanotoxin congeners. MCRR had a max concentration of 10.9 µg/L (3-year mean = 1.7 +/- 2.2 µg/L) and MCLR max was 6.0 µg/L (3-year mean = 1.0 +/- 1.0 µg/L). Neither MCLR nor MCRR were significant by depth (MCLR: $p > 0.05$ and MCRR: $p > 0.05$). MCYR (0.5 +/- 0.6 µg/L), MCLA (0.1 +/- 0.2 µg/L), and dmLR (0.02 +/- 0.07 µg/L) were not significant by depth either ($p > 0.05$).

Individual AP congeners were measured at relatively low (less than 1.0 µg/L) concentrations for the majority of samples. Mean AP-B from 0 m was 0.5 µg/L, 0.5 µg/ from 1 m and 0.4 µg/L from 3 m. Concentrations of AP-B were not significantly different by depth ($p > 0.05$). Interestingly, the max AP-B, 2.7 µg/L, was measured from 1 m, and was the only cyanotoxin congener whose 1 m sample exceeded the concentration of 0 m. Mean AP-F was 0.2

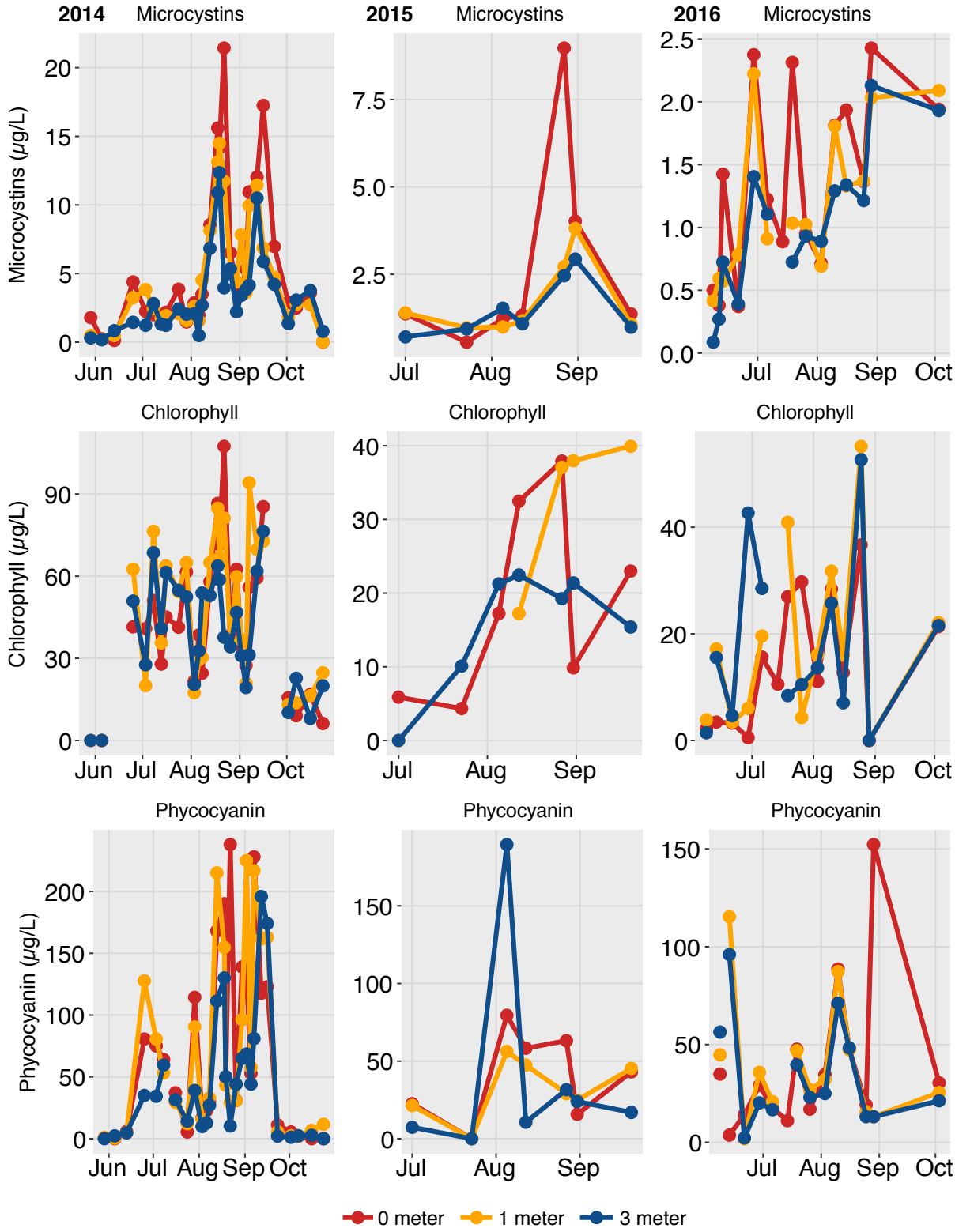

Figure 3.3: Time series analysis of microcystins (top), chlorophyll (middle) and phycocyanin (bottom) by depth: 0 m = red, 1 m = orange, and 3 m = blue.

µg/L from 0 m, 0.1 µg/L from 1 m and 0.1 µg/L from 3 m and concentrations were not significant by depth ($p > 0.05$).

CPs were measured at less than 1.0 µg/L for the majority of samples. Mean CP-1007 from 0 m was 0.5 µg/L, 0.3 µg/L from 1 m and 0.3 µg/L from 3 m; concentrations were not significantly different by depth ($p > 0.05$). The occurrence of CP-1041 was rare, with a mean concentration of 0.1 µg/L at 0 m and less than 0.1 µg/L at 1 m and 3 m; concentrations were not significantly different by depth ($p > 0.05$). CP-1020 was not detected from 2014 – 2016.

MG-690 was present sporadically at low concentrations (less than 0.1 µg/L) in 2015 and in August of 2016 and was not present at all in 2014. NOD was not detected in any sample.

Cyanotoxins and Advisory Thresholds

Recreational advisory limits were established by the U.S. Environmental Protection Agency and recommend 8.0 µg/L MCs as the limit or threshold for safe recreation (51). The EPA also published national drinking water health advisories for microcystins, which state an adverse health risk for children 6 and older consuming drinking water with at 1.3 µg/L MCs for 10 day and 0.3 ug/L MCs for infants. MCs were above the recreational threshold 7 times from surface water (24% of the samples collected), 6 times from 1 m (21%) and 3 times from 3 m (10%) in 2014 (Figure 3.3). SumMCs exceeded the drinking water threshold 26 times from surface water (90% of samples collected), 6 times from 1 m (21%) and 3 times from samples at 3 m (10%). It is important to note that from June 6 to October 16, 99% of all depth samples that were collected were above the drinking water threshold, with a mean concentration of

5.27 ug/L MCs, indicating that significant treatment strategies are necessary to remove MCs.

The recreational guideline was exceeded once in 2015 from 0 m (14% of the samples collected) and not at all in 2016. In 2015, the drinking water threshold was exceeded 6 times from surface water (86% of samples collected), 6 times from 1 meter (86%) and 4 times from 3 m (57%). In 2016, samples did not exceed the recreational threshold, but exceeded the drinking water threshold 9 times from surface water (60% of samples collected), 9 times from 1 m (60%) and 8 times from 3 m (53%).

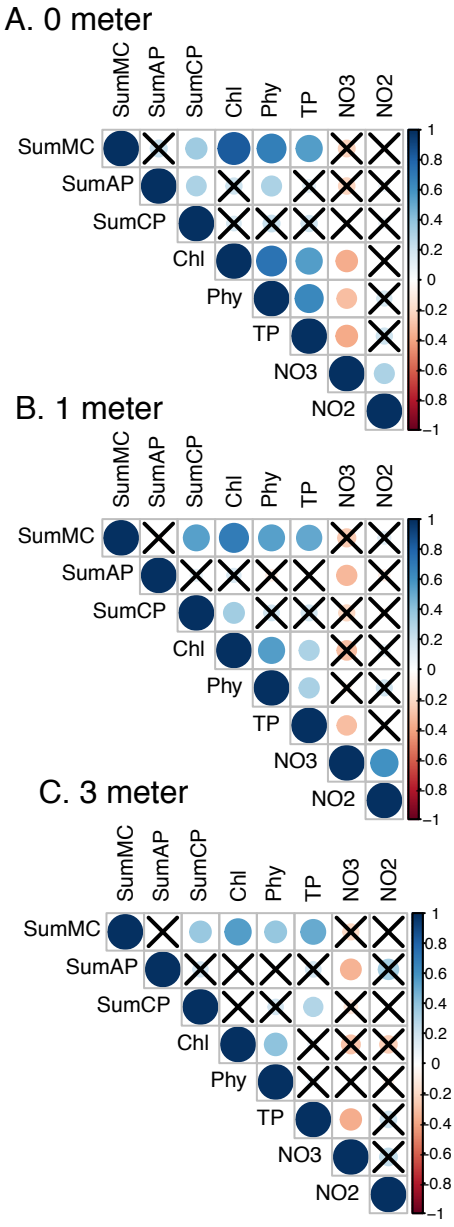

Figure 3.4: Correlational matrices comparing the correlation or dependence among the variables: SumMCs, SumAPs, SumCPs, chlorophyll (Chl), phycocyanin (Phy), Total Phosphorus (TP), Nitrate (NO3), Nitrite (NO2). An 'X' indicates the two variables are not correlated.

3.2 Relationships between toxins and pigments

Chlorophyll and phycocyanin were both significantly correlated with SumMCs ($R = 0.6$, $p < 0.001$ and $R = 0.5$, $p < 0.001$, respectively) (Figure S3.2). Correlation strength decreased between SumMCs and the pigments as water column depth increases (Figure 3.4). When considering all depths, chlorophyll and phycocyanin were significantly and strongly correlated to each other within the water column ($R = 0.60$, $P = < 0.001$). Similar to SumMCs, the strength of the pigment correlation is significant at all depths and is stronger at 0 m and decreases with

increasing depth. Chlorophyll was correlated to SumAPs ($R = 0.3$, $p < 0.001$) and SumCPs ($R = 0.2$, $p = 0.05$) and phycocyanin was correlated to SumAPs ($R = 0.4$, $p < 0.001$) and SumCPs ($R = 0.3$, $p < 0.001$).

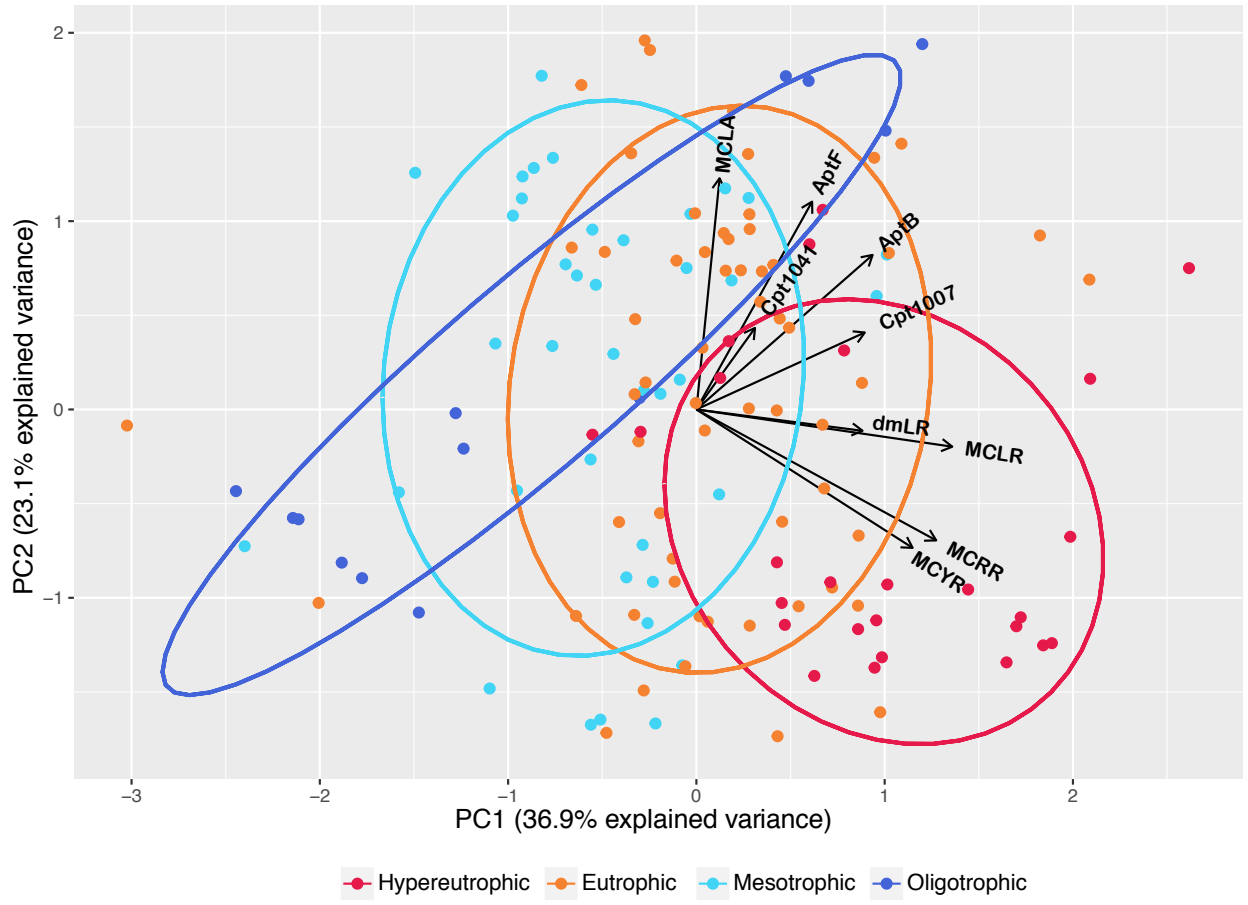

Figure 3.5: A principal component analysis was performed on log transformed samples. Each circle represents the cyanotoxin diversity and is colored by trophic state, identified by measured chlorophyll concentrations. Microcystin congeners -LR, -RR and -YR can be associated by hypereutrophic chlorophyll concentrations while the occurrence of MCLA and AP-F are associated more with mesotrophic conditions.

Calculated chlorophyll concentrations were used as a proxy for designating the trophic state of the water e.g. mesotrophic, eutrophic. Individual cyanotoxin and TBP congeners were log transformed and used in a principal component analysis. Sample points represent the specific diversity of toxins and TBPs of a sample, and sample are colored by trophic status

(Figure 3.5). The presence of some AP congeners and MCLA can be explained by mesotrophic conditions, whereas MCLR, MCRR, MCYR and dmLR dynamics can be explained by eutrophic or hypereutrophic conditions. The presence of MCLA can be supported by recent literature describing this relationship with mesotrophic waters as well (193). An ANOSIM revealed the trophic states groups were significantly not different ($R = 0.32$, $P = 0.001$).

3.3 Relationships between toxins and nutrients

TP median values were greatest in 2014, and peak chlorophyll biomass was also measured in 2014. Total phosphorus can be considered a proxy for algal biomass and was not included in further analysis as an environmental driver. TP correlated with SumMCs ($R = 0.6$, $p < 0.001$), not significantly correlated with SumAPs ($p > 0.1$) and significantly correlated with SumCPs ($R = 0.3$, $p = 0.001$). None of the other limnological variables were strongly correlated with SumMCs, SumCPs, or SumAPs (Figure 3.4).

3.4 Environmental Drivers

Using chlorophyll in a linear regression as an associated variable for SumMCs, the pigment was significant at 0 m ($p < 0.001$). Additional measured environmental variables (pigments, TP, as well as chlorophyll:phycocyanin, TP:chlorophyll) were included into a multiple linear regression as associated variables for SumMCs. The following variable was significant at 0 m: Phycocyanin ($p = 0.002$). Using the same approach for 3 m, Chlorophyll as the only variable in a linear regression was significant ($p < 0.001$). Including pigments, TP, as well as chlorophyll:phycocyanin, TP:chlorophyll, all variables were significant ($p </= 0.05$) (Table 3.3).

Previous correlations between cyanotoxin classes (e.g. SumAP, SumCP) and the measured environmental variables revealed few variables were strongly correlated to SumAPs

and SumCPs and indeed, multiple linear regression revealed no strong significance among either cyanotoxin class. The same variables that were used to predict SumMCs, were used to predict SumAPs from 0 m and 3 m. At 0 m, phycocyanin was significant as an associated variable ($p = 0.002$). Significant associated variables at 3 m were all but chlorophyll ($p </= 0.05$) (Table 3.3) For SumCPs, phycocyanin was significant at 0 m ($p = 0.002$) and there were no significant associated variables from 3 m.

4. Discussion

Several environmental variables known to be associated with cyanobacterial blooms (e.g. pigments and nutrients) were assessed as variables associated with cyanotoxins in an important drinking water and recreational water body. Although sampling frequency (i.e. number of samples/month) was different between each cyanobacterial growing season, sampling occurred during July and August for all 3 years, and June, September, and October for 2 years. Only 1 sample was collected in May for this project. Of the environmental drivers assessed, chlorophyll and phycocyanin were the most correlative to cyanotoxins (Figure 3.3). This correlation between cyanotoxins and pigment fluorescence was specific to MCs. APs and CPs were weakly correlated to pigment fluorescence and overall, the two cyanotoxin class concentrations seemed to be at low i.e. less than or equal to 1 µg/L levels, regardless of the month of sample collection.

CP and AP congeners accounted for 25-48% of the cyanotoxin profile in 2015 and 2016 when the mean SumMCs were lower (4.2 µg/L in 2014 vs. 1.8 and 1.2 µg/L in 2015 and 2016) (Figures 3.2). Including other cyanotoxin classes like CPs and APs into the discussion of cyanotoxin blooms and cyanotoxin monitoring is extremely important as the ecological

implication of TBPs is still an area of research. While MCs were the dominant cyanotoxin class, if you consider CPs and APs as a singular class i.e. TBPs, there were several occasions when TBPs were in similar abundance (in µg/L) with MCs (Figure 3.2). As inhibitors of carboxypeptidase A and PP1, APs may have human health effects (194) and CPs may have ecological impacts to aquatic organisms for additional inhibitory actions (195). Collectively, the TBPs should be considered within the cyanotoxin pool for monitoring programs. CPs and APs can be toxic in the aquatic environment to other organisms and assessing the toxicity of a cyanoHAB should extend beyond MCs.

Although cyanobacterial species composition was not analyzed in this study, historical and recent records indicate dominant cyanobacterial species in Lake Winnebago are *Microcystis,* A*phanizomenom* and *Dolichospermum (Anabaena)* (92, 196, 197). These cyanobacterial species have all been shown to produce the classes of cyanotoxins targeted in this study – MC, AP, and CP – although few studies have investigated the collective co-occurrence of these cyanotoxin and TBPs (134-136). The difference in cyanotoxin class dominance throughout the years could be due to the species present, which could be further related to the nutrients available.

Timing of cyanobacterial blooms and cyanotoxins are important factors to consider from a monitoring and modeling perspective. We would expect chlorophyll and phycocyanin to be correlated to cyanotoxins because both pigments are indicators of algal biomass. However, as observed in a previous study of Lake Winnebago (185), pigment blooms and cyanotoxin blooms don't always occur at the same time cyanotoxins (Figure 3.4). It is possible the pigments precede cyanotoxins production and act as a precursor or warning for a possible cyanotoxin

bloom, in which case the sampling frequency of this study would not have been robust enough to capture the cyanotoxin dynamics. It is also possible to observe a pigment bloom in the absence of cyanotoxins because not all cyanobacteria species produce cyanotoxins (198, 199). Variables that have been associated with toxic strain production over non-toxic strains include elevated water temperatures around 25 C and increased N and P, however results have varied by lake and/or contradict previous studies, and cyanoHAB drivers may be lake specific (113, 200, 201). Nutrient concentrations were at their greatest in 2014 as were chlorophyll, phycocyanin, and SumMCs. An area for further research would be to determine the percent of a cyanoHAB community that is toxin producing.

