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# Cyanobacteria and Cyanotoxin Ecology in Lakes and Drinking Water

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CYANOBACTERIA AND CYANOTOXIN ECOLOGY IN LAKES AND DRINKING

WATER


by

Chelsea Weirich


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ABSTRACT
CYANOBACTERIA AND CYANOTOXIN ECOLOGY IN LAKES AND DRINKING
WATER

by

Chelsea Weirich


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Under the Supervision of Professor Todd Miller, PhD


Freshwater harmful algal blooms (FHABs) present a threat to ecological and public

health in inland lakes. Problems associated with FHABs include human and animal

illness, production of taste and odor compounds, and declining water quality and

property values. Despite increased awareness and research on FHABs in recent

decades, questions remain regarding long-term growth and diversity of FHABs in lakes

that have taken measures toward slowing or reversing eutrophication, environmental

factors impacting toxin occurrence, the most appropriate toxin analysis methods for

protecting public health, and evidence for cyanotoxins detected in finished drinking

water as a connection to exposure for potentially causing disease; this dissertation

research seeks to add evidence to the field for answering such questions. Chapter 1

proposes maximum acceptable concentrations for a variety of cyanotoxins, in advance

of recent US Environmental Protection Agency guidelines, and reviews childrens' risk to

cyanotoxin exposure, as well as possible clinical biomarkers. A long-term analysis of

major FHAB-forming cyanobacteria and cyanobacterial community composition (CCC)

in Chapter 2 demonstrates connections to organic nitrogen, changes in ice cover, total

phosphorus, Schmidt stability and rainfall correlating with cyanobacterial abundance

and CCC. Chapter 3 presents novel preservation methods for cyanotoxins in lake water samples for intercontinental shipping or storage, as well as describes patterns of occurrence for 13 cyanopeptides (e.g. microcystins, anabaenopeptins, cyanopeptolins, nodularin, microginin) in 22 lakes on a semi-global scale using liquid chromatography with tandem mass spectrometry quantification methods. These patterns in cyanotoxin occurrence are then analyzed based on characteristics of their lakes, including mussel invasion, dominant surrounding land usage, trophic status, and lake size. Finally, Chapter 4 characterizes amounts and types of cyanopeptides in raw and finished drinking water and efficiency of their removal among four drinking water treatment process trains. The resulting work demonstrates that other peptides are as common as microcystins in lakes and drinking water, and a variety of cyanopeptides at low doses are detectable in finished water from modern water treatment plants, while providing novel storage, extraction, and detection methods and defining parameters of interannual cyanobacterial dominance in a eutrophic lake with suggested future directions for monitoring.

Dedication

I dedicate these writings to my parents, Joseph Weirich and Catherine Breider, who encouraged me to study science with passion and persistence, and to my boyfriend, Nathaniel Kuhl, my source of endurance and inspiration.

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## LIST OF ABBREVIATIONS

AC – activated carbon

ACE – angiotensin-converting enzyme

ACN – acetonitrile

Adda – ((2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic

acid)