Another goal of this study was to assess the effect water column depth had on cyanotoxins and cyanotoxins drivers. Lake Winnebago is a large, shallow lake, and as such, it can quickly respond to temperature and nutrient changes which is in contrast to deep lakes (202). Given the potential for the lake to mix and stratify quickly, the lack of significance between cyanotoxin classes and depth is unsurprising. This information is important from a lake management perspective. Lake Winnebago is an important drinking water and recreation resource and surface water and bottom water has important implications for each resource. Based on results from this study, we know that if there are MCs at the surface of the water, there will likely be MCs at lower concentrations at the bottom water (Figure 3.4).

Cyanotoxins can be taken up by exposure via inhalation and ingestion (50). Ingestion can be a common exposure route through recreation and children are at particular risk given their recreational behavior and lower tolerance for cyanotoxins due to higher body burden (as opposed to adults) (137). The recreational guideline of 8 µg/L would have been exceeded on

seven consecutive occasions in 2014 from surface water samples and again in 2015 during the month of August, which is usually a peak recreational period. Alternatively, drinking water production occurs 24/7. SumMC concentrations from 3 m ranged from 0.08 – 12.4 µg/L and exceeded the drinking water advisory 90% of the time in 2014 from surface samples and 10% of the bottom water samples. Given the consecutive samples that exceeded the drinking water threshold in 2014 (mean concentration was 4.27 ug/L greater than the advisory limit) significant treatment strategies are necessary to remove MCs.

MCs are stable compounds (159, 177) and can persist in the environment when there is little cyanoHAB biomass (178). Their persistence in the environment is an important factor to consider for public health, when monitoring strategies rely on the visual aspect of a bloom as an indicator for bloom toxicity. Indeed, the effects of temperature, light, and availability of nutrients play important roles in the growth and potential dominance of cyanobacteria and determining the drivers for cyanotoxin dominance remains an area of active research. Key takeaways include summer cyanoHAB dominance, as well as MC blooms concentrating at the surface while also blooming at depths near bottom waters. Dominance between all three cyanotoxin classes occurred at different times and depths throughout the study; as more is learned about the combined health effects of these cyanotoxins, more emphasis will need to be put on other cyanotoxin classes than just MCs.

Table 3.1: Environmental variables measured in the study.

Phosphorus	Nitrogen	Carbon	Pigments
TP, TDP, PP	NO3, NO2, NH3, DIN	TOC	Chl, Phy

Table 3.2: Mean and max values for individual cyanotoxin classes by depth and year.

Year	Depth	SumMCs Mean	SumMCs Max	SumAPs Mean	SumAPs Max	SumCPs Mean	SumCPs Max
3-year	0 m	4.00	21.43	0.66	3.02	0.57	3.79
	1 m	3.29	14.50	0.63	3.26	0.33	1.47
	3 m	2.53	12.35	0.45	2.90	0.27	1.54
2014	0 m	5.65	21.43	0.60	2.45	0.43	2.22
	1 m	4.68	14.50	0.56	3.20	0.30	1.37
	3 m	3.51	12.35	0.35	1.62	0.18	1.37
2015	0 m	2.69	9.00	0.58	1.53	1.34	3.79
	1 m	1.74	3.81	0.83	2.21	0.76	1.47
	3 m	1.52	2.93	0.50	1.52	0.77	1.54
2016	0 m	1.38	2.42	0.83	3.02	0.48	1.57
	1 m	1.21	2.22	0.69	3.26	0.18	0.51
	3 m	1.03	2.13	0.64	2.90	0.20	0.53

Table 3.3: Variables for cyanotoxins as determined by multiple linear regression

Cyanotoxin Class	**Predictor Variable**	**P – value 0 m**	**P – value 3 m**
SumMCs	Chl	0.18	0.02*
	Phy	0.002*	0.01*
	TP	0.19	< 0.001*
	Chl/TP	0.79	< 0.001*
	Chl/Phy	0.23	0.005*
SumAPs	Chl	0.15	0.003*
	Phy	0.002*	0.19
	TP	0.54	0.015*
	Chl/TP	0.78	0.008*
	Chl/Phy	0.11	0.001*
SumCPs	Chl	0.11	0.73
	Phy	0.01*	0.78
	TP	0.41	0.19
	Chl/TP	0.15	0.73
	Chl/Phy	0.23	0.75

* = significant p-value

Chapter 4:

Spatial analysis of toxic or otherwise bioactive cyanobacterial peptides in Green Bay, Lake Michigan

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Abstract

Cyanobacterial harmful algal blooms (cyanoHABs) are a growing problem in freshwater systems worldwide. CyanoHABs are well documented in Green Bay, Lake Michigan but little is known about cyanoHAB toxicity. This study characterized the diversity and spatial distribution of toxic or otherwise bioactive cyanobacterial peptides (TBPs) in Green Bay. Samples were collected in 2014 and 2015 during three cruises at sites spanning the mouth of the Fox River north to Chambers Island. Nineteen TBPs were analyzed including 11 microcystin (MC) variants, nodularin, three anabaenopeptins, three cyanopeptolins and microginin-690. Of the 19 TBPs, 12 were detected in at least one sample, and 94% of samples had detectable TBPs. The most prevalent TBPs were MCRR and MCLR, present in 94% and 65% of samples. The mean concentration of all TBPs was highest in the Fox River and lower bay, however, the maximum concentration of all TBPs occurred in the same sample north of the lower bay. MCs were positively correlated with chlorophyll and negatively correlated with distance to the Fox River in all cruises along a well-established south-to-north trophic gradient in Green Bay. The mean concentration of MC in the lower bay across all cruises was 3.0 +/- 2.3 μg/L. Cyanopeptolins and anabaenopeptins did not trend with the south-north trophic gradient or varied by cruise suggesting their occurrence is driven by different environmental factors. Results from this study provides evidence that trends in TBP concentration differ by congener type over a trophic gradient.

INTRODUCTION

Cyanobacterial harmful algal blooms, or cyanoHABs, are a growing problem in freshwater systems worldwide including the Laurentian Great Lakes due to excessive nutrient pollution (203-205). Although cyanoHABs are naturally occurring, excess proliferation can have significant impacts on ecological health, as well as on the socioeconomics and human health of surrounding regions. Every year, toxins produced by cyanoHABs (cyanotoxins) are responsible for animal deaths, including pets and livestock (206) and in some cases have caused human illness and fatalities (207-209). Furthermore, decaying cyanoHAB biomass creates hypoxic/anoxic conditions harmful to fish and other aquatic life (155, 210).

Toxin-producing cyanoHABs have been described in some of the Great Lakes, although most studies have focused on the lower Lakes. Toxin-producing blooms are documented in Lake Erie (211, 212), Huron (213, 214) and Ontario (215, 216), where *Microcystis* and *Planktothrix* have been shown to be the major genera producing microcystins (MCs) (217, 218). Lake Erie is often used as a model ecosystem for Great Lakes cyanoHAB events, but it is currently unknown if trends found in Lake Erie extend to other cyanoHAB impacted areas, such as Green Bay. Surprisingly, there is a lack of information on cyanotoxins in Green Bay, a highly productive region in the Laurentian Great Lakes (154).

One of the most commonly observed or measured cyanotoxins in the Great Lakes region is microcystin (MC), a peptide where more than 200 different variants have been detected (54). A potent liver toxin (62, 63), MC acts by inhibiting protein phosphatases 1 and 2A (64, 65). The general structure of MC is a cyclic heptapeptide containing the unique Adda (3-Amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) side chain, plus four additional non-

protein amino acids and two variable amino acids (53). Variations in the MC structure are numerous, due to substitutions and modifications of its amino acid residues although MC variants with leucine and arginine (MCLR) or arginine and arginine (MCRR) are often the dominant congeners. Nodularin is a peptide with similar structure to MCs primarily occurring in brackish waters but is increasingly detected in freshwaters. It contains five amino acids and has the same mode of toxicity as MCs (66, 68, 187).

Cyanobacteria produce hundreds of other toxic or otherwise bioactive peptides (TBPs). These TBPs inhibit various proteases and may be beneficial for commercial or medicinal uses, such as antifungals, antimicrobials or antivirals (93, 94). Microginins (Mgn), for example, are inhibitors of proteases including an angiotension converting enzyme and may be useful in treating high blood pressure (110). Anabaenopeptins (APs) are also inhibitors of phosphatase 1 and 2A like microcystin (160) as well as inhibitors of carboxypeptidases (101, 102). At least 96 variants of APs have been reported and as such, the pharmacological effects of these peptides is an emerging area of study (103, 104). Cyanopeptolins (CPs) are cyclic serine protease inhibitors and may have pharmaceutical value as they may be applied in treatment of asthma or viral infections (106). Alternatively, a CP variant, CP-1020, has been shown in recent studies to be toxic to the crustacean *Thamnocephalus platyurus* and a neurotoxin in zebrafish (96, 97). Ecologically, TBPs other than MCs including APs, and CPs have been implicated in a variety of phenomena including inhibiting parasitic infections from chytrid fungi (219), preventing digestion of cyanobacteria by inhibiting zooplankton digestive enzymes (220, 221), and allelopathic competition (222). Thus, TBP diversity likely has implications for the ecology of cyanobacteria and their predators as well as for human health.

Despite decades of research, the causes, consequences and complexities of cyanoHABs remain too poorly understood to fully inform remediation, management and policy. As such, more information is needed about the occurrence of cyanotoxins, and collectively, TBPs. In this study, we focused on a suite of TBPs including eleven microcystins – MCLR, MCRR, MCYR, MCLA, desmethyl MCLR (dmMCLR), MCLF, MCLY, MCLW, MCWR, MCHtyR, MCHilR, three anabaenopeptins – AP-B, AP-F, and AP-A, three cyanopeptolins – CP-1007, CP-1041, CP-1020, one microginin analog – Mgn690 and nodularin. The spatial variability of these cyanotoxins was assessed in Green Bay, a large, shallow and eutrophic embayment in Lake Michigan. The bay experiences persistent nutrient pollution from point and nonpoint sources, including storm water and urban runoff, wastewater effluent and agriculture runoff, which can fuel cyanoHABs. There is a great need for information about cyanoHABs, their toxins, and other bioactive metabolites in this area that may pose recreational risk to swimmers, particularly children (137, 138). While there are no recreational beach monitoring programs in lower Green Bay, the EPA does have provisional guidelines in place for recreation with regards to total microcystin concentrations (223). Given the city of Green Bay plans to revitalize Bay Beach in lower Green Bay which may include reopening a swimmable beach (224) in addition to the expansive size of Green Bay and its role as a popular recreational hub, assessing the spatial variability of cyanotoxins is crucial. This is the first study of its kind to assess the spatial diversity of cyanotoxins in Green Bay, Lake Michigan.

METHODS

Study site

Lower Green Bay (an area of 55 km^2 of southern Green Bay) is listed as an Area of Concern (AOC) by the International Joint Commission and the State of Wisconsin (225). Unlike western Lake Erie and Saginaw Bay, very little is known about cyanobacterial bloom toxicity in this system. Previous studies have shown that the Lake Winnebago – lower Fox River – to – Green Bay corridor contributes approximately 1/3 of all phosphorus in Lake Michigan (154, 163) while the Fox River contributes approximately 70% of the nutrient and sediment loading although most of this is entrained in the lower portion of the bay (226, 227), giving Green Bay estuarine-like qualities as the transition zone from the Fox River to Lake Michigan. As such, the sampling sites in this study are spatially segregated along a series of east-west transects from north to south, divided into five geographic zones defined by water quality and trophic status (228) (Figure 4.1).

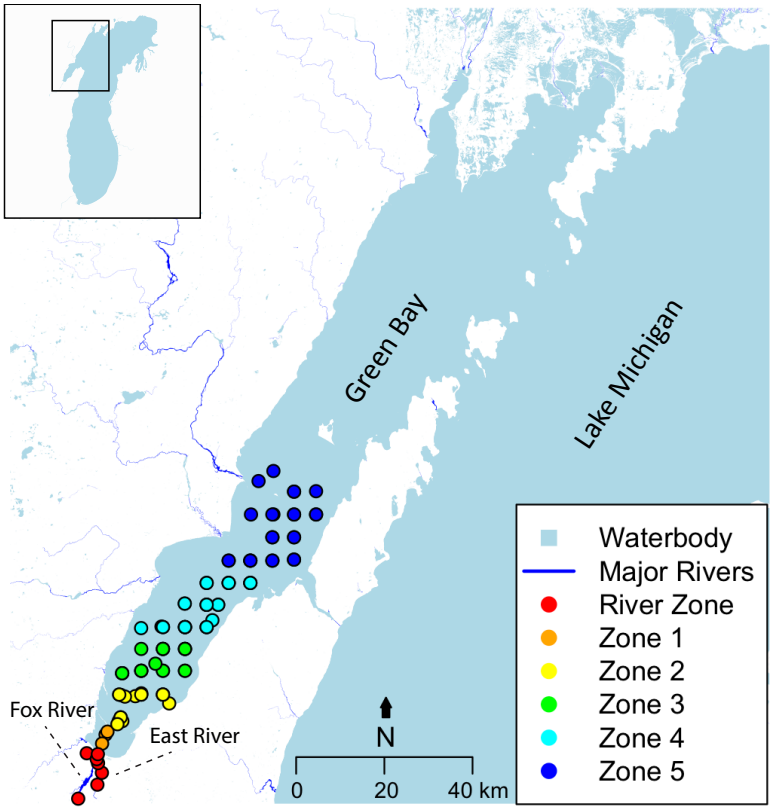


Figure 4.1: Sampling sites in Green Bay, Lake Michigan. Color indicates sampling zone.

Sample collection

Green Bay was sampled from the RV *Neeskay* during three cruises – August 2014, and July and August 2015. The sites were based on a 5x5 km grid that has been used in previous Green Bay studies (154, 229, 230). Samples were collected from the water column at 0 meter (m) and 1 m depths in 2014 and at 1 m depth

during both 2015 cruises. Samples collected at 0 m during the August 2014 cruise will be specifically referred to as such, whereas all other cruises with samples collected from 1 m will be referenced by their month and year (e.g. August 2014). Samples were collected via a submersible pump (flow rate ≈ 40 liters per minute) into 25 mL sterile plastic Vulcan® vials. Immediately following collection, 5 mL of sample water was pipetted out for shipboard fluorometer measurements using a Turner® handheld fluorometer. The remaining sample was sealed and placed in a freezer within 10 minutes of collection for TBP extraction and analysis.

Additional sites including the Fox River, East River (a tributary to the Fox River), and zones 1-3, were sampled from the Bay Guardian with NEW Water, the Green Bay Metropolitan Sewerage District, during the July 2015 cruise. These samples were taken from 1 m depth via a submersible pump into Nalgene bottles. Samples were kept on ice until processing immediately upon return to the lab. Samples were subsampled for TBP and chlorophyll analysis. For chlorophyll, water was filtered through 0.7 µm, 47 mm diameter Whatman GF/F filters (GE Healthcare, Pittsburgh, PA, USA). Filters were transferred to 15 mL tubes, amended with 90% acetone, sonicated and refrigerated overnight before spectrophotometric analysis (231, 232). Whole water was frozen at −20 °C until TBP extraction and analysis.

Extraction and analysis of TBPs

Frozen whole water samples (10 mL) were lyophilized and the dried mass was resuspended in 1 mL of 0.1% formic acid and subjected to three freeze-thaw cycles at -80 °C and 55 °C, respectively. After adding 2 mL of 100% methanol, samples were placed in a sonicating water bath at 45 °C for 10 minutes and then centrifuged at 10,000 x g for 15

minutes. One mL portions of the supernatant were transferred to liquid chromatography (LC) vials and stored at -20 °C until analysis.

TBPs were measured via 20 µL injections using liquid chromatography tandem mass spectrometry (LC-MS/MS) with electrospray ionization on an ABSciex 4000 QTRAP equipped with a Shimadzu Prominence HPLC. Cyanotoxins were separated using gradient elution on a reverse phase C18 column (Luna 3 µm C18 100 Å, LC Column 150 x 3 mm, Phenomenex, Torrance, CA, USA) where the mobile phase consisted of buffer A (0.1% formic acid and 5mM ammonium acetate in HPLC grade water) and buffer B (0.1% formic acid and 5mM ammonium acetate in 95% acetonitrile). The gradient began at 30% buffer B for 3 minutes, increasing over a linear gradient to 95% buffer B at 9 minutes, and held at 95% buffer B until 15 minutes at which point buffer B was returned to the starting condition until 20 minutes.

TBPs eluted from the column were detected on the mass spectrometer using a scheduled multiple reaction monitoring method. Compound specific parameters including ionization and collision energies were optimized for each compound by syringe infusion of reference standards at 1000 µg/L in 50% acetonitrile with 0.1% formic acid. Single charged ion species [M+H] were targeted for all MCs except MCRR, which preferentially takes on a double charge [M+2H]. Compound non-specific parameters including gas flows and ionization temperatures were optimized using flow injection analysis of standards in 70% methanol. Further details of the LC-MS/MS method are provided in Electronic Supplementary Material (ESM) Table S4.1 and have also been described previously (92).

TBP standard materials

Whenever possible, certified reference standards were used. Nodularin, MCLR and dmMCLR were certified reference materials from the National Research Council of Canada Biotoxins program (Halifax, Nova Scotia). Microcystin standards – MCLA (> 95%), MCRR (> 90%), and MCYR (> 90%) were purchased from Sigma-Aldrich (Milwaukee, WI) and MCLF (> 95%), MCLY (> 95%), MCWR (> 95%), MCLW (>95%), MCHtyR (> 95%), (> 95%), and MCHilR (> 95%) were purchased from Enzo Life Sciences (Farmington, NY, USA). AP-A (> 95%), B (> 95%) and F (> 95%), CP-1007 (> 95%), 1020 (> 95%), and 1041 (> 95%), and Mgn690 (> 95%) were purchased from MARBIONC (Wilmington, NC, USA).

Statistical Analysis

All statistics were performed using R statistical software (166). Pearson Moment correlations were used to compare the concentration of TBPs and chlorophyll to a spatial gradient (distance to the Fox River). Distance of sampling sites to the Fox River was calculated using the distCosine function in the R stats package 'geosphere' (233). Correlation matrices were visualized using the R stats package 'corrplot' (233). Correlations were considered significant at $P < 0.05$. Mann-Whitney U tests were used to test for significant differences in mean concentrations of TBPs, and an Analysis of Variance (ANOVA) was used to test for significant differences between the mean concentration of MCs by sampling zone.

RESULTS

Summary of TBPs Detected

Of the 19 TBPs targeted in this study, 12 were detected in at least one sample from Green Bay or the Fox River, including seven MCs, all three APs, and two of three CPs. The most prevalent TBPs were MCRR and MCLR, present in 94% and 65% of samples, respectively (Figure 4.2). The average MCRR concentration (0.53 µg/L) was slightly higher than that of MCLR (0.47 µg/L), but the concentrations were not significantly different ($P > 0.05$) (Table 4.1). AP-B was

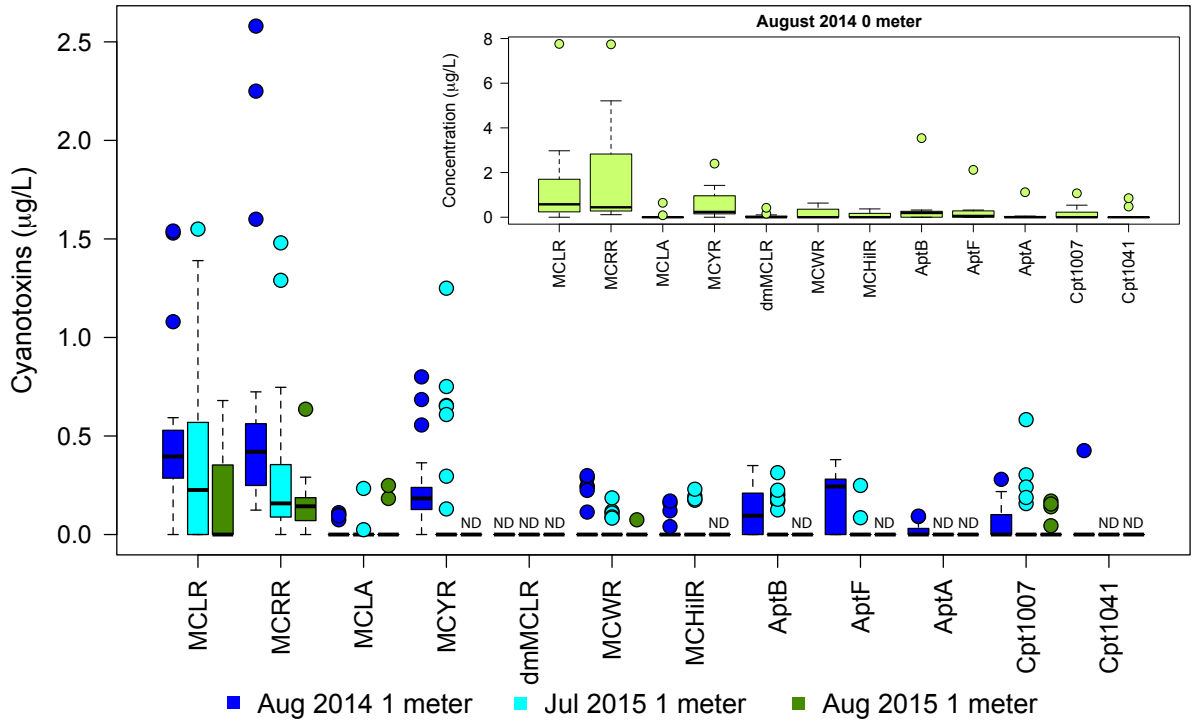


Figure 4.2: Concentration of toxic or otherwise bioactive peptides (cyanotoxins) detected on all cruises. The central line represents the median. The top and bottom of the box represents the 25th and 75th quartiles, respectively. The whiskers extend to data points that are not considered outliers, and solid circle symbols are outliers. ND = not detected.

the most abundant of the three APs followed closely in abundance by AP-F, present in 30% and 27% of samples, respectively. The mean concentrations of these two APs were similar at approximately 0.1 µg/L. The third AP targeted, AP-A, was detected in 12% of samples. CP-1007 was the dominant CP, present in 24% of samples with an average concentration of 0.06 µg/L. The other CPs targeted in this study were either detected infrequently (CP-1041) or not detected (CP-1020). The mean concentration for each CP was less than 0.1 µg/L. The maximum

concentration for all TBPs was measured in the sample from site 17 on August 27, 2014. Site 17 is approximately 34 km northeast of the mouth of the Fox River and the location of the UW-Milwaukee Green Bay water quality data buoy (Great Lakes Observing System; station 45014).

TBP Dynamics by TBP Type and Cruise

Microcystins

Among all cruises, the 0 m samples in August 2014 had the greatest number of sites where the sum of all MCs detected (SumMCs) was higher than 4 µg/L (4.98 µg/L ± 5.90 standard deviation (S.D.)), the provisional EPA recreational guideline value. Within this set of samples at 0 m, the four sites with SumMCs above 4 µg/L were in zones 1, 2, and 3 following a northeasterly line from the Fox River to site 17 in mid-bay (Figure 4.3). The greatest diversity of MC congeners was also observed in the 0 m samples from 2014 where 7 of the 11 MC congeners were detected. Interestingly, there were differences in the spatial distribution of individual MC congeners. dmMCLR was detected from zones 1, 2, and 3. MCWR, and MCHilR were also detected in zones 1, 2, and 3 only, whereas MCRR, MCLR, and MCYR were detected in all 5 zones. MCLA was detected twice, but only in zones further north, zones 3 and 5.

During the 2014 cruise, samples were taken at 1 m depth. Among these samples SumMCs in 2014 showed the greatest variability in concentration compared to all other samples and/or cruises (Figure 4.4). Two samples, both from zone 2, had SumMCs greater than 4 µg/L. The overall mean concentration of SumMCs across all 1 m samples in 2014 was 1.38 µg/L ± 1.29 S.D. and ranged from 0.12 - 5.27 µg/L spanning zones 2 - 5., MCLR, MCYR, and MCWR were detected in all the zones, whereas MCLA was detected only in northern zones 4 and 5, and MCHilR was only detected in zone 2. Interestingly, dmMCLR was not detected in

samples from 1 m, but was detected at 0 m. The max SumMCs was measured from zone 2 (5.27 µg/L) and the mean SumMCs were significantly different between zones (P = 0.002; ANOVA).

The July 2015 cruise included samples from all 5 zones and the Fox and East River (therein referred to as the river) (Figure 4.5). SumMCs ranged from below detection limits to 4.70 µg/L. The overall mean concentration of SumMCs during the July 2015 cruise was 0.86 µg/L ± 1.16 S.D. across all sampling stations (Table 4.2). MCRR, MCLR, and MCYR were detected in all five zones and the river, whereas MCWR and MCHilR were not detected north of zone 1. As in 2014, MCLA was detected in only northern zones. The max SumMCs was measured from the river samples (4.70 µg/L), following a gradient of high SumMCs closest to the river with decreasing max concentrations further from the river. The mean SumMC between zones were significantly different (P < 0.001; ANOVA).

Samples from the August 2015 cruise had the lowest mean and max SumMCs (0.32 and 1.40 µg/L, respectively) (Table 4.2) of all cruises, which spanned zones 2 - 5 (Figure 4.6). Similar to all other cruises, MCRR and MCLR were the dominant MC congeners with similar mean and max toxin concentrations (0.15 µg/L mean and 0.64 µg/L max for MCLR vs. 0.15 µg/L and 0.68 µg/L for MCRR). Unlike previous cruises MCLA was detected twice in zone 2 in addition to northern zone 4. MCWR was detected once from zone 3. The max SumMC was measured from zone 3 (1.4 µg/L) and the mean SumMCs among zones were significantly different during the August 2015 cruise (P = 0.002; ANOVA).

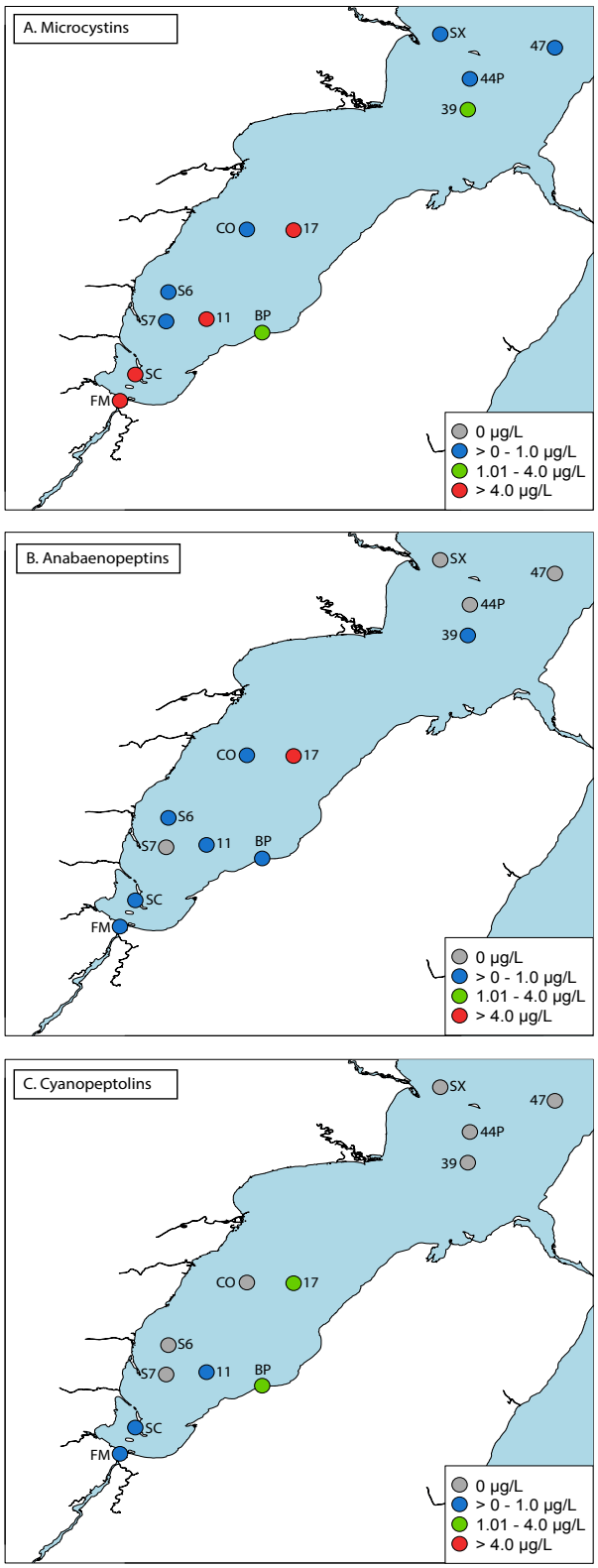


Figure 4.3: Spatial distribution of toxic or otherwise bioactive peptides during the August 2014 cruise at 0 meters.

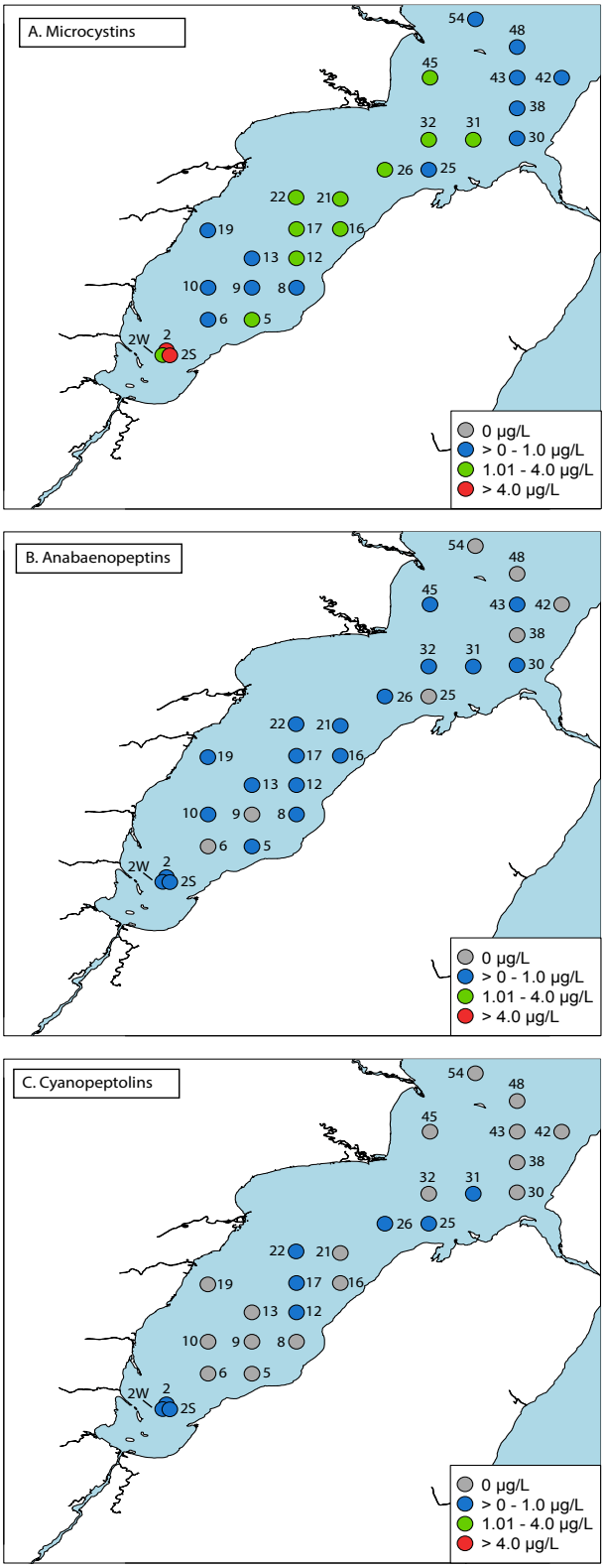
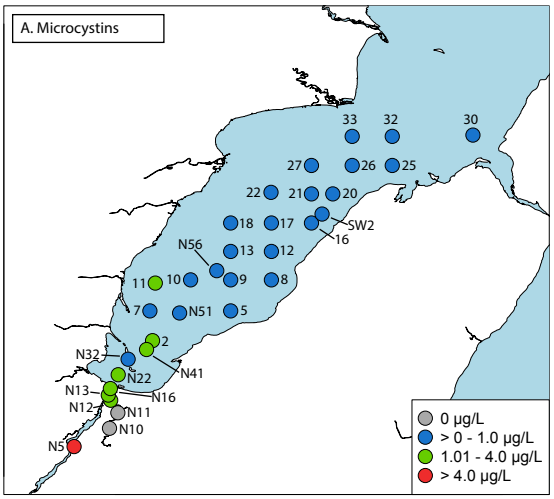
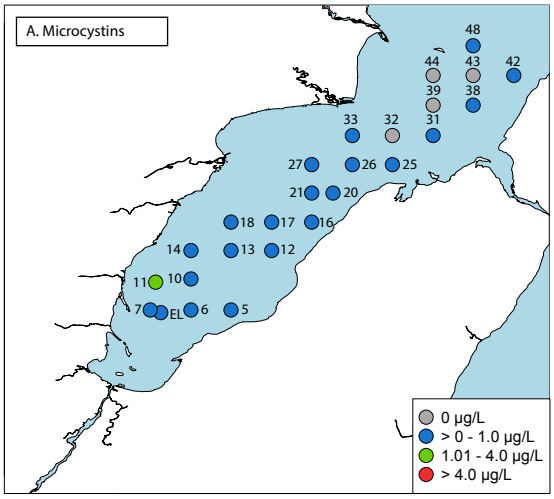
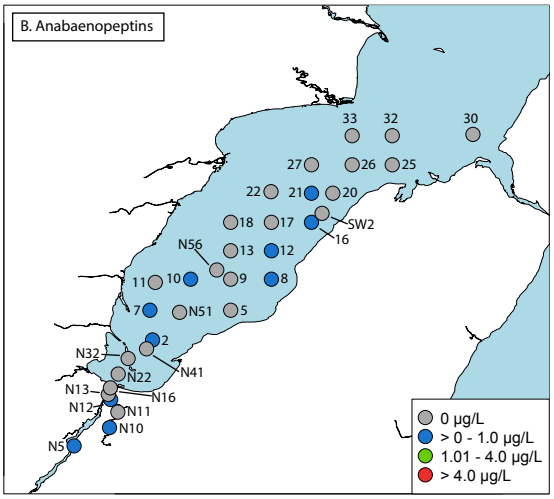
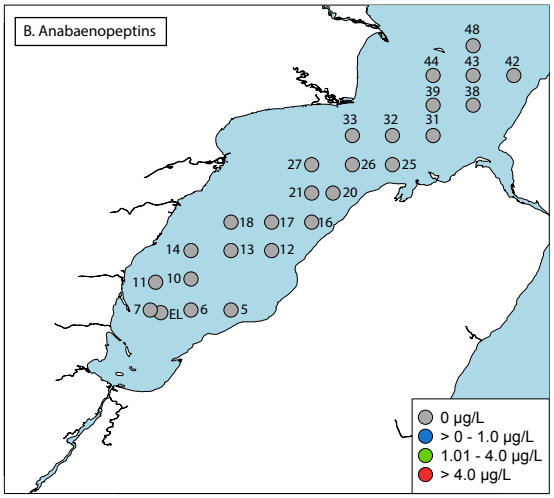


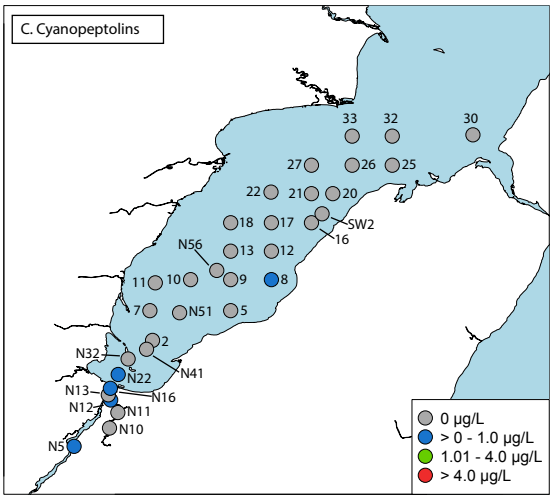
Figure 4.4: Spatial distribution of toxic or otherwise bioactive peptides during the August 2014 cruise at a depth of 1 meter.











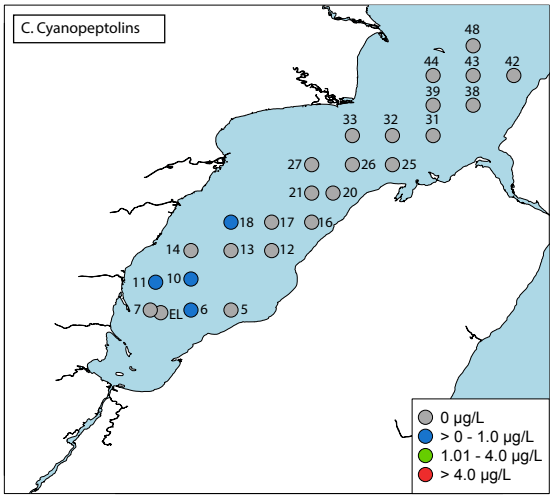


Figure 4.5: Spatial distribution of toxic or otherwise bioactive peptides during the July 2014 cruise at a depth of 1 meter. Samples denoted 'N' were collected by NEW Water.

Figure 4.6: Spatial distribution of toxic or otherwise bioactive peptides during the August 2014 cruise at a depth of 1 meter.

Anabaenopeptins

Similar to SumMCs, the max sum of all APs detected (SumAPs) occurred in August 2014 from 0 m. This max (6.78 µg/L) was from a zone 4 sample, specifically at site 17 (Figure 4.3) and was comprised of the three AP congeners targeted in this study – AP-B, F, and A. Among all 0 m samples, AP-B was most dominant, detected in 58% of samples with a mean concentration of 0.42 µg/L followed by AP-F (50% detection and 0.28 µg/L), and AP-A (33% detection and 0.10 µg/L).

Of the three AP congeners targeted, AP-F was most abundant during the August 2014 cruise. AP-F was detected in 69% of samples from all the zones sampled, zones 2 - 5, with a mean concentration of 0.19 µg/L (Figure 4.4). AP-B was also detected in zones 2 - 5 with a mean of 0.11 µg/L; whereas AP-A was detected in zones 3 - 5 with a mean of only 0.02 µg/L. The mean SumAPs was 0.32 µg/L ± 0.25 S.D. (Table 4.2).