ALP – alkaline phosphatase

ALT – alanine aminotransferase

Apts – anabaenopeptins

Arg – arginine

AST – aspartate aminotransferase

ATX – anatoxin-a

BMAA – β-methylamino-L-alanine

BMP – best management practices

BV – biovolume

b.w. – bodyweight

CASPER – Community Needs Assessment for Public Health Emergency Response

CCC – cyanobacterial community composition

CDC – Centers for Disease Control and Prevention

CGS – cyanobacterial growing season

Chl-*a* – chlorophyll-*a*

Cpts – cyanopeptolins

$C_v$ – coefficient of variation

cyanoHABs – cyanobacterial harmful algal blooms

CYN – cylindrospermopsin

DBP – disinfection byproducts

DCA – Detrended Correspondence Analysis

Dha – dehydroalanine

DON – dissolved organic nitrogen

DRP – dissolved reactive phosphorus

DWA – Drinking Water Advisory

DWTP – drinking water treatment plant

EFSA – European Food Safety Authority

ELISA – enzyme-linked immunosorbent assay

EPA – Environmental Protection Agency

EtOH – ethanol

$Fe_2(SO_4)_3$ – ferric sulfate

FHABS – freshwater harmful algal blooms

GAC – granular activated carbon

GLEON – Global Lakes Ecological Observatory Network

GGT – gamma-glutamyltransferase

Gpd – gallons per day

HOCl – hypochlorous acid

J – Joules

KJDL N – Kjeldahl nitrogen

$KMnO_4$ – potassium permanganate

LC – liquid chromatography

LOD – limit of detection

LPS – lipopolysaccharide

MAC – maximum acceptable concentration

MC-LR – microcystin-leucine, arginine

Mcgns – microginins

MeOH – methanol

metaT – epilimnion depth

MMPB – 3-methoxy-2-methyl-4-phenylbutyric acid

MS – mass spectrometry

MS/MS – tandem mass spectrometry

N – nitrogen

NaCl – sodium hypochlorite

NC – not colonized by dreissenid mussels

$NH^+_4$ – ammonium

$NH_3$ – ammonia

NOAEL – no-observed-adverse-effect-level

NOD – nodularin

NOM – natural organic matter

$NO^-_3NO^-_2$ – nitrate + nitrite

NTL-LTER – North Temperate Lakes Long Term Ecological Research

OZO – ozonation

P – phosphorus

PAC – powdered activated carbon

PAR – photosynthetically active radiation

PAR:Epi – ratio of depth of the photic zone divided by depth of the epilimnion

PDA – photodiode array

PDC – Parkinson's disease complex

PH – Public Health

$PO_4$ - phosphate

PP – protein phosphatases

PSP – paralytic shellfish poison

ROS – reactive oxygen species

SD – standard deviation

St – Schmidt stability

STX – saxitoxin

TDI – tolerable daily intake

TN – total nitrogen

US – United States

UV-Vis – ultraviolet-visible spectrophotometry

WHO – World Health Organization

WSP – Water Safety Planning

Wtemp – water temperature mean from 0-8 meters

ACKNOWLEDGEMENTS

**CHAPTER 1:**

**Freshwater Harmful Algal Blooms: Toxins and Children's Health**

**Introduction**

An increasing number of waterways in the United States support frequent and intense freshwater harmful algal blooms (FHABs). These large accumulations of cyanobacteria (often called "blue-green algae") contain toxins harmful to humans, pets, fish, birds, and other wildlife. Decaying algae release noxious odors and reduce dissolved oxygen levels resulting in a loss of biodiversity [1]. As the incidence of FHABS increases there is greater probability that humans may be exposed to FHAB toxins in recreational environments through accidental ingestion of natural waters or intended ingestion of drinking water and food (e.g. sport fish) containing the toxins. Exposure through skin contact may also result in dermatitis [2, 3]. The toxicology of the most common FHAB toxins is well known and most are effective at very low doses (parts-per-billion). Multiple cases of acute illness, primarily in children and young adults has been reported [4, 5]. Frequent low-level exposures are associated with chronic illnesses such as liver cancer [6-9]. There are no laws requiring regular testing for FHAB toxins in recreational settings, drinking water or fish in the United States and only recent laws in a few states (e.g. Oklahoma Senate Bill 259) require posting advisories at recreational areas warning of the presence of FHAB toxins, but without any mandate for regular testing of recreational waters. While FHAB poisonings are likely rare compared to other diseases, the rate of FHAB related illnesses is essentially unknown and anecdotal reports of FHAB poisonings are common in the United States and elsewhere, particularly during summer months.

The apparent increase in FHAB events in U.S. waterways is due to a variety of factors including increased agricultural productivity, land use change, and global climate change. Algal productivity in freshwater environments is most often limited by phosphorus and sometimes nitrogen. Addition of these nutrients allows the algae to replicate in the presence of other optimal conditions (e.g. temperature, lack of predators, etc.). Thus, runoff from fertilizers used on croplands is a major contributing factor to the occurrence of FHABs [10]. Agricultural pressures on U.S. lands have increased in recent decades and farmers are now getting more food from the same amount of land. For example, the U.S. farm output has grown at a steady rate of 1.63 percent per year since 1948, yet from 1945 to 2002 the total cropland in the United States has decreased from 451 to 442 million acres [11]. At the same time annual phosphorus and nitrogen application to these lands has increased by 1.5 and 9.5 million pounds, respectively from 1960 – 2010 [12]. The end result is greater fertilization per acre of cropland, making it more difficult to stem the tide of non-point source nutrient runoff.