During the July 2015 cruise, APs were detected in 33% of samples, specifically in zones 2, 3, 4, and the river (Figure 4.5). Specifically, AP-B was the dominant congener detected in zones 2, 3, 4 and the river, AP-F was detected in zone 3 and the river, and AP-A was not detected. The mean SumAPs was 0.06 µg/L ± 0.10 S.D. (Table 4.2)

Interestingly, no AP congener was detected in samples from the August 2015 cruise (Figure 4.6) even though they (SumAPs) were detected frequently in the August 2014 (71% of samples) and July 2015 (17%) cruises.

Cyanopeptolins

Among all cruises, the 0 m samples in August 2014 had the greatest mean sum of CPs (SumCPs) detected, equal to 0.30 µg/L ± 0.59 S.D. (Table 4.2). Max SumCPs was 0.53 µg/L and

was measured from zone 4 (site 17) (Figure 4.3). CP-1007 was the dominant congener and detected in zones 1, 2, 4, and the river, while CP-1041 was detected twice, in zones 2 and 4.

CP-1007 was also the most abundant CP in samples collected during the August 2014 cruise, present in 35% of sites spanning zones 2 - 5 (Figure 4.4). CP-1041 was detected in one site from zone 4. The mean SumCPs was 0.06 µg/L ± 0.12 S.D. (Table 4.2).

During the July 2015 cruise, CP-1007 was the only CP congener detected, present in 15% of sites spanning zones 1, 3 and the river (Figure 4.5). The mean SumCPs was 0.04 µg/L ± 0.12 S.D. (Table 4.2). CP-1007 was also the only congener detected in samples collected during the August 2015 cruise, present in 15% of sites spanning zones 2, 3, and 4 (Figure 4.6). The mean SumCPs was 0.02 µg/L ± 0.06 S.D. (Table 4.2).

TBP Trends with Trophic Gradients

Previous research has established that Green Bay is characterized by a trophic gradient from a eutrophic or hypereutrophic environment in the Fox River and zone 1 (i.e. the AOC) transitioning to a mesotrophic environment in zone 2 and all zones north (234, 235). Our chlorophyll results confirmed a chlorophyll gradient was present on all three cruises (ESM

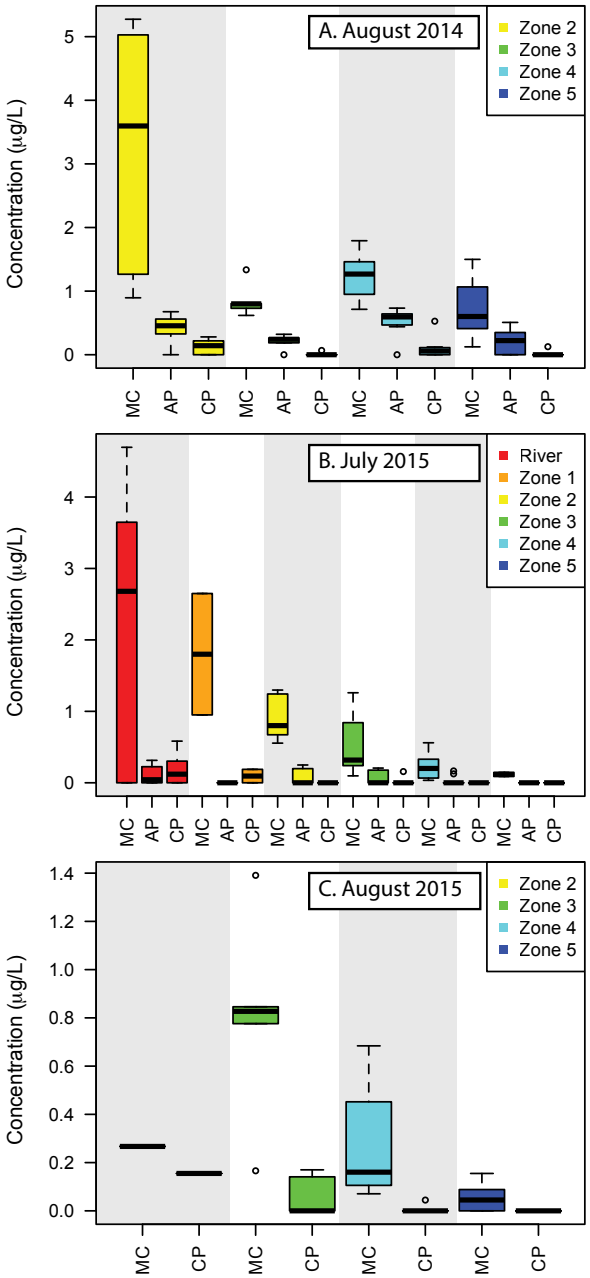


Figure 4.7: Concentration of the sum of all congeners of MC, APs, and CPs for samples taken at a depth of 1 m; A. August 2014, B. July 2015, and C. August 2015. The central line represents the median. The top and bottom of the box represents the 25th and 75th quartiles.

Figure S4.1). The July 2015 cruise included sites throughout all zones as well as the river in order to determine whether TBPs follow a similar gradient using chlorophyll as a trophic state indicator (Figure 4.7). As expected, chlorophyll decreased significantly (R = -0.59, P = 0.0042) with increased distance from the mouth of the Fox River (lat = 44.53778 lon = -88.03889), as did MCs (R = -0.60, P = 0.00026) (Figure 4.8). The August 2014 cruise (1 m samples) and August 2015 cruise (1 m samples) did not include samples from zone 1 or the river. However, significant correlations were still observed between chlorophyll (R = -0.59, P = .002) and MC (R = -0.91, P = <0.0001) and distance to the Fox River in 2014 as well as in 2015 (R = -0.70, P = .0002 for MC; R = -.80, P = <0.0001 for chlorophyll). These correlations suggest that trends in MC concentration along the trophic gradient persist into zones beyond the AOC.

In August 2014, CP and chlorophyll were not significantly correlated (R = 0.28, P = 0.16) nor were CPs significantly correlated with respect to distance from the Fox River (R = -0.22, P =

0.27) (Figure 4.8). However in 2015 CP was correlated with distance to the Fox River in samples from the July 2015 and August 2015 cruises, (R = 0.84, P = <0.0001 and R = 0.76, P = <0.0001, respectively), and strongly correlated with chlorophyll (R = -0.37, P = 0.04 and R = -0.62, P = 0.002, respectively). APs did not decrease significantly with distance to the Fox River on any cruise and was not correlated with chlorophyll (P > 0.05). Thus, only MCs showed a consistent trend with trophic gradients in Green Bay on these cruises whereas other TBPs did not trend with the trophic gradient or showed a variable response. This suggests that the production of MCs and other TBPs are not driven by the same ecological conditions.

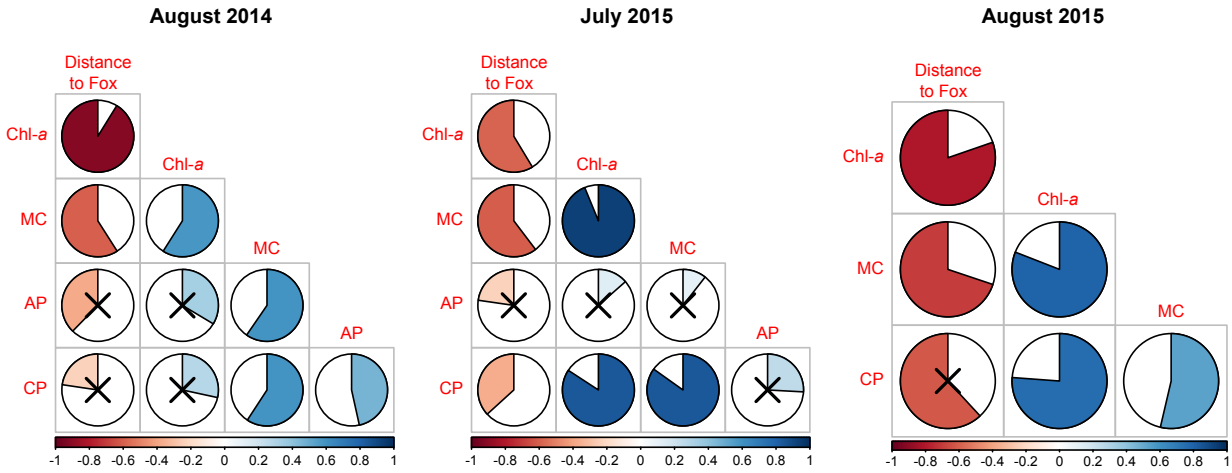


Figure 4.8: Results from pair-wise correlations among the variables: SumMCs, SumAPs, SumCPs, chlorophyll (Chl-*a*), and Distance to the Fox River for samples taken at a depth of 1 m. An 'X' indicates the two variables are not significantly correlated (P<0.05). Positive correlations are represented in blue and negative correlations in red; correlation coefficient is represented by the size and color of the pie.

DISCUSSION

CyanoHABs have been frequently observed in Green Bay (236-239) fueled by excessive nutrient runoff from the Fox-Wolf watershed. While much is known about the biogeochemistry and phytoplankton ecology in Green Bay, this is the first spatial analysis of cyanoHAB toxins and other metabolites (i.e. TBPs) of human health concern in Green Bay, from the mouth of the Fox

River to south of Chambers Island. To date, very few cyanotoxin studies have taken place in Green Bay, despite this being the largest freshwater estuary in the world and highly eutrophic. The influx of nutrients combined with shallow waters in the lower bay creates an ideal environment for the proliferation of cyanobacteria and formation of cyanoHABs. This study describes congener- specific changes in cyanotoxin profiles over a trophic gradient.

One limitation of this study is the lack of data on cyanobacterial community composition. In Green Bay, early reports from 1939 described blooms of *Aphanizomenom* beginning in early June followed by *Microcystis* dominance in mid-July with *Anabaena* (now *Dolichospermum*) present but in low abundance (239). More recent work confirms all three genera are still the dominant cyanobacteria taxa seasonally in Green Bay in moderate to high abundance (240). All three genera are known to produce a variety of TBPs (241). Of those TBPs targeted in this study, MCs, CPs, APs, and microginins have been detected in both *Microcystis* and *Dolichospermum* taxa as well as genes for their biosynthesis (53, 66, 242, 243) while *Aphanizomenom* taxa have been shown to produce APs (94, 101). Whether these genera are responsible for production of the TBPs targeted in this study in Green Bay is unknown. Answering that question is complicated by the fact that multiple genera have been shown to produce individual TBP congeners, the genes for TBP synthesis can be mutated and/or lost, potentially gained through horizontal gene transfer, and transcriptional/translational regulation may increase or decrease TBP synthesis according to cyanobacterial physiological status. Thus, TBP producers cannot be identified through a microscopic examination. An analysis of TBP RNA transcript abundance may provide one avenue for the identification of TBP producers but was beyond the scope of this study.

Data from this study informs the development of beneficial use impairments in Green Bay. Green Bay is an important recreational resource, supporting many sport fisheries and is a popular destination for summer water activities. EPA's draft recreational water quality criteria state water should not exceed 4 μg/L MCs for safe recreation. In 2014, 16% of samples exceeded 4 μg/L and in 2015, 2% exceeded the guideline. Most of the exceedances were located in the AOC.

Use of Lower Green Bay as a drinking water resource is considered impaired under the AOC guidelines. According to the Lower Green Bay and Fox River Area of Concern remedial action plans from 1988 to 2017, beneficial use of Green Bay for recreation and drinking water is impaired due to cyanobacteria and recent action plans cite an absence of sufficient data on concentration and type of toxins present. Thus, the results of this study directly addressed this need.

Currently, 1 μg/L of MCLR equivalents (MCs) is used as the standard for listing lower Green Bay as impaired for use as a drinking water resource under the AOC listing (244), which is the same guideline established by the World Health Organization (WHO) (245) for drinking water. However, historically there has been a lack of data describing MC concentrations in Green Bay including the lower Green Bay AOC making this beneficial use impairment questionable. This study provides some baseline data to inform the AOC guidelines. We report here that of all the samples, 50% exceeded 1 μg/L MC in 2014 and 14% of samples exceeded the threshold in 2015, for samples from all sites in the study, not just those in the AOC. Thus, impairment of the Lower Green Bay AOC for drinking water production is warranted. While one municipality (Marinette) uses Green Bay as a drinking water source, it is located far north of the

AOC. However, it is important to consider that cyanoHAB toxicity is highly variable from site to site and from year to year (92, 246, 247). Indeed, the highest TBP concentrations were measured in a sample well north of the AOC.

MC concentrations reported here in Green Bay are comparable to other eutrophic water bodies. SumMCs in Green Bay ranged from <1 – 20 μg/L, with an average of 1.27 ± 2.52 μg/L, which is similar to Bay of Quinte, Lake Ontario (2.40 ± 0.5 μg/L) (248) and Sodus Bay, Lake Ontario (<1 – 20 μg/L) (249). MCs in Lake Erie vary from extreme concentrations of 3,144 μg/L and 570 μg/L measured from surface or shallow water scum samples, to an average of 1-3 μg/L in open water (as reviewed in (133)). A robust study in the early 2000's describes MC concentrations in New York lakes (including Lakes Erie, Ontario and Champlain) ranging from not-detected to > 20 μg/L (204). Thus, MC concentrations in Green Bay are similar to other eutrophic environments in the Great Lakes region that have been impacted by cyanoHABs.

Currently, recreational and drinking water guideline values do not exist for CPs and APs in the United States. These bioactive peptides are considered "nontoxic" and little is known about the pharmacological effects of these peptides either from exposure to individual TBPs or as a mixture of APs, CPs, and MCs, which is common in nature and in this study. Some of these TBPs exhibit similar modes of toxicity as MCs, but yet do not exhibit similar toxicity, one example being AP-F (160). From an ecological standpoint, AP-F and AP-B are interesting because they have been shown to lyse certain cyanobacterial species (222), and as protease inhibitors it has been suggested that they may function to inhibit digestive enzymes in crustaceans, making cyanobacteria that produce them a poor food source. Indeed, CPs have been found to be highly toxic to freshwater crustaceans, and they have also been classified as a

potential neurotoxin in zebra fish. CPs were detected in approximately 24% of samples from 2014 and 2015. The co-occurrence of APs, CPs, and MCs was common at these Green Bay sites, although it is interesting that the different TBP classes either correlated (MC) or did not (CP and AP) with the trophic gradient. Future work to elicit TBP-specific drivers is needed.

One objective of this study was to observe relationships between a trophic state indicator (i.e. chlorophyll) in Green Bay and TBP concentrations. In addition to being a trophic state indicator, chlorophyll data were also used for the context of cyanobacterial bloom presence. MC showed the strongest correlations with chlorophyll and both were significantly negatively correlated with distance from the Fox River. In the three separate cruises, MCs followed the strong south-north trophic gradient previously described in Green Bay (250).

A concurrent study to the August 2014 cruise using the same samples at 1 m examined phosphorus species from the same spatial gradient (229). By August, all forms of phosphorus (P) measured (dissolved inorganic P, dissolved organic P, particulate inorganic P, particulate organic P) were in highest abundance in the lower bay, localized to the eastern shore. Similarly, in this study TBPs were also most abundant in the lower bay and along the Eastern shore. Indeed, our analysis of TBPs at 1 m showed the highest concentrations of toxins were measured in the lower bay, specifically in zone 2 (samples were not collected south of zone 2 at 1 m in 2014) with two samples exceeding 4 μg/L. Thus, P species, like chlorophyll and MC, follow a south-north gradient. Both P and MC showed extensive entrainment in the lower bay with pockets of accumulation along the eastern coast of the bay.

In conclusion, this study provides a necessary baseline on spatial distribution of TBPs in Green Bay. We identified the most abundant TBPs and congener- specific changes in TBP

diversity along a trophic gradient. Future studies should examine the most abundant TBPs identified here alongside a compendium of limnological variables (e.g. taxonomic community composition) in order to identify a suite of possible environmental drivers of TBP production.

ACKNOWLEDGEMENTS

This work was funded by grants from the National Science Foundation (Award#'s 1256200 and 1313936), the National Institutes of Environmental Health Sciences, Oceans and Human Health program (R01 ES022075-01), and the NOAA CSCOR Coastal Hypoxia Research Program (NA10NOS4780139). SLB was funded by a graduate fellowship from the Cooperative Institute for Great Lakes Research.

Table 4.1: Statistics for TBPs detected of 19 targeted in 2014 and 2015 from 3 cruises with samples collected at 0 and 1 m (2014) and 1 m only (2015). TBPs not detected include MCLF, MCLY, MCHtyR, MCLW, CP-1020, Mgn690, and NOD. SumMC = the sum of all microcystin congeners; SumAP = the sum of all anabaenopeptin congeners; SumCP = the sum of all cyanopeptolin congeners; MC = Microcystin; AP = Anabaenopeptin; CP = Cyanopeptolin; Mgn = Microginin; NOD = Nodularin.

TBP	Mean (µg/L)	Max (µg/L)	Frequency of Detection	Coefficient of Variation
SumMC	1.28	19.97	94%	1.97
SumAP	0.20	6.78	38%	3.45
SumCP	0.07	1.92	24%	3.20
MCLR	0.47	7.76	65%	1.91
MCRR	0.66	7.74	53%	1.97
MCYR	0.19	2.40	18%	2.03
MCLA	0.04	0.65	2%	4.17
dmMCLR	0.01	0.42	1%	6.62
MCWR	0.07	0.63	4%	2.63
MCHilR	0.03	0.37	2%	2.80
AP-A	0.08	1.12	2%	6.44
AP-B	0.09	3.54	10%	3.74
AP-F	0.10	2.12	9%	2.70
CP-1007	0.06	1.07	6%	2.63
CP-1041	0.02	0.85	2%	5.93

Table 4.2: Max and mean concentrations of all TBPs measured from samples taken on 0 m from August 2014 and 1 m from August 2014, July 2015, and August 2015 cruises. SumMC = the sum of all microcystin congeners; SumAP = the sum of all anabaenopeptin congeners; SumCP = the sum of all cyanopeptolin congeners.

Transect	Analyte	Mean (µg/L)	Max (µg/L)
August 2014	SumMCs	4.98	20.0
0 meter	SumAPs	0.92	6.78
n = 12	SumCPs	0.36	1.92
	Chl-*a*	NA	NA
August 2014	SumMCs	1.38	5.28
1 meter	SumAPs	0.32	0.73
n = 26	SumCPs	0.06	0.53
	Chl-*a*	15.3	32.5
July 2015	SumMCs	0.86	4.70
1 meter	SumAPs	0.06	0.31
n = 33	SumCPs	0.04	0.58
	Chl-*a*	15.8	84.3
August 2015	SumMCs	0.32	1.40
1 meter	SumAPs	0.00	0.00
n = 26	SumCPs	0.02	0.17
	Chl-*a*	15.0	43.6

CONCLUSION

This dissertation addressed several major questions about the dynamics of cyanobacterial toxins (cyanotoxins) and toxic or otherwise bioactive peptides (TBPs) in two connected eutrophic waters – Lake Winnebago and Green Bay, Lake Michigan. From an environmental, regulatory or even funding perspective, Lake Winnebago and Green Bay are often treated differently despite their connectedness, the former as an inland water body and the latter as a coastal water body. These two water bodies have experienced intense eutrophication and have history of high chlorophyll concentrations likely due to the presence of cyanobacteria during the summer months and still, little was known about the cyanotoxins between the two systems.

1. Using a high-resolution sampling strategy, how do cyanotoxins vary over time within a cyanobacterial growing season?
2. Focusing on an important recreational and drinking water resource, how do cyanotoxin dynamics vary by depth and are any environmental variables associated with the cyanotoxins?
3. In a eutrophic embayment with a long history of cyanoHABs, are cyanotoxins present in the water body and how do cyanotoxins dynamics vary by space and time?

To begin to assess a temporal variability of cyanotoxins, **Chapter 2** used a proven technology, a Teledyne ISCO water sampler, deployed to a water quality-monitoring buoy, to achieve a high-resolution sampling strategy for cyanotoxins. This study took place in Lake Winnebago, Wisconsin, at the site of a drinking water intake pipe. Samples were collected every 6-hours and analyzed for a suite of cyanotoxins, including several microcystin (MC)

congeners. Microcystins (MCLR/MCRR) were detected in 100% of samples and had the highest mean and max concentrations. Interestingly, the max microcystin concentration (17.86 µg/L) was recorded in a midnight sample during October. Of further interest, the highest cyanotoxin concentrations occurred during non-bloom periods i.e. in the absence of a pigment bloom as recorded by the in-situ fluorometers. A typical sampling strategy such as sampling once per week during daylight hours or even sampling due to the presence of a cyanoHAB would not have captured these two phenomena. The high variability of cyanotoxin levels measured from this single location means a lower sampling frequency would underestimate maximum microcystin levels by greater than 3-fold. Challenges remain for devising a sampling strategy for drinking water production and recreation that can take into consideration these dynamics.

Following the high-resolution study in 2013, **Chapter 3** presented a multi-year analysis of possible environmental variables associated with cyanotoxins over multiple depths from the same fixed monitoring station in Lake Winnebago used in Chapter 2. This study focused on a suite of cyanobacterial secondary metabolites including MCs and TBPs over three cyanobacterial growing seasons, 2014 – 2016. The temporal variability of MCs and TBPs along with pigments and nutrients were assessed from three different depths (0, 1, and 3 meter (m)) spanning the water column. Concentrations of SumMCs, APs, and CPs were not significant with depth, however detectable concentrations of cyanotoxins were measured throughout the water column. Given the possibility for the shallow lake to stratify and mix quickly due to wind and wave dynamics, this is unsurprising. The lack of significance in depth further reiterates the need for a robust monitoring and treatment plan for drinking water. Of the environmental variables assessed, chlorophyll and phycocyanin were most correlative to the cyanobacterial

secondary metabolites, although this relationship was specific to microcystins, and was weak with anabaenopeptins and cyanopeptolins. Timing of pigment blooms and toxin peaks (i.e. toxins in the absence of visual blooms) will be important factors to consider from a monitoring and modeling perspective.

Lake Winnebago feeds into lower Green Bay through the Fox River. Despite the connectedness of the system, these two systems are often treated as separate water bodies. CyanoHABs are well documented in in Lake Winnebago and Green Bay, Lake Michigan but much less is known about cyanoHAB toxicity. **Chapter 4** characterized the diversity and spatial distribution of cyanotoxins in Green Bay. Samples were collected in 2014 and 2015 during three cruises at sites spanning the mouth of the Fox River north to Chambers Island. Nineteen different cyanotoxins were analyzed and of that, 12 were detected in at least one sample including a mixture of MCs and TBPs. Similar to Lake Winnebago, the most prevalent cyanotoxins were MCRR and MCLR. Green Bay is characterized by hyper/eutrophic conditions in the lower bay that improves as distance from the mouth of the Fox River increases. Cyanotoxins followed this trophic gradient. The mean concentration of all cyanotoxins was highest in the Fox River and lower bay, and MCs were negatively correlated with distance to the Fox River in all cruises along a well-established south-to-north trophic gradient in Green Bay. Cyanopeptolins and anabaenopeptins did not trend with the south-north trophic gradient or varied by cruise suggesting their occurrence is driven by separate environmental factors. This study provides evidence that trends in cyanotoxin concentration differ by congener type.

Results from the above studies provide crucial information in a void of cyanotoxin dynamics, specifically when discussing cyanotoxin monitoring strategies. Among the 12

cyanotoxins that were measured in every sample, microcystins were detected in 100% of samples. The cyanotoxin gradient followed a seasonal trend with most of the cyanotoxins, except MCLA, having max abundance later in the season (August – October), rather than the early months (May – July) (Figure 5.1). This could be due to cyanobacterial dominance over

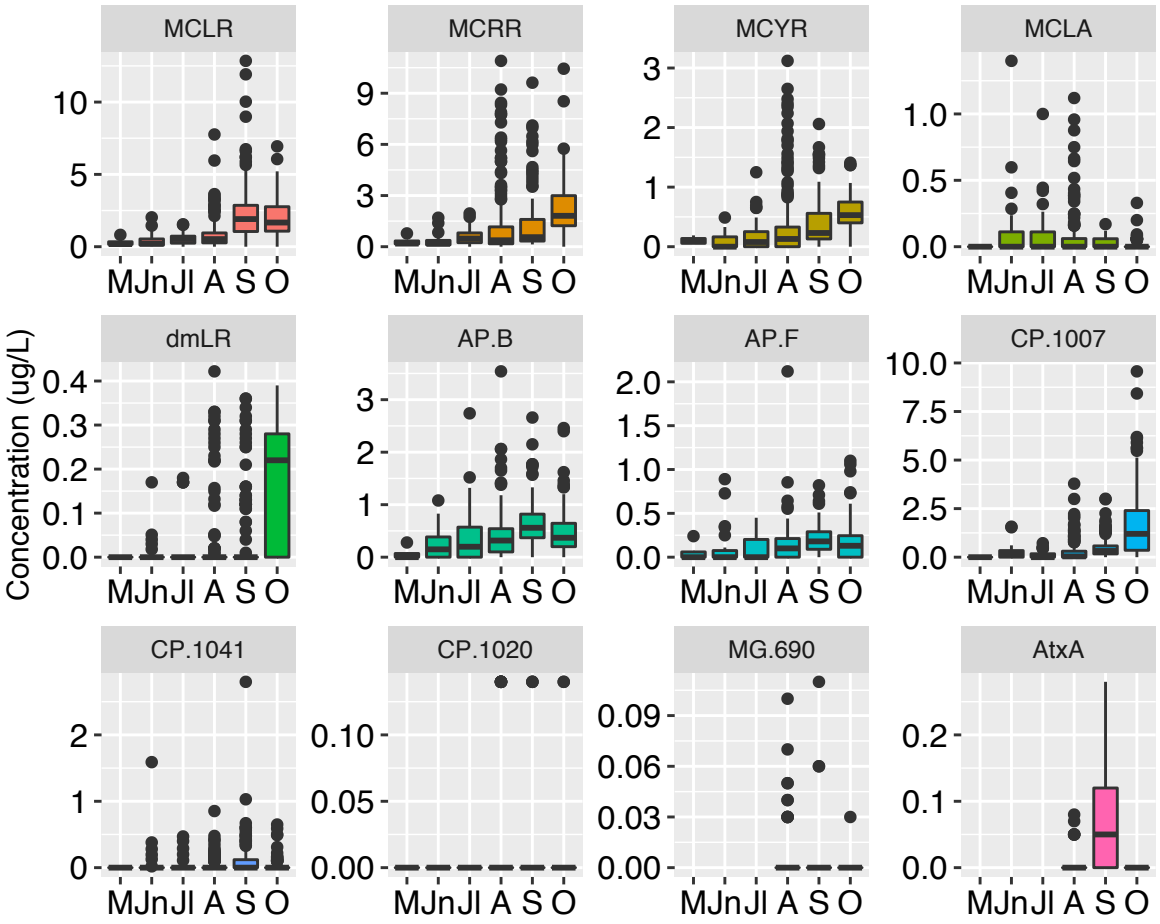


Figure 5.1: Cyanotoxin abundance increased throughout the cyanobacterial growing season for all congeners except MCLA. Boxplots represent the median (middle horizontal line). =

other phytoplankton assemblages coupled with warm water temperatures later into the summer growing season that can create optimal conditions for cyanobacteria to proliferate. If increased water temperature is persisting later into the cyanobacterial growing season, it will be imperative that recreational monitoring extend past Labor Day, which is sometimes a final end date for Great Lakes Beach Monitoring programs.

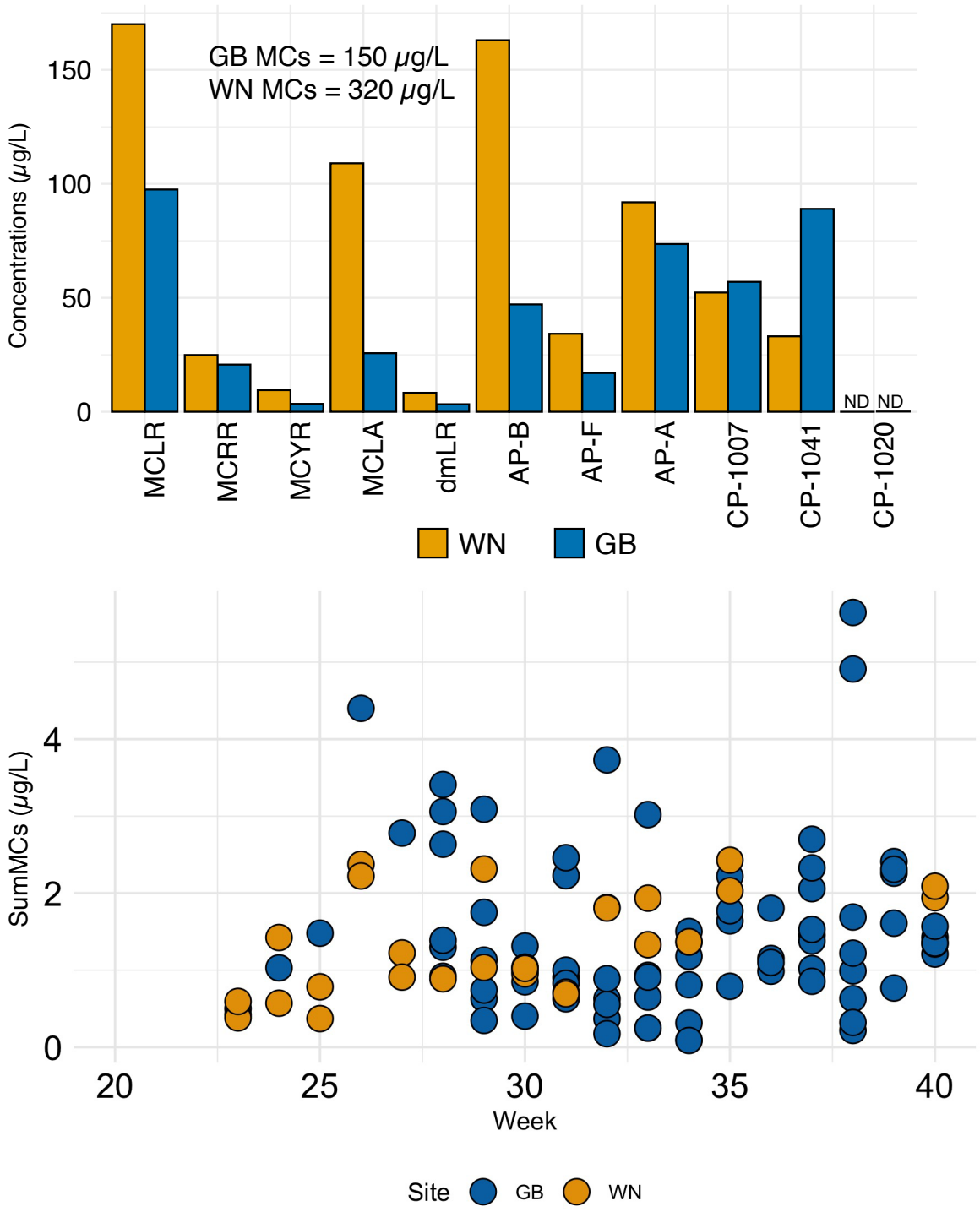


Figure 5.2: Samples were collected from Lake Winnebago (orange) and Green Bay (blue) in 2016. (Top). A scum sample was collected on the same day from each water body and concentrations were magnitudes higher than ambient concentrations measured throughout the season (Bottom). Overall, the cyanotoxin and TBP congeners were similar between the two water bodies in the scum sample, and similar concentrations were measured throughout the year.

Lake Winnebago and Green Bay have both faced intense eutrophication and both water bodies have a history of high chlorophyll concentrations, likely due to the presence of cyanobacteria, during the summer months. As such, nutrient limitation in these two systems does not appear to play a role when considering bloom formation. While sample depth was not a significant variable associated with cyanotoxin abundance, an additional depth attribute to consider is scum, which forms on the water surface. CyanoHABs can produce scum, a layer of biomass on the immediate water surface which may form near the shorelines when wind and waves provide calm conditions for formation. Scum samples can often be magnitudes higher in cyanotoxin concentrations and can be host to more rare congeners (Figure 5.2). In 2016, samples were collected from sites in Lake Winnebago and Green Bay. Overall, ambient levels of MCs were detected from each water body in 2016 and the two water bodies revealed similar profiles. In August, both water bodies had blooms at the site of sample collection and scum samples were collected from each on the same day (Figure 5.2). These samples were magnitudes higher than the ambient weekly samples collected - Winnebago MCs max 320 µg/L, Green Bay MC max 150 µg/L versus max MCs 2-5 µg/L from samples collected throughout the season. While cyanotoxin and TBP concentrations were greater in Lake Winnebago than Green Bay, overall cyanotoxin congeners dynamics revealed MCs as the cyanotoxin class in greatest abundance. Interestingly, CPs were in greater concentrations from Green Bay and CP-1020 was not detected in either water body scum.

Over a span of 4 years, 572 samples were collected between Lake Winnebago and Green Bay. The recreational guideline of 8 µg/L was exceeded 55 times (10% of the total samples collected) whereas the drinking water guideline was exceeded 397 times (69% of

samples). The average concentration of samples exceeding recreational guidelines was 11.3 µg/L of MCs and 4.2 µg/L of MCs for drinking water. It is important to recall samples collected would be considered raw water and would still go through a treatment process prior to needing to meet the drinking water guidelines. However, it is concerning that more than half of the samples were above the guideline as many of these samples were consecutive. These data further would provide a basis for a permanent monitoring program. In 2013, a concurrent study assessed cyanotoxins and the drinking water treatment processes in Lake Winnebago, and results revealed treatment removed cyanotoxins and TBPs to levels below the drinking water guideline for MCs (251). Overall cyanotoxin removal during drinking water treatment would depend on the specific processes as some intermediate processes showed increased levels of cyanotoxins or TBPs, and drinking water treatment are not the same amongst all plants.

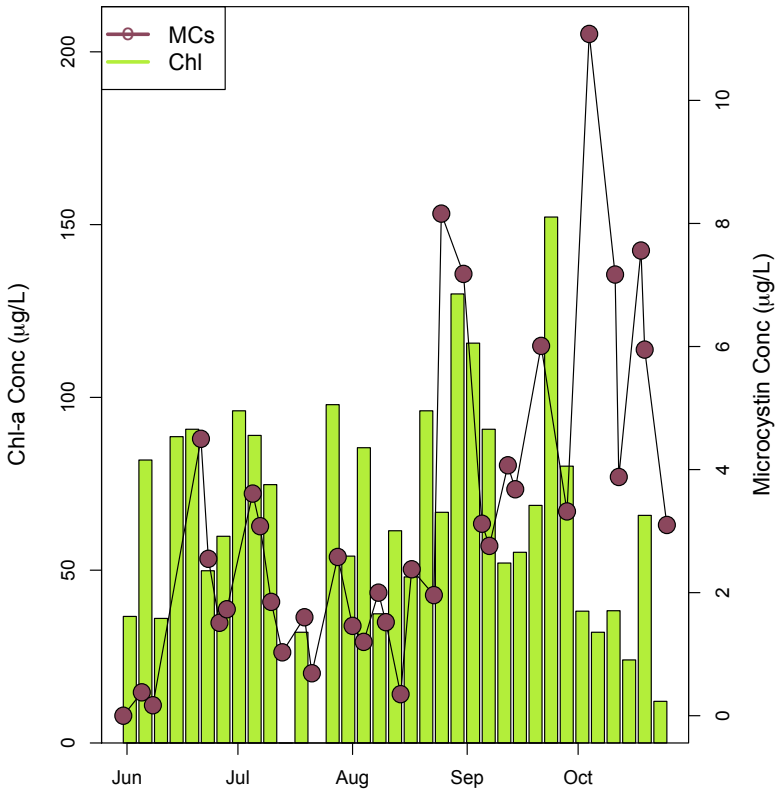


Figure 5.3: Time series of microcystins (MCs) and chlorophyll and the positive correlation between the two variables for much the cyanobacterial season. The two can disassociate towards the end of the growing season, possibly due to the bloom dying.

The relationship between pigments and MCs e.g. green colored water is laden with toxins, is not always valid. There were moments in this study when the relationship broke apart and the two are no longer correlated,

although this was specifically observed with Lake Winnebago as temporal data were not available in 2013 and 2014 to assess this relationship. However, an additional study occurred in Green Bay (data unpublished) and MCs and chlorophyll from this site revealed MCs in abundance, and even over the recreational limit in the absence of a chlorophyll bloom, although the sample was collected in October which would be outside the standard recreational season (Figure 5.3). Determining when or what causes this disassociation is still an area for further research. One obvious disassociation occurred in the high-resolution sampling strategy when max SumMC abundance occurred in the absence of a bloom. The seasonal timing of this event was September. There are other instances of cyanotoxins occurring in the absence of blooms which can happen towards the end of the sampling season This could be attributed to the bloom dying (Figure 5.3). From a monitoring standpoint, it may be difficult to provide guidance for safe recreation if relying on fluorescence as an indicator for cyanotoxins.

A recreational monitoring program should extend past September if weather conditions continue to permit recreation. Monitoring should utilize *in-situ* sensors including water temperature and chlorophyll/phycocyanin probes. These probes can be a real-time warning system to ensure if the water is green, recreators should stay out. This defense strategy is in line with recommendations from the Wisconsin State Department of Health and the DNR, to stay out of the water when it is green. MCs and Chl were correlative in Green Bay and Lake Winnebago, despite periods of cyanotoxins in the absence of blooms, so this is good advice if providing a minimal amount of monitoring, but it shouldn't be the only tool. Samples should be collected at least weekly, which is likely frequent enough to capture the cyanotoxin dynamics. Once a bloom has been detected, it will become important to increase the number of water

samples collected to test for microcystins. Daily sampling is the minimum, and unfortunately might also be the most realistic maximum when the sample collection and analysis will require manpower until *in-situ* MC monitoring is a reality. As discussed in Chapter 2, cyanotoxins can vary greatly over the course of a day. Working towards technology that will monitor microcystins in real-time will continue to grow in demand as cyanoHAB reporting increase.

Taking into consideration the EPA guidelines, drinking water and recreational guidelines were exceeded every cyanobacterial growing season of this study. The need for a monitoring program and stringent drinking water treatment plans should not be overlooked. Future studies should examine the most abundant cyanotoxins and TBPs identified here alongside a collection of variables (e.g. taxonomic community composition including toxic and non-toxic strain information) and in order to identify a suite of possible environmental drivers of cyanotoxin production that can be used in modeling cyanotoxins, in the absence real-time monitoring.