While agricultural productivity and fertilizer usage has increased, so too have pressures for residential development around lakes and rivers. Lakefront property values continue to increase. For example, in 2002, adding a view of Lake Erie to a home added over $250,000 to the sale price [13]. Development around lakes has also increased. Between 1992 and 2001, 33.5% and 7.5% of lands in the U.S. Great Lakes watershed were converted to residential property and roads, respectively [14]. Similar levels of development have been observed to degrade littoral, nearshore habitats [15].

Residential housing development and creation of roads decreases vegetation in nearshore habitats that would otherwise reduce nutrient runoff [16].

The Clean Water Act, section 303(d) requires states to identify water bodies that are unsafe and that do not meet current water quality standards, and take action to clean them up. This especially applies to non-point source pollution such as nutrient runoff that is responsible for FHABs. While largely ignored by states, a reduction in nutrient loading to lakes and rivers from non-point sources is required in order to be in compliance with the Clean Water Act. A variety of best management practices (BMP) have been employed to control storm water and agricultural runoff. These BMPs are aimed at reducing nutrients and other contaminants in the storm water before it enters lakes and rivers. These include diverting runoff into grasslands or wetlands, retention ponds, water treatment systems, trenches, and rain barrels, among others [17, 18]. In agriculture, the implementation of buffer strips, ~50 foot wide strips of grass, reduces nutrient runoff from croplands [19]. A reduction in nutrient runoff may also be achieved by maintaining proper manure storage areas and reducing liquid manure spreading and spreading of manure on hard or frozen soils [20]. Some areas of the country have observed decreases in nutrient loading to lakes and rivers as a result of these practices including modest reductions in the Northeastern U.S. [21]. However, across the entire country currently over 90% of rivers in 12 out of 14 EPA ecoregions are considered nutrient enriched or eutrophic and thus capable of supporting FHAB activity [22].

Returning over-enriched lakes to pre-enrichment status will likely require decades of intense management efforts. However, even if excess phosphorus inputs to lakes were to completely stop today, many lakes contain sufficient levels of phosphorus

stored in sediments to fuel FHABs for decades [23, 24]. Major reductions are needed in order to observe any significant decreases in algal production [23]. Current mandates for total maximum daily phosphorus loadings are already difficult for municipalities to obtain, particularly in agriculturally impacted areas. In the meantime, global climate change may be increasing the intensity and frequency of FHAB events since FHAB species, primarily cyanobacteria, grow fastest at higher water temperatures (>20 C) [25]. In addition, toxic tropical algal species have spread into temperate regions, presumably as a result of global climate change. The result is a greater diversity of toxins, not previously present in temperate areas and more intense and/or frequent FHAB events [26].

There has been an increase in the number of lakes and rivers in the United States that are capable of supporting FHAB toxin production (i.e. eutrophic environments) [22]. This is problematic since most of the municipal drinking water in the United States (63%) is produced from surface waters (lakes, rivers, reservoirs) and the number of homes obtaining water from a municipal supply has steadily increased at a rate of 0.5% per year since 1950 [27]. Drinking water treatment processes have been shown to remove some, but not all FHAB toxins [28]. Low levels of FHAB toxins have been detected in U.S. finished drinking water [28]. The steady increase in use of surface waters for drinking water production combined with an increase in the number of lakes and rivers capable of supporting FHAB toxin production indicates that greater pressure will be placed on water treatment plants to produce safe drinking water in the future. Substantial upgrades at cost to consumers will likely be required in order to maintain a safe drinking water supply.

Children, as opposed to adults are most likely to be affected by FHAB toxins due to a combination of smaller size, risky behaviors, and developmental toxicity. Herein we describe the major FHAB toxins children are most likely to encounter, the mode of action of FHAB toxins, symptoms they produce, and potential methods for detecting FHAB toxin exposure in the human population. We also review some anecdotal reports of children's exposure to FHAB toxins in the United States and elsewhere, as well as epidemiological investigations, specifically with regards to children's health and FHAB-related illnesses.