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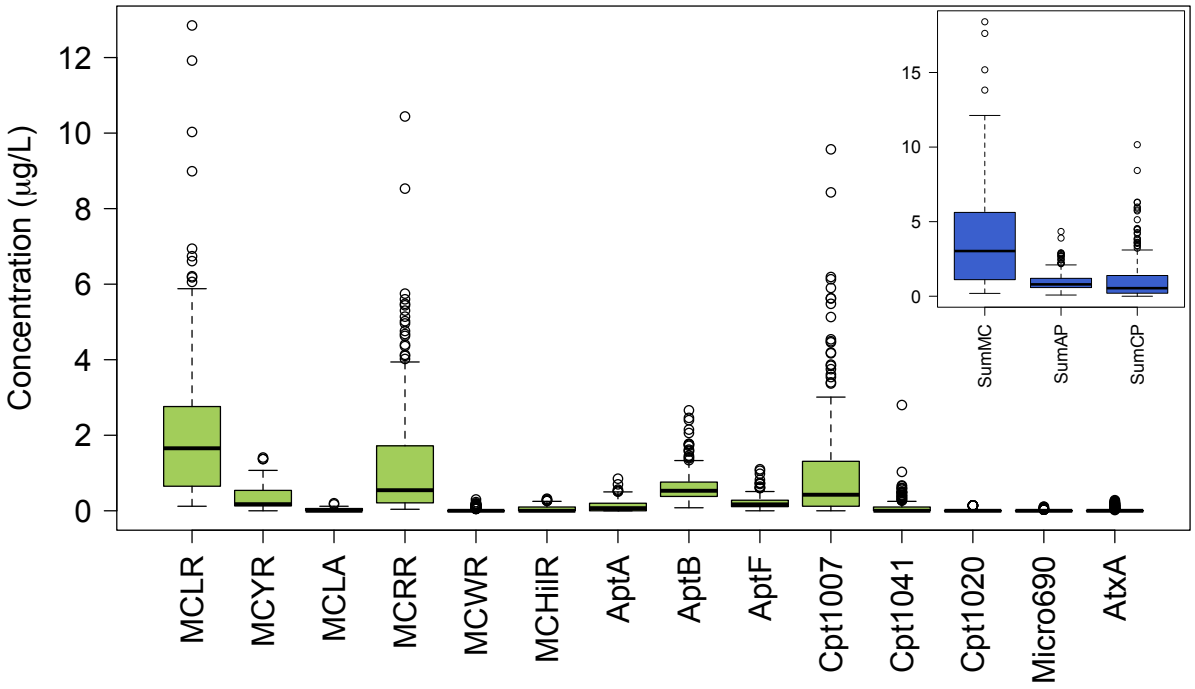

Figure S2.1 Distribution of cyanotoxins throughout the 2013 high-resolution sampling season. SumMC = the sum of all microcystin congeners; SumAP = the sum of all anabaenopeptin congeners; SumCP = the sum of all cyanopeptolin congeners

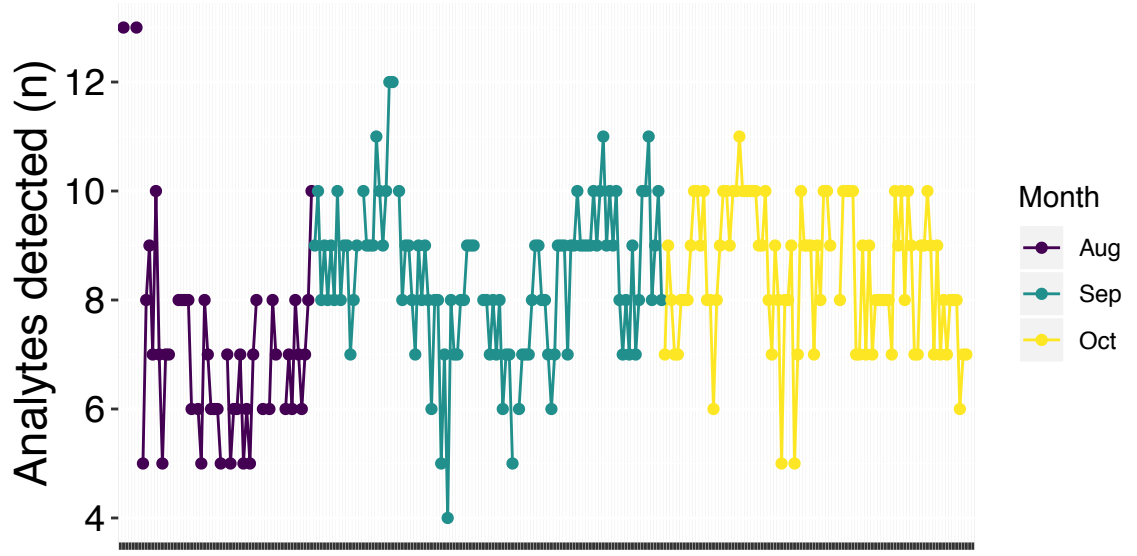

Figure S2.2 Cumulative number of analytes detected in each individual 6-hour sample. Mean number of analytes were greater in September and October, although the most diverse samples with the max number of analytes occurred at the start of the sampling in August.

Table S2.1: Compound specific parameters for mass spectrometer

Analyte	Parent	Fragment	DP	EP	CE	CXP	RT (min)
MCLR	995.6	135.3	126	10	115	26	8.6
	995.6	127.1	126	10	115	26	8.6
MCRR	520.0	135.1	81	10	43	8	7.8
	520.0	70.1	81	10	129	10	7.8
MCYR	1045.6	135.3	141	10	107	8	8.6
	1045.6	127.1	141	10	123	8	8.6
MCLA	910.6	776.4	106	10	27	8	10.2
	910.6	135.2	106	10	87	8	10.2
dmLR	981.5	135.3	126	10	101	22	8.5
	981.5	103.2	126	10	129	6	8.5
MCWR	1068.5	135.3	161	10	109	22	8.6
	1068.5	159.4	161	10	103	8	8.6
MCHilR	1009.6	135.3	126	10	99	22	8.6
	1009.6	107.2	126	10	129	18	8.6
MCHtyR	1059.6	135.3	136	10	105	8	8.3
	1059.6	107.3	136	10	127	18	8.3
MCLF	986.5	135.3	91	10	91	8	10.7
	986.5	375.3	91	10	49	10	10.7
MCLY	1002.5	135.3	96	10	89	8	9.9
	1002.5	107.2	96	10	129	18	9.9
MCLW	1025.5	135.3	101	10	99	8	10.5
	1025.5	107.2	101	10	129	16	10.5
AP-B	837.5	201.4	106	10	57	14	4.3
	837.5	70.0	106	10	129	12	4.3
AP-F	851.7	201.0	121	10	53	12	5.3
	851.7	175.1	121	10	53	12	5.3
AP-A	844.5	84.3	81	10	129	14	8.2
	844.5	637.4	81	10	37	29	8.2
CP-1007	1007.5	989.6	131	10	51	32	8.1
	1007.5	776.3	131	10	59	22	8.1
CP-1041	1042.5	1024.5	136	10	51	28	8.4
	1042.5	70.1	136	10	123	12	8.4
CP-1020	1021.6	989.6	131	10	57	32	8.6
	1021.6	776.4	131	10	63	22	8.6
MG-690	691.4	510.2	96	10	31	16	4.9
	691.4	343.1	96	10	37	10	4.9
NOD	825.5	103.2	116	10	83	16	8.1
	825.5	135.3	116	10	129	8	8.1
^{13}C-Phe	172.1	126.2	41	10	19	8	1.50
	172.1	109.2	41	10	39	6	1.50
CYN	416.2	194.0	71	10	49	10	1.60
	416.2	336.2	71	10	31	10	1.60

DP = Declustering Potential (volts); EP = Entrance Potential (volts); CE = Collision Energy (volts); CXP = Collision Cell Exit Potential (volts); RT = retention time; LOD = limit of detection in the lake

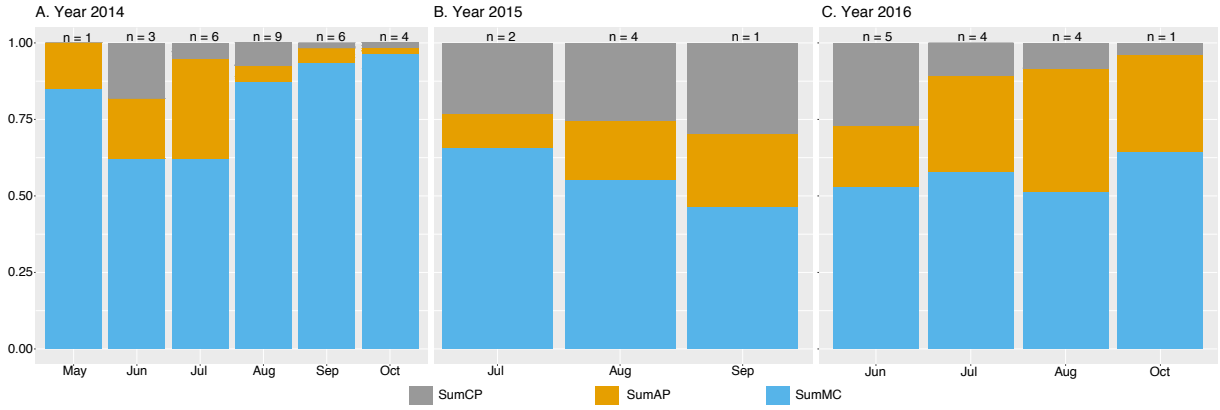

Figure S3.1: Relative abundance of cyanotoxin classes by year and sampling month. Sample collection quantity (n) indicated on each monthly bar graph. CP = cyanopeptolin, AP = anabaenopeptin, MC = microcystsin

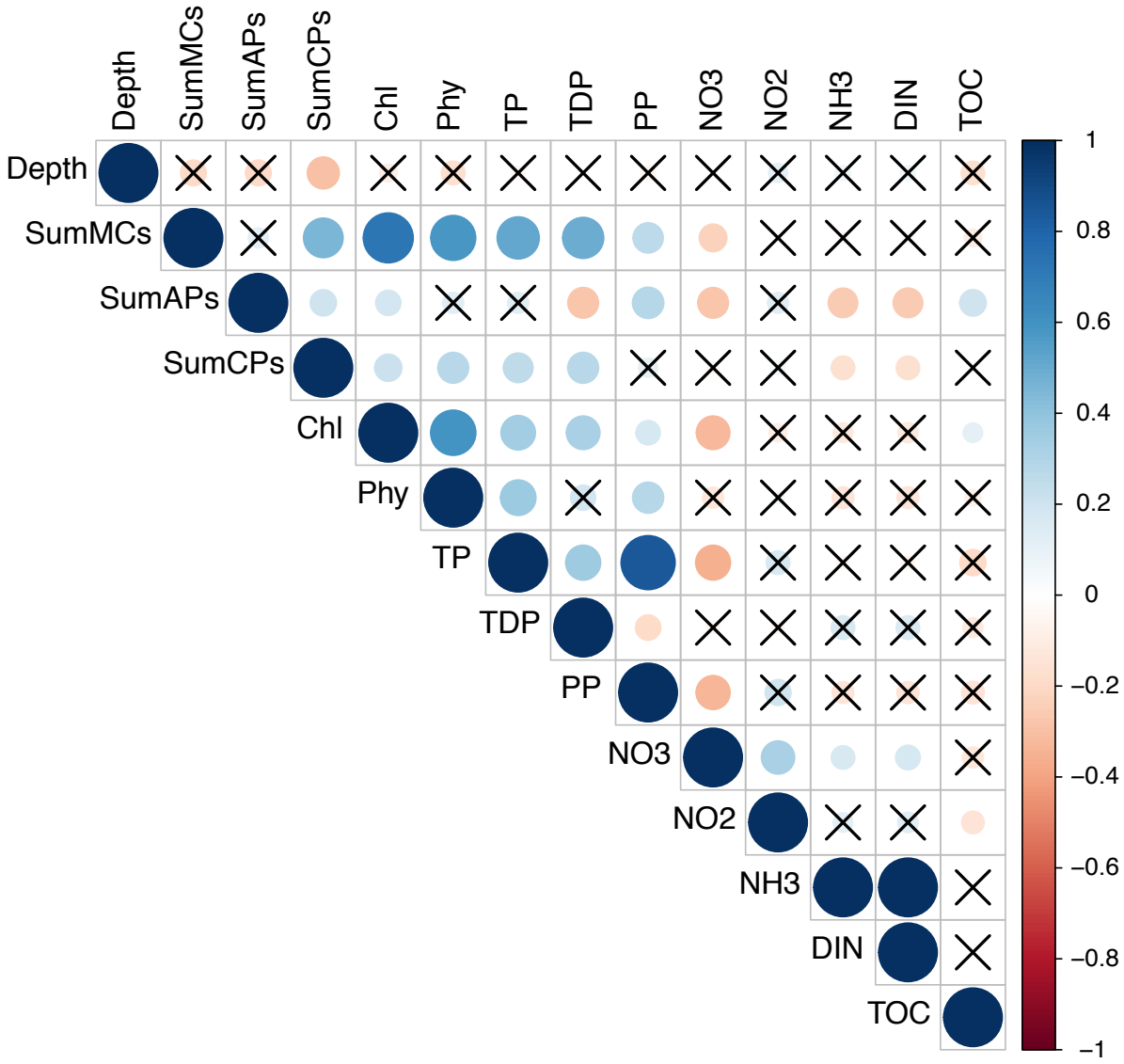

Figure S3.2: Results from pair-wise correlations among the cyanotoxins and environmental variables for all depths: SumMCs, SumAPs, SumCPs, chlorophyll (Chl), phycocyanin (Phy), Total Phosphorus (TP), Total Dissolved Phosphorus (TDP), Particulate Phosphorus (PP), Nitrate (NO3), Nitrite (NO2), Ammonia (NH3), Dissolved Inorganic Nitrogen (DIN), and Total Organic Carbon (TOC). An 'X' indicates the two variables are not correlated. Circles that are larger in size and more closely color-coded to '1' indicate the variables are strongly correlated, and charts that are more closely coded to '-1' indicate the variables are anti-correlated.

Appendix C: Chapter 4 Supplemental Data

Figure S4.1: Chl and MC boxplot

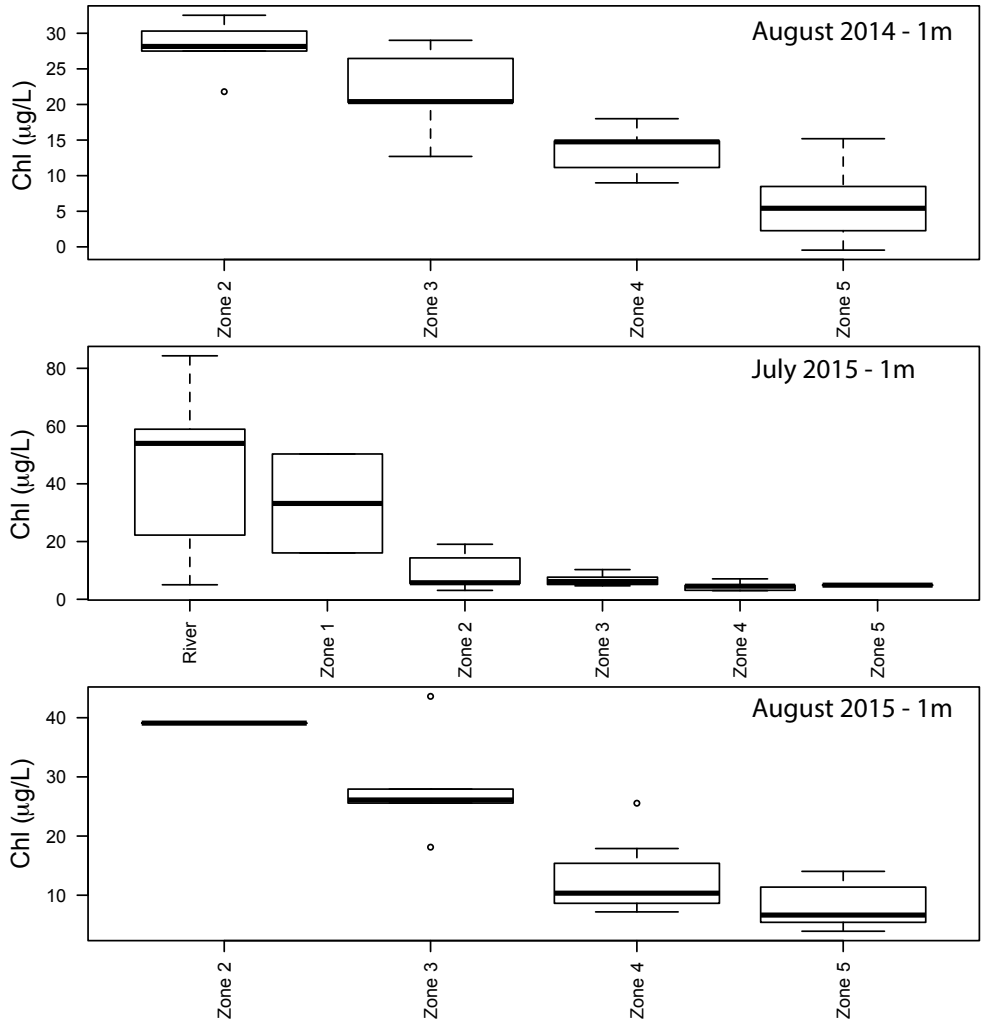

Table S4.1: Compound specific settings for mass spectrometer optimization.

Analyte	Parent	Fragment	DP	EP	CE	CXP	RT (min)
MCLR	995.6	135.3	126	10	115	26	8.6
	995.6	127.1	126	10	115	26	8.6
MCRR	520.0	135.1	81	10	43	8	7.8
	520.0	70.1	81	10	129	10	7.8
MCYR	1045.6	135.3	141	10	107	8	8.6
	1045.6	127.1	141	10	123	8	8.6
MCLA	910.6	776.4	106	10	27	8	10.2
	910.6	135.2	106	10	87	8	10.2
dmMCLR	981.5	135.3	126	10	101	22	8.5
	981.5	103.2	126	10	129	6	8.5
MCWR	1068.5	135.3	161	10	109	22	8.6
	1068.5	159.4	161	10	103	8	8.6
MCHilR	1009.6	135.3	126	10	99	22	8.6
	1009.6	107.2	126	10	129	18	8.6
MCHtyR	1059.6	135.3	136	10	105	8	8.3
	1059.6	107.3	136	10	127	18	8.3
MCLF	986.5	135.3	91	10	91	8	10.7
	986.5	375.3	91	10	49	10	10.7
MCLY	1002.5	135.3	96	10	89	8	9.9
	1002.5	107.2	96	10	129	18	9.9
MCLW	1025.5	135.3	101	10	99	8	10.5
	1025.5	107.2	101	10	129	16	10.5
AP-B	837.5	201.4	106	10	57	14	4.3
	837.5	70.0	106	10	129	12	4.3
AP-F	851.7	201.0	121	10	53	12	5.3
	851.7	175.1	121	10	53	12	5.3
AP-A	844.5	84.3	81	10	129	14	8.2
	844.5	637.4	81	10	37	29	8.2
Cpt1007	1007.5	989.6	131	10	51	32	8.1
	1007.5	776.3	131	10	59	22	8.1
Cpt1041	1042.5	1024.5	136	10	51	28	8.4
	1042.5	70.1	136	10	123	12	8.4
Cpt1020	1021.6	989.6	131	10	57	32	8.6
	1021.6	776.4	131	10	63	22	8.6
Mgn690	691.4	510.2	96	10	31	16	4.9
	691.4	343.1	96	10	37	10	4.9
NOD	825.5	103.2	116	10	83	16	8.1
	825.5	135.3	116	10	129	8	8.1

DP = Declustering Potential (volts); EP = Entrance Potential (volts); CE = Collision Energy (volts); CXP = Collision Cell Exit Potential (volts); RT = retention time

Appendix D: Detailed Protocols

Procedure for Quantitative Analysis of LC-MS Sample Runs in Analyst

1. Select Analyst (make sure correct Project Folder is selected in upper middle dropdown menu).

Step #1: Building Quantitation Method

2. Select "Build Quantitation Method" from lefthand tab.
3. Find Datafile in "Select Sample" box (use batch name – ex: datePPCP.wiff or dateC18.wiff).
4. Select one of the standards (select a single run), usually the highest, and click "Okay".
5. Click on the Integration tab
    a. This process will create a quantitation method giving the program a template based on the analytes detected in the standard.
    b. Analyte Box – select an analyte from the dropdown menu
        i. When looking at analytes – all quantitative and confirmatory ions (ex: BMAAq and BMAA1, BMAA2) should be present at the same retention time (ex: 5.19 minutes)
    c. If an analyte is at a different Retention Time and the peak is not highlighted:
        i. Check that the other ion transitions are present at the same time
        ii. Highlight the peak at the given time
        iii. Select peak icon (on right side at top)

iv. If there is a peak at a different retention time from normal, highlight peak at the consistent retention time (maybe it will not be the highest peak for all analytes). Analyst will automatically select the highest peak, but some analytes may have same/similar enough ion transitions that one peak will be the same but the confirmatory ions will have slightly different retention times.

d. Some runs may say "no peak" – this could be an issue with the standard (i.e. compound not included in standard mix), method (if a scheduled method, retention time window incorrect/not large enough to include retention time shifts for the analyte), and or mobile phases (if made incorrectly, can affect retention time of compounds from column.

6. Select File on top toolbar in Analyst
7. Select "Save As" and save the quantitation method using the same name as the datafile/batch name.

<u>Step #2: Creating a Results File</u>

8. Double-click on "Quantitation Wizard" in the left-hand panel.
9. Scroll through the left-hand window and single click on the name of the datafile you are analyzing samples from.

a. Select all samples for quantitation by single-clicking and highlighting them. Do not use the first high standard ran as a check before the full standard curve was run.
b. Once all samples are highlighted, select the > arrow; the names of the sample runs will go into the furthest righthand box under “Available Samples” (“Selected Samples”).
c. Note: If several people run samples related to different projects, different batches/datafiles may be associated with the same standard curve. For example, two batches may be created to analyze algal toxins from drinking water samples and fish tissues. The standard curve may have been included in one batch/datafile with the fish tissue data, while the drinking water data could be in a different datafile. This is okay, as long as the samples and standard curve were run with the same method.

10. Hit “Next” button twice
11. Create Quantitative set – choose the existing quantitation method that you just created (will be a .gif file)
12. Click “Finish”. Analyst should open up a spreadsheet with each analyte for the first standard run in your selected data.

Step #3: Organizing Your Data

13. Save your Results File immediately by clicking on File -> Save As in top left corner of Analyst, using same name as datafile.
14. Right-click in the tan area right above the spreadsheet.
15. From the dropdown menu that appears, select “Analyte” and choose the first analyte you want to analyze.
    a. Note: If you have spiked an internal standard into your samples (i.e. 13C-phenylalanine for cyanopeptides analysis), you will want to analyze this data first to determine if ion suppression occurred throughout your sample runs.
16. In the Analyst spreadsheet, fill out the following for each sample run:
    a. Sample Type:
        i. Blanks = “double blanks” without internal standards; “blanks” with internal standards
        ii. Standards (i.e. have known concentrations of chemical reference materials; typically listed as 0.1 – 100, depending on spread of standard curve) = “standards”
        iii. Samples = “unknowns”
    b. Analyte Concentration: Enter values for the standards (ex: 0.1, 0.5, 1, 5, 10, 25, 50, 100), but not for the unknowns. (Analyst shouldn’t allow you to type values in this box for samples.)
    c. Use Record
        i. If this column does not automatically appear, right-click at the top of the spreadsheet and select “Edit Table”, then click on “Columns”. There will

be a dropdown in the upper left of a new small box that appears, select "Record" from this dropdown menu. Check the box that says "Use Record" so that it will appear in your spreadsheet.

ii. This will automatically get checked for all of your standards. You can remove this checkmark for standards where no peak height shows so that it is not included as a standard with no peak area (i.e. concentration = 0 when it should = 0.1 or = 0.5). Removing these checkmarks will improve the accuracy of your standard curve.

d. (Calculated Concentration: calculated automatically)

e. Click on the floppy disk icon in the upper left of Analyst to save your spreadsheet at this point, or click on File -> Save As. This should be done periodically to save your analysis in case Analyst crashes ☹

<u>Step #4: Calculating Percent Peak Area for Noting Ion Suppression</u>

Note: Skip this step if you are not analyzing microcystins/cyanopeptides. If you are analyzing your extracts for microcystins and cyanopeptides, you should have added 5 uL 1000 ug/L $13C_6$-phenylalanine immediately before LC-MS/MS analysis to each of your samples and your blank in your standard curve. $13C_6$-phenylalanine is thus being used as an internal standard an analyte added to a sample at a constant concentration for calibration and quantitation.

17. In Analyst, right-click in the tan area above the spreadsheet and select 13C-Phe from the Analyte dropdown menu.

18. Go through procedures detailed in Step #5 below to accurately evaluate peak area for 13C-phenylalanine in your blanks and samples. 13C-phenylalanine will not be a true peak in your standards (methanol spiked with toxin reference materials) – Analyst may try to select a peak, but it should just be part of the background.
19. Copy and paste the following information from Analyst into an Excel spreadsheet: all sample names and peak areas for 13C-Phe for each vial.
20. In a single cell, calculate the average peak area of 13C-Phe in the blanks by typing =avg($PA_{cell1,}PA_{cell2}$…). Each separate peak or group of peaks selected needs to be separated by a comma so that Excel doesn't add any of the values together.
21. For each sample, calculate the ratio of 13C-Phe peak area in the sample to that the average of 13C-Phe in the blanks using the following formula: =($PA_{sample1}/PA_{avgblank}$)*100.
22. If any samples have a peak area ratio for 13C-Phe below ~80%, remove that sample(s) from the analysis, dilute 1:10 in 70% methanol, and re-run in a future datafile. There is potentially sample matrix interference from one or more samples, if this is the case.

<u>Step #5: Calculating Concentrations of Analytes in Your Samples</u>

23. Examine the chromatogram for each analyte (toxin, PPCP, etc.) in each standard and sample (unknown) – correct any jagged lines or tailing included in the peak areas of the selected peaks.
24. Double click on the first "Blank" cell in the spreadsheet under "Sample Name" to pull up the first chromatogram.

a. Instead of one chromatogram, four separate chromatograms may pop up in the window underneath the spreadsheet. If this is the case, right-click in one of the four sub-windows, and click on “Options”. Select from the dropdowns 1 as the # of rows and columns and zoom the Y-axis to 100% of largest peak.

25. Buttons: In the tan area above the chromatogram, click on the button with the counterclockwise arrow to show options for smoothing, manual integration, etc. Note that these are options for adjusting your peak areas to get a better quantitative measurement from your sample – you may not need to smooth or manually integrate each sample.

    a. In the tan area above the chromatogram, click on the 3rd icon (from the left, excluding arrow buttons) which is the “Manual Integration” – this allows you to draw a line across the bottom of the peak manually.

    b. In the tan area above the chromatogram, you can also adjust the smoothing width from a dropdown menu – this will average the lines across a specific number of points across the peak you are selecting. Your peak must be highlighted to smooth it. The lowest number you have to use to get a good peak, the better. (4th option from left, not including arrows)

        i. Click “Apply” after changing the smoothing width.

        ii. Note: In unscheduled MRM methods, there is a value in the mass spec details for “Time (msec)”. This is referred to as “dwell time” and is the amount of time that the MS spends looking for the analytes all at once. This is calculated when the method is created based on the total number

of analytes in the method and the number of points we want per peak (usually 12-15). These points are the ones being smoothed, or averaged. In scheduled MRM methods, the same points are being averaged, but the number of points across each peak is determined elsewhere in the method.

c. Highlight on Y-axis and/or X-axis (outside of the graph area) in order to zoom in to smooth or manually integrate accurately.

26. When identifying your peaks, visually set the minimum peak height to be twice the baseline height for a signal to noise ratio of 2:1. You can accept peak heights at 3:1 and higher. This prevents Analyst from calling background noise a peak in blanks or samples.
27. To remove sample listings, click on the 1st icon in the toolbar with +/-. The same window will pop up from when you created your Results Table. Highlight the samples you want to remove and click on the < arrow.
28. To change your analyte, right-click in the tan area above the spreadsheet – click on "Analyte" and select the next one to scroll through chromatograms.
    a. Before changing each analyte, remember to transfer the respective data (sample name – only once; peak area, peak height, and calculated concentration) for each analyte into your Excel spreadsheet (described in Step #6 below).
29. Remember to continue saving your Results Table after each analyte. To do this, make sure you click on the upper half-window with the spreadsheet so that a blue box appears around it. Save by either clicking on the floppy disk icon in upper left or by going to File – Save/Save As (if you didn't save prior to going through analytes).

Step #6: Transferring Data to Excel

30. Building your Excel table:
    a. Create a Sample column
    b. Create the following columns for each analyte (may want to create a merged heading column above the following with each analyte name):
        i. Peak Area (PA)
        ii. Peak Height (PH)
        iii. Calculated Concentration
        iv. % Recovery (must be manually calculated – for spiked samples)
        v. Concentration in sample after extraction (ug/L)

31. Formula for calculating % Recovery:
    a. = (PA cell of sample/PA cell from standard at expected concentration)*100
        i. Ex: =(B13/B9)*100

Extraction of Microcystins and Cyanobacterial Bioactive Peptides using 70% Ethanol

Reagents:

Water with 0.1% formic acid

100% Methanol

1. Lyophilize 10 ml of each water sample for 24-48 hours:
    a. Put 10 ml into a 50 ml conical or glass test tube.
    b. Wrap one-and-a-half squares of Parafilm around the top of each tube.
    c. Use a small pipet tip to poke holes in the tops of the Parafilm.
    d. Add to a freeze flask (2 in each small flask, up to 10 in each large flask)
    e. Freeze for ~1 hour, until all water is solid ice.
    f. Attach flasks to lyophilizer one at a time. Wait until lyophilizer is down to 0.040 psi (-50 C) until adding each flask.
2. Cut Parafilm circles with razor blade so that they fall into the tubes; discard Parafilm wrapped around tubes
3. Add 1 ml of water/0.1% formic acid (vortex).
4. Three 30-minute freeze-thaw cycles between the -80 freezer and 50 C water bath; turn on sonicating water bath during freeze-thaw cycles so it warms up.
5. Add 2 ml 100% MeOH (vortex; final concentration MeOH ~70%).
6. Sonicate in the 45 C sonicating water bath for 10 minutes – make sure it is at least 2/3 of the way full with distilled water (vortex).
7. Centrifuge at maximum speed for 15 minutes.

8. Transfer 1000 µl of the supernatant to a labeled LC vial; make sure not to suck up any particulates as these could clog the LC lines.
9. Add standard: 5 uL of 1000 ug/L 13C-Phe to each sample in LC vial. Vortex.
10. Store at -20C until analysis on LC/MS-MS

Extraction of HILIC cyanotoxins for Analysis with LC/MS/MS

1. Transfer 1 mL of sample to a 1.5mL tube.
    a. Add 10 uL of 0.5 ug/mL 13C-phenylalanine in 0.1% Formic Acid.
    b. Add 1 uL of Formic Acid.
    c. Vortex to mix.
2. Freeze samples in -80C for 30 minutes; thaw in 55C water bath for 5 min.
    a. Perform 3 cycles of freeze/thaws.
    b. Vortex after each thaw.
3. Place samples in sonicating water bath (45C) for 10 minutes.
4. Vortex.
5. Centrifuge for 15 minutes at max speed.
6. Transfer the top 500 uL of supernatant to an LC Vial for analysis.
7. Store at -20C until analysis on LC/MS-MS

Extraction and Spectrophotometric measurement of Chlorophyll-a using UV-Spectrometry

Procedure:

If sample is filtered with MF-millipore filters, skip step #7

If sample is filtered with GF/F filters, perform step #7

1. Filter sample through MF-millipore filters until filtration begins to slow down, then filter in 100mL increments until filtration slows/stops. Fold filters and store in blue-top 50mL falcon tubes at -35C. Record volume of water filtered.
2. Add 1mL of ddH20 to falcon tubes
3. Perform three rounds of freeze/thaw cycles to lyse the cells.
    a. Freeze at -80C for 30 min
    b. Thaw at room temperature
    c. Vortex vigorously after each thaw
4. Add 9ml of reagent grade acetone to tube.
5. Sonicate for 5 minutes at 55C. (Turn sonicator on during F/T cycles to allow it to warm-up).
    a. Vortex vigorously
    b. Repeat sonication and vortex two more times
6. Centrifuge at max speed for 15 minutes.
7. Transfer supernatant to new conical, passing supernatant through a syringe containing a pinch of glass wool to obtain a sample free of filter debris.
8. Turn spectrophotometer on and allow to warm for 20 min.

9. Add 1 ml of each sample to a 1cm acetone resistant cuvette.
10. Pipette 1 mL of buffered acetone to a cuvette to be used as a blank.
11. Zero the spec at 750nm with the blank.
12. Measure absorbance of each sample at 750 nm. Absorbance at 750nm should be minimal.
13. Measure absorbance of each sample at 663, 645, and 630, making sure to blank when changing wavelengths.
14. For acid correction: Immediately after measuring the absorbance, add 0.1 mL of 0.1 N HCl to the spectrophotometer cell, mix, wait 90 seconds and measure the absorbance at 750 and 665.

Calculations

Subtract the absorbance at 750 nm from the 630, 645, and 663 nm values (turbidity

$$\text{Uncorrected Chlorophyll } a\ (\mu g/L) = \frac{[11.64\ (Abs663) - 2.16\ (Abs645) + 0.10\ (Abs630)]\ E(F)}{V(L)}$$

Where F = *Dilution Factor (i.e., if the 663 Abs is >0.99 with the 1 cm cell, dilute, re-analyze and insert the dilution factor in the equation)*
E = *The volume of acetone used for the extraction (mL)*
V = *The volume of water filtered (L)*
L = *The cell path length (cm)*

Acid Correction:

Subtract the absorbance at 750 nm from the absorbance at 665 nm (turbidity correction).

$$\textit{Corrected Chlorophyll a } (\mu g/l) = \frac{26.73(663_b - 665_a)\ E(F)}{V(L)}$$

$$\textit{Pheophytin a } (\mu g/l) = \frac{26.73(1.7\ x\ [665_a] - 663_b)\ E(F)}{V(L)}$$

Where F = *Dilution Factor (if the extract requires dilution)*
E = *The volume of acetone used for the extraction (mL)*
V = *The volume of water filtered (L)*
L = *The cell path length (cm)*
665_a = *The turbidity corrected Abs at 665 nm after acidification*
663_b = *The turbidity corrected Abs at 663 nm before acidification*

Extraction and Measurement of Phycocyanin using UV-Spectrometry

1. Remove black sample tubes from the freezer. Record label and volume listed into a notebook.
2. Transfer filter to a clear 15 ml (Blue Top) Falcon tube using clean forceps.
3. Add 10 ml 0.2 M sodium acetate buffer (pH 5.5) to the black sample tube, vortex, and pour into the Blue Top tube containing the filter.
4. Perform three freeze/thaw cycles:
    a. Place tubes in -80 C freezer for 10 min.
    b. Transfer tubes to 50 C water bath for 5 min.
    c. Shake tubes vigorously.
    d. Make sure filter material is at the bottom of the tube.
    e. Repeat two more times.
5. Centrifuge tubes for 20 min. at 4,000 x g at 4°C in the swinging bucket rotor. Turn on the spec while running.
6. Pipet 1 ml of fluid from centrifuged samples into cleaned, labeled 1 cm cuvettes. Be sure not to suck up filter debris. If noticeable filter debris is present then centrifuge 1.5 ml of the extract in a microcentrifuge tube at max speed for 5 minutes before transferring to the cuvette.
7. Pipet 1 ml of sodium acetate buffer into another cleaned cuvette labeled “blank.”
8. Insert the blank and five samples into the spec carousel holder.
9. Set spec to 620 nm, insert the blank and zero the instrument by pressing “B,” then “Measure Blank” to zero the instrument.

10. Measure and record the absorbance of all samples at 620 nm by pressing #1 - #5.
11. Set spec to 650 nm, insert the blank and zero the instrument as previously.
12. Measure and record absorbance of all samples at 650 nm, as previously.
13. Run a scan from 300 nm – 1100 nm on the sample with the highest absorbance at 620 nm.
14. Wash cuvettes out with distilled water and allow to dry upside down on paper towels.
15. Record absorbance data.
16. Enter sample label, absorbance data, and volume of lake water filtered into an Excel spreadsheet.
17. Use the following formula to calculate the concentration of phycocyanin in the extract:

    $P_{extract}$ (mg/ml) = (Abs.620 – (0.7 x Abs. 650))/7.38

    To calculate concentration in lake water:

    P_{lake} (ug/L) = $P_{extract}$ x Volume Buffer (10 ml) / Volume Lake Water Filtered (ml) x 10^6

Measuring Total Organic Carbon Using a Phoenix 8000 TOC Analyzer

How total organic carbon (TOC) is measured: TOC refers to carbon bound to an organic compound. It can be used as a non-specific indicator of water quality. The water sample is injected into the sparger; N_2 gas then flows into the sparger to purge the water sample of inorganic carbon (IC), which is vented out of the instrument. Once IC is removed, the sample is transferred to the UV reactor with persulfate reagent. The persulfate and UV light together oxidize carbon in the sample to carbon dioxide. CO_2 is then measured by an infrared detector inside the instrument. So, the measurement of TOC involves 1) oxidizing organic carbon in a sample, 2) detecting and quantifying the oxidized product, and 3) presenting the result in units of mass of carbon per volume of sample[1]. The limit of detection for this instrument is ~0.2 mg/L.

Other sites of equipment to note:

- Chlorine scrubber: removes chlorine from carbon dioxide before sample gas goes to detector. Halogen can damage the detector, so it's important to remove this to prevent analytical errors.
- Moisture control system: Moisture is removed from the sample because the detector can confuse water vapor and CO_2. Condensation may occur when sample is carried through tubing after being oxidized; a low heat is generated by the UV reactor. The gas/liquid separator (visible) removes most condensation. This is followed by the sample going through a mist filter and permeation tube.

[1]More information on the process of carbon analysis can be found in the Phoenix 8000 manual.

Check solvents before starting instrument:

1. Reverse osmosis (RO) water: fill bottle with RO water from sink on northwest side of lab. Fill separate 500 mL bottle with RO water to use for blanks and creating standards.
2. Sodium Persulfate*: dissolve 25 g in 213 mL water and 9 mL phosphoric acid[2]; can be made every 2-3 weeks if instrument is in continuous use
3. 21% Phosphoric Acid (by volume)*: make new solution when it runs out if instrument is in continuous use
4. Waste bucket*: check pH of solvents; neutralize and dispose of solution if pH not between 5-9 (should have been done at end of previous analysis)

*Neutralize acidic solutions (old acidic mobile phases or waste) with baking soda to pH 5-9 before putting down the drain.

Starting the Phoenix 8000

1. Turn on the On/Off switch at back of instrument.
2. Turn on N_2 gas to flow ~34 psi.
3. Open TOC Talk 3.0 on Desktop. Select “Instrument Setup”, then select “Ready” for Instrument Status. This will turn on the UV lamp, which needs to be on for ~15 minutes to warm up.