**FHAB toxins, mechanism of action and symptoms**

Cyanobacteria are the major producers of FHAB toxins. These photosynthetic bacteria are ancient organisms, responsible for oxygenation of the planet over 3.5 billion years ago. They have evolved the capability to produce a wide range of compounds known as secondary metabolites. These metabolites are not essential for normal functioning of the cell, but provide some benefit to the organism. Although it is not readily known why cyanobacteria produce these compounds studies have suggested that they may harm or ward off predators, act as signaling compounds between cell types, act as iron scavengers, adsorb oxygen radicals produced during photosynthesis, provide osmotic balance, or act as nitrogen and/or carbon storage molecules among other functions [29-31]. The diversity of such compounds in nature and their pharmacological activity are a rich area of study. Some cyanobacterial secondary metabolites, including some FHAB toxins may be useful for human health as antibacterial, anti-fungal, or anti-cancer agents when prescribed and used as intended.

However, in the case of FHAB events, the exposure is accidental, unintended, and the dose is unknown. Therefore it is important to recognize that cyanobacteria produce a complex suite of compounds that may produce some effect or symptom in the human body, whether desired or not. Except for endotoxin, ingestion of water containing the toxin is required for poisoning to occur.

The most commonly encountered FHAB toxins can be divided into three groups including hepatotoxins, neurotoxins, and endotoxin. The symptoms of exposure to these different types of toxins are summarized in Table 1.1. These algal toxins pose risk to human health in waters used both for recreational activity and purifying drinking water. Most drinking water standards are based on the 1 µg/L recommended limit of microcystin-LR from the World Health Organization (WHO) [32], but often no regular testing or penalties are imposed until poisoning events occur. Recreational water standards are determined locally in the United States, along with algal cell counts for determining low-, medium-, high-, and extremely high-risk levels based on the WHO recommendations for public health risk monitoring. Until very recently most states in the U.S. completely ignored or were unaware of the threat of FHAB toxins to the safety of recreational environments and drinking water. Internationally, many countries have adopted the WHO standard for microcystins in drinking water [33].

**Microcystins and related hepatopeptides**

Microcystins and nodularin are the most commonly found hepatotoxins in fresh and brackish waters. Microcystin production has been reported from multiple species of

Table 1.1 Major FHAB toxins, target organs, symptoms, effect levels, and observed environmental concentrations

| Toxin | Primary Chemical Target | Symptom(s) | NOAEL[1] (µg/kg b.w.) | Environmental Concentrations | Ref. |
|---|---|---|---|---|---|
| Microcystins | Serine/threonine-specific phosphatases in liver | Diarrhea, vomiting, goosebumps, weakness; liver hemorrhaging /toxicity | 40 | 48.6 µg/L microcystin-LR, Salto Grande Dam, Argentina; 30-2480 µg/L Total microcystins, Hirosawa-no-ike Pond, Kyoto, Japan; 2.5-7,600 µg/L microcystin-LR, various water bodies, Wisconsin, USA; 0.03-16,000 µg/L microcystin-RR, various water bodies in Iowa | [34-36] [37-39] |
| Nodularin | Serine/threonine-specific phosphatases in liver | Diarrhea, vomiting, goosebumps, weakness; liver hemorrhaging | Not enough toxicological data for specific NOAEL - refer to microcystins | 42,300 µg/L Gulf of Gdańsk, Poland | [40] |
| Cylindrospermopsin | Protein synthesis | Gastroenteritis, kidney failure, liver failure | 30-150 | 4-173 µg/L Gazan Dam Lake, Saudi Arabia; 0.41-18.43 µg/L Lake Albano, Italy; 0.082-12.1 µg/L in German lakes | [41-44] |

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[1] NOAEL is the "no-observed-adverse-effect-level," an exposure level based on empirical data at which there is no statistically significant change in observed detrimental health effects when compared to healthy controls.