Making TOC Standards

1. Using fresh RO water, rinse out and fill up a 50 mL conical. Run this as a blank 3x while creating TOC standards.
2. Rinse out five other tubes with RO water and then pipet 50 mL RO water to each tube.
3. DOC Standard (2.123 g dried KHP/1 L H_2O at ~1000 mg/L) stored in fridge. Remove the amount of water shown in Table 1 for each standard (labeled on each tube), then replace that volume of water with the DOC stock. Cap and invert each standard to mix.
4. Samples that exceed the highest standard in the 'Low' standard curve should be set aside and rerun with the higher standard curve.

Table 1. DOC Standard Curve (Low)

Concentration DOC (mg/L)	Volume DOC Standard (uL)
1	50
2	100
5	250
10	500
20	1000

Table 2. DOC Standard Curve (High)

Concentration DOC (mg/L)	Volume DOC Standard (mL)
5	.250
20	1
40	2
80	4
140	7
200	10

Total Phosphorus and Soluable Reactive Phosphorus Persulfate Digestion in Test Tubes

Reagents/solutions:

1. Stock phosphorus: dissolve 0.438g of KH_2PO_4 in 20 ml of ddH_20 then dilute 10-fold in ddH_2O. Add 10 µl of concentrated H_2SO_4. Store in acid washed solvent bottle.
2. Valderrama's Reagent: Tare a beaker and add 15g $K_2S_2O_8$ followed by 7.5g Boric Acid. Add 50 ml ddH_2O, stir to dissolve with stir bar then add 70 ml 1.5 M NaOH. Bring to 250 ml in a graduated cylinder.
3. Combined reagent (made fresh every time):
    a. Sulfuric acid, 14%
    b. Potassium antimonyl tartrate (dissolve 1.37g $K(SbO)C_4H_4O62H_2O$ and bring up to 500 ml ddH_2O in a graduated cylinder; store in fridge)
    c. Ammonium molybdate (dissolve 4g $(NH_4)_6Mo_7O_{24}4H_2O$ and bring up to 100 ml in graduated cylinder; store at 4°C
    d. Ascorbic acid (dissolve 1.76g ascorbic acid in 100 ml ddH_2O. Good in 4° refrigerator for up to one week.)
4. Concentrated hydrochloric acid

Note – for Soluble Reactive Phosphorus (SRP) measurement: Conduct same procedures as below for total phosphorus (TP), <u>except do not add Valderrama's reagent or autoclave (steps 6-9)</u>

Procedure:

1. To prepare standards first dilute 0.4 ml of Stock P Solution in 200 ml of ddH_2O using a graduated cylinder to give 1 mg/L working stock P (calculated as amount of "P" in KH_2PO_4).
2. Add 10 mL of de-ionized water with a volumetric pipet into each standard test tube.

   Note: If the digestion from TP will also be used for total nitrogen, then 20 ml of DI water should be added to test tubes.

3. Pipet out the necessary amount of de-ionized water to allow addition of Stock P according to Table 1.
4. Add 10 ml of sample to test tubes (20 ml sample volume if also using for TN)
5. Add 10 µl of hydrochloric acid to each standard and sample.
6. Add 0.4 ml of Valderrama's reagent to each test tube. Invert.

Table 1. Phosphorus standards

Sample Label	Working Stock P (1 mg/L; 32.258 µM) for SRP	Working Stock P (1 mg/L; 32.258 µM) **for TP/TN**	Standard Concentration (mg/L)
Blank	0	0	0
S1	0.1	0.2	0.01
S2	0.3	0.6	0.03
S3	0.5	1	0.05
S4	1	2	0.1
S5	2	4	0.2
S6	3	6	0.3
S7	4	8	0.4
S8	5	10	0.5

7. Loosen lids and autoclave for 60 minutes liquid cycle.
8. Remove test tubes from autoclave and allow them to cool to room temp.

   Note: If using samples for TN analysis, at this point, transfer 10 ml of each sample and standard to 15 ml Falcon tubes and save in -20 freezer.

9. Look for loss of volume from samples or standards. Remove samples or standards with significant loss from the set.
10. Remove ascorbic acid and other reagents from 4°C refrigerator.
11. To make 50 mL of Combined Reagent follow Table 2

    Note: Calculate amount of combined reagent needed and make more than 50 mL if necessary

12. Pipet reagents into a 150 mL beaker with stir bar inside (**Note**: Must add reagents in order.)
13. Add 4 ml of combined reagent to each test tube, starting with standards.
14. Turn spectrophotometer on to wavelength 880 nm 10 minutes before measuring samples.
15. Allow reaction to go for 30 minutes before measuring.
16. Rinse the cuvette 3x with de-ionized water.
17. Set spectrophotometer to 0 with the Blank (880 nm).
18. Run standards through spectrophotometer. Record absorbance. No need to rinse between standards if they are read from lowest to highest.
19. Read samples, rinse cuvette with ddH_2O between each sample.
20. Measure standards again, starting with Blank – DO NOT RESET THE BLANK.

Table 2: Combined Reagent

Amount (ml)	Reagent
25	Sulfuric acid
2.5	Potassium antimonyl tartrate
7.5	Ammonium molybdate
15	Ascorbic acid
Total 50	

Clean-up:

Acid-wash all tubes and caps used for analysis: use two washes of 1% HCl followed by two washes of Millipore water to wash all tubes and caps used for analysis. Pour 1% HCl into the test tubes in a blue rack; place another blue rack on top of that one and tip over into a waste bin in the sink to catch the acid wash. Recycle the same acid wash and pour back into the tubes for the 2nd rinse. Save the labeling tape on a space out of the way in the lab.

Miller Laboratory Nitrite Protocol

Principle

Nitrite reacts with sulfanilamide and "Ned", N-1-napthylethylene diamide dihydrochloride (similar to EDTA) to produce an azo dye that is red in color with max absorbance near 543 nm. Sulfanilamide consists of a sulfonamide group, ($SO_2(NH_2)$) attached to aniline. In an acidic solution nitrite is converted to nitrous acid (gives pale blue color), which reacts with the primary amine group of the aniline moiety of sulfanilamide producing the diazo (two linked nitrogen atoms) compound. This reacts with the primary amine of "Ned" producing the red dye.

Preparation of Reagents – Store reagents in 4C

1. Sulfanilamide: dissolve 1g in 90ml distilled water. Bring to 100ml with concentrated HCl. Wrap tinfoil around container.

2. N-1-napthylethylene diamide dihydrochloride: dissolve 2g in 1 liter of water. Wrap tinfoil around container.

3. 1 mg/L-N sodium or potassium nitrite standard

Preparation of Standards

1. To prepare standards, first dilute 1ml of Stock Potassium-Nitrite (1mg/L) into 9 ml of ddH_20 to give a working stock standard of 0.1mg/L.

Prepare standards according to table below. Add the following to each of the seven tubes.

Distilled Water (ml)	0.1 mg/L-N nitrite (ml)	Final Concentration (ug/L)
3.6	0.4	100
3.8	0.2	50
4	0.04	10
4	0.02	5
4	0.01	2.5
4	0.004	1
4	0	0

Procedure

1. Transfer 100 ul of each standard to a 1cm cuvette followed by 900 ul of distilled water. This is a 1:10 dilution, which provides a linear range of absorbance of the standards from 1 ug/L – 100 ug/L. Transfer 1000ul of each sample to a 1cm cuvette. For more or less concentrated samples, increase or decrease the dilution.
2. Add 0.2ml of sulfanilamine solution and incubate at room temp for 5 min
3. Add 0.2ml of NED solution
4. Blank the UV spec and measure the absorbance of each standard at 543 nm.
5. Measure the absorbance of all samples.
6. Measure absorbance of all standards again. Record all measurements.

Spectrophotometric determination of ammonium by conversion to indophenol

Reagents needed

1) Phenol-alcohol solution: dissolve 10g phenol in 100mL 95% ethanol
2) 0.5% nitroprusside: dissolve 1g of nitroprusside in 200mL ddH_2O
3) Alkaline solution: dissolve 100g trisodium citrate and 5g sodium hydroxide in 500mL ddH_2O
4) Hypochlorite solution (bleach): use a bleach that is at least 8% hypochlorite
5) To be made fresh daily, combine alkaline with hypochlorite solutions 4:1 (e.g. 100mL alkaline solution with 25mL bleach)

Method

1) Add 4% phenol-alcohol solution (e.g. 2mL to 50mL sample); mix
2) Add 4% nitroprusside solution (e.g. 2mL to 50mL sample); mix
3) Add 10% alkaline/hypochlorite solution (e.g. 5mL to 50mL sample); mix
4) Let color develop for at least 1hr and up to 24hrs
5) Read absorbance at 640nm using 10cm cuvette

Blank and standards

-For the blank and standards, use ddH_2O with solutions added. I made a 1g/L N-NH_4^+ stock (3.85g ammonium chloride in 1L ddH_2O = 1g N-NH_4^+/L).

-The working range using the 10cm cuvette is around 1µg/L up to 1000µg/L. Anything over that gets close to maxing out the spectrophotometer.

CURRICULUM VITAE

Sarah Bartlett

EDUCATION

PhD Freshwater Sciences and Technology, School of Freshwater Sciences

University of Wisconsin-Milwaukee

Graduation: December 2019

M.S. Freshwater Sciences, School of Freshwater Sciences

University of Wisconsin-Milwaukee

Graduation: May 2013

B.S. Natural Sciences, University Wisconsin-Madison

Certificate: Environmental Studies

Graduation: May 2010

Capstone: *Drip Tip Length vs. Presence of Epiphylls in Tropical Forest Understory Plants*

RESEARCH EXPERIENCE

Water Resource Specialist, April 2017-present

NEW Water, the brand of the Green Bay Metropolitan Sewerage District

Graduate Research Assistant, May 2013 – May 2019

Aquatic Chemistry and Microbiology Laboratory of Dr. Todd Miller,

Zilber School of Public Health, University of Wisconsin-Milwaukee**,** Milwaukee, WI.

- Cyanobacterial harmful algal bloom ecology and cyanotoxins in the eutrophic Lake Winnebago-Green Bay water system

Volunteer Field Assistant, August – October, 2011

Organization for Tropical Studies, La Selva Biological Station, Sarapiqui, Costa Rica.

- Mutualistic interaction between Myrmelachista ants and their obligate host plants

ACADEMIC HONORS AND FUNDING

Fellowships

- 2016-2017, CILER-GLERL Great Lakes Long-term Fellowship Award

  Cooperative Institute for Limnology and Ecosystems Research and NOAA's Great Lakes Environmental Research Lab

  PI: Dr. Todd Miller, UW-Milwaukee, Co-PI: Dr. Timothy Davis, NOAA-GLERL
- 2015-2016, Global Lake Ecological Observatory Network Fellow,

  U.S. National Science Foundation under Science Across Virtual Institutes

  Advisors: Dr. Paul Hanson, UW-Madison, Dr. Kathleen Weathers, Cary Institute of Ecosystem Studies

Honors

- NSF Early Career Travel Award

December 2018, U.S. National Science Foundation

Full travel support to attend the Global Lake Ecological Observatory Network (GLEON) All-Hands Meeting, held December 2-7, 2018 in Rottnest Island, Australia

- SFS Student Scholarship

  August 2016, School of Freshwater Sciences Student Scholarship, UW-Milwaukee

  Funding to attend and present at the 2016 Ecological Society of America conference held in Fort Lauderdale, FL in August 2016

- GLEON 17 Travel Award

  October 2015, U.S. National Science Foundation under Science Across Virtual Institutes

  Full travel support to attend the Global Lake Ecological Observatory Network (GLEON) All-Hands Meeting, held October 16-20, 2015 (Chuncheon, South Korea)

- SFS Student Scholarship

  June 2015, School of Freshwater Sciences Student Scholarship, UW-Milwaukee

  Funding to attend and present at the 2015 Gordon Research Conference: Mycotoxins and Phycotoxins held in June 2015.

- Graduate Student Travel Award

  June 2015, UW-Milwaukee

  Funding to attend and present at the 2015 Gordon Research Conference: Mycotoxins and Phycotoxins in June 2015.

- Jeffrey K. Kunkel Scholarship

  March 2015, School of Freshwater Sciences, UW-Milwaukee

- GLEON 16 Travel Award

October 2014, U.S. National Science Foundation under Science Across Virtual Institutes

Full travel support to attend the Global Lake Ecological Observatory Network (GLEON) All-Hands Meeting, held October 27-31, 2014 in Orford, Québec, Canada

- SFS Student Scholarship

  November 2014, School of Freshwater Sciences, UW-Milwaukee

  Funding to attend and give an oral presentation at the 2014 Great Lakes Beach Association conference held November 2014, in Toronto Canada.

- MAPMS Student Poster Award

  2014 Midwest Aquatic Plant Management Annual Conference

  Best Poster Presentation awarded at the annual conference held in Lombard, IL

- Graduate School Travel Award

  October 2013, UW-Milwaukee

  Travel support to attend the Global Lake Ecological Observatory Network held in October 2013 in Bahia Blanca, Argentina.

- Great Lakes National STEM Scholarship Recipient

  August 2012

- Nelson Institute Community Scholar

  April 2010, UW-Madison Nelson Institute

PUBLICATIONS

1. Miller, T. R., Bartlett, S., Weirich, C. A., & Hernandez, J. (2019). **Automated Sub-Daily Sampling of Cyanobacterial Toxins on a Buoy Reveals New Temporal Patterns in Toxin Dynamics**. Environmental Science & Technology.
2. **Bartlett, S. L.**, Brunner, S. L., Klump, J. V., Houghton, E. M., & Miller, T. R. 2018. Spatial analysis of toxic or otherwise bioactive cyanobacterial peptides in Green Bay, Lake Michigan. *Journal of Great Lakes Research*, *44*(5), 924-933.
3. Beversdorf, L.J., Rude, K., Weirich, C.A., **Bartlett, S.L.**, Seaman, M., Kozik, C., Biese, P., Gosz, T., Suha, M., Stempa, C. and Shaw, C., 2018. Analysis of cyanobacterial metabolites in surface and raw drinking waters reveals more than microcystin. *Water research*, *140*, pp.280-290.
4. McCullough, I.M., Dugan, H.A., Farrell, K.J., Morales-Williams, A.M., Ouyang, Z., Roberts, D., Scordo, F., **Bartlett, S.L**., Burke, S.M., Doubek, J.P. and Krivak-Tetley, F.E., 2018. Dynamic modeling of organic carbon fates in lake ecosystems. *Ecological Modelling*, *386*, pp.71-82.
5. Dugan, H.A., **Bartlett, S.L.**, Burke, S.M., Doubek, J.P., Krivak-Tetley, F.E., Skaff, N.K., Summers, J.C., Farrell, K.J., McCullough, I.M., Morales-Williams, A.M. and Roberts, D.C., 2017. Salting our freshwater lakes. *Proceedings of the National Academy of Sciences*, *114*(17), pp.4453-4458.
6. Dugan, H.A., Summers, J.C., Skaff, N.K., Krivak-Tetley, F.E., Doubek, J.P., Burke, S.M., **Bartlett, S.L**., Arvola, L., Jarjanazi, H., Korponai, J. and Kleeberg, A., 2017. Long-term chloride concentrations in North American and European freshwater lakes. *Scientific data*, *4*, p.170101.

7. Miller, T., Beversdorf, L., Weirich, C. and **Bartlett, S**., 2017. Cyanobacterial toxins of the Laurentian Great Lakes, their toxicological effects, and numerical limits in drinking water. *Marine drugs*, *15*(6), p.160.
8. Steinman, A.D., Cardinale, B.J., Munns Jr, W.R., Ogdahl, M.E., Allan, J.D., Angadi, T., **Bartlett, S.**, Brauman, K., Byappanahalli, M., Doss, M. and Dupont, D., 2017. Ecosystem services in the Great Lakes. *Journal of Great Lakes Research*, *43*(3), pp.161-168.
9. Beversdorf, L.J., Weirich, C.A., **Bartlett, S.L.** and Miller, T.R., 2017. Variable cyanobacterial toxin and metabolite profiles across six eutrophic lakes of differing physiochemical characteristics. *Toxins*, *9*(2), p.62.

PUBLICATIONS in Review, Preparation, or Submitted

1. **Bartlett, S.L.,** Perello, M., Baumert K., Smyth, R., Borre, L. Perceived ecological risk of eutrophication and cyanobacterial harmful algal blooms. In preparation for *Ecological Applications*
2. **Bartlett, S.L.,** Weirich, C. A., Miller, T. R. A multi-year analysis of cyanotoxins and toxic or otherwise bioactive peptides over a drinking water intake and possible environmental drivers. In preparation for *Environmental Science & Technology*
3. Doubek, J.P., Burke, S.M., Summers, J.C., Dugan, H.A., Krivak-Tetley, F.E., Skaff, N.K., **Bartlett, S.L.** Salt and phytoplankton and zooplankton community relationships in lakes and reservoirs across the United States. In preparation for *Ecosphere*

TEACHING EXPERIENCE

1. *Undergraduate Mentor*

Teach and supervise undergraduate students to obtain general laboratory skills related to aquatic environmental microbiology chemistry and guide students in independent research projects.

2013 – 6 students

2014 – 6 students

2015 – 5 students

2016 – 5 students

2017 – 1 student

2. *Invited Lecturer*

ECOLOGY OF ALGAL BLOOMS Field Course, Iowa State University. 11 July - 22 July 2016

PRESENTATIONS AT SCIENTIFIC MEETINGS

1. State of Lake Michigan, Green Bay, WI (2017): Assessing cyanoHAB toxicity in lower Green Bay, Lake Michigan
2. Society for Freshwater Sciences Annual Meeting, Milwaukee, WI (2015): Temporal Dynamics of Toxic and Non-Toxic Cyanobacterial Peptides in a Eutrophic Lake
3. Great Lakes Beach Association 14th Annual Meeting, Toronto, Canada (2014): High-Resolution Monitoring of Pharmaceuticals and Personal Care Products in Lake Michigan and Comparison to Microbial Indicators

ORGANIZED WORKSHOPS

Student insights on a model for team science and interdisciplinary research training. Ecological Society for America, August 2016 - Fort Lauderdale, FL

INVITED TALKS

"High Resolution Monitoring of Cyanobacterial Toxins in Lake Winnebago, WI " Iowa State University, Iowa Lakeside Lab, July 12, 2016.

CONFERENCE POSTER PRESENTATIONS

1. GLEON All-Hands Meeting, Rottnest Island, Australia (2018): Local knowledge and expert opinion of increasing eutrophication and cyanoHABs
2. GLEON All-Hands Meeting, Lake Mohonk, New York (2017): A macroscale study of global chloride trends, drivers, and ecological impacts in lakes
3. GLEON All-Hands Meeting, Chuncheon, South Korea (2015): A macroscale study of global chloride trends, drivers, and ecological impacts in lakes
4. Gordon Research Conference (GRC), Mycotoxins and Phycotoxins, Stonehill College, MA (2015): Temporal Dynamics of Toxic Cyanobacterial Peptides
5. GLEON All-Hands Meeting, Quebec, Canada (2014): Temporal Dynamics of Toxic Cyanobacterial Peptides in Drinking Water Sources.
6. Midwest Aquatic Plant Management Society 34th Annual Conference, Lombard, Illinois (2014): Influence of lake metabolism on toxic cyanobacteria bloom production.
7. GLEON All-Hands Meeting, Bahia Blanca, Argentina (2013): Influence of lake metabolism on toxic cyanobacteria bloom production.