Table 1.1 Major FHAB toxins, target organs, symptoms, effect levels, and observed environmental concentrations

| | | | | | |
|---|---|---|---|---|---|
| Anatoxins | Nicotinic receptors or acetylcholinesterase in neuromuscular junctions | Muscle twitching, salivation (-a(S)), cramping, paralysis, possible death | 500 (Not enough toxicological data available for NOAELs of homoanatoxin-a and anatoxin-a(S)). | 0.7-1750 µg/L Anatoxin-a, various water bodies, Wisconsin, USA; 0.01-13.1 µg/L Anatoxin-a, various water bodies in Germany; 24 µg/L Homoanatoxin-a, Lough Sillan, Co. Cavan, Ireland | [39, 45-48] |
| Aplysiatoxins | Protein kinase C; tumor promoters | Skin irritation; asthma-like symptoms | Not enough toxicological data for specific NOAEL | 0.01-85.96 µg/g (toxin/algae, dry-weight) debromoaplysiatoxin, Hawaiian Islands, USA | [35, 49] |
| Lyngbyatoxins | Protein kinase C; tumor promoters | Smooth muscle contraction leading to irritation, dermatitis, poisoning; asthma-like symptoms | Not enough toxicological data for specific NOAEL | 5.97 µg/g (toxin/algae, dry-weight) Lyngbyatoxin-A, Moreton Bay, Australia; 0.02-30.64 µg/g (algae, dry-weight) Lyngbyatoxin-A, Hawaiian Islands, USA | [35, 49] |
| Saxitoxins | Sodium channels at neuromuscular junctions | Muscle twitching | 0.5 | 215 µg/g (toxin/algae, dry-weight) *Lyngbya wollei* toxin-1, St. Lawrence River, Canada; 50-3400 µg/g (algae, dry-weight) Tullaroop Reservoir, Australia | [50-52] |
| Lipopolysaccharide | Irritant – exposed tissue | Gastroenteritis; Skin irritation, eye irritation, allergic reactions, hay fever, asthma | | | |

Table 1.1 Major FHAB toxins, target organs, symptoms, effect levels, and observed environmental concentrations

| | | | | | |
|---|---|---|---|---|---|
| | NMDA excitotoxicity; | No specific acute clinical symptoms; | Not enough toxicological data for | 17-25 µg/L in South African water supplies | |
| BMAA | NMROS production | ALS/PDC with long-term exposure | specific NOAEL | | [53, 54] |

cyanobacteria including *Microcystis, Anabaena, Oscillatoria, Planktothrix, Chroococcus*, and *Nostoc*, whereas nodularin is primarily produced by *Nodularia spumigena* [55, 56]. Microcystins' and nodularin's common cyclic structure (Figure 1.1, A and B) consists of an Adda ((2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) chain [57], and then either six or four amino acids, respectively. Together, microcystins and nodularin have 80 different structural variants [58]. These cyanobacterial hepatotoxins bind serine/threonine-specific protein phosphatases PP1A and PP2A in animal liver cells [59]. Microcystins are able to covalently bind to these enzymes after entering animal cells via bile acid transporters called organic anion transporting polypeptides, evidence of which has been found in liver and brain cells [60, 61]. Phosphatase binding causes hyperphosphorylation of cell infrastructure and hence destruction of the cells, resulting in tissue hemorrhaging. Other mechanisms of microcystin toxicity include production of reactive oxygen species (ROS), mitochondrial interaction, cell apoptosis, and influence of transcription factor and protein kinase activity, affecting cell proliferation pathways, as reviewed by Campos and Vasconcelos [62]. Both microcystins and nodularin act as initiators and promoters of tumor growth as indicated by several animal studies [63-66]. Tumor initiation and growth in animal studies have formed the basis for epidemiologic investigations of microcystin exposure correlating with epidemic liver and colorectal cancers in China and other countries in Eastern Europe [6-9]. Clinical symptoms of acute microcystin or nodularin exposure include diarrhea, vomiting, "goosebumps", weakness, and pale skin [67, 68].

Attempts have been made to determine what levels of exposure would be "safe" for microcystins and other toxins. A no-observed-adverse-effects-level (NOAEL) of 40

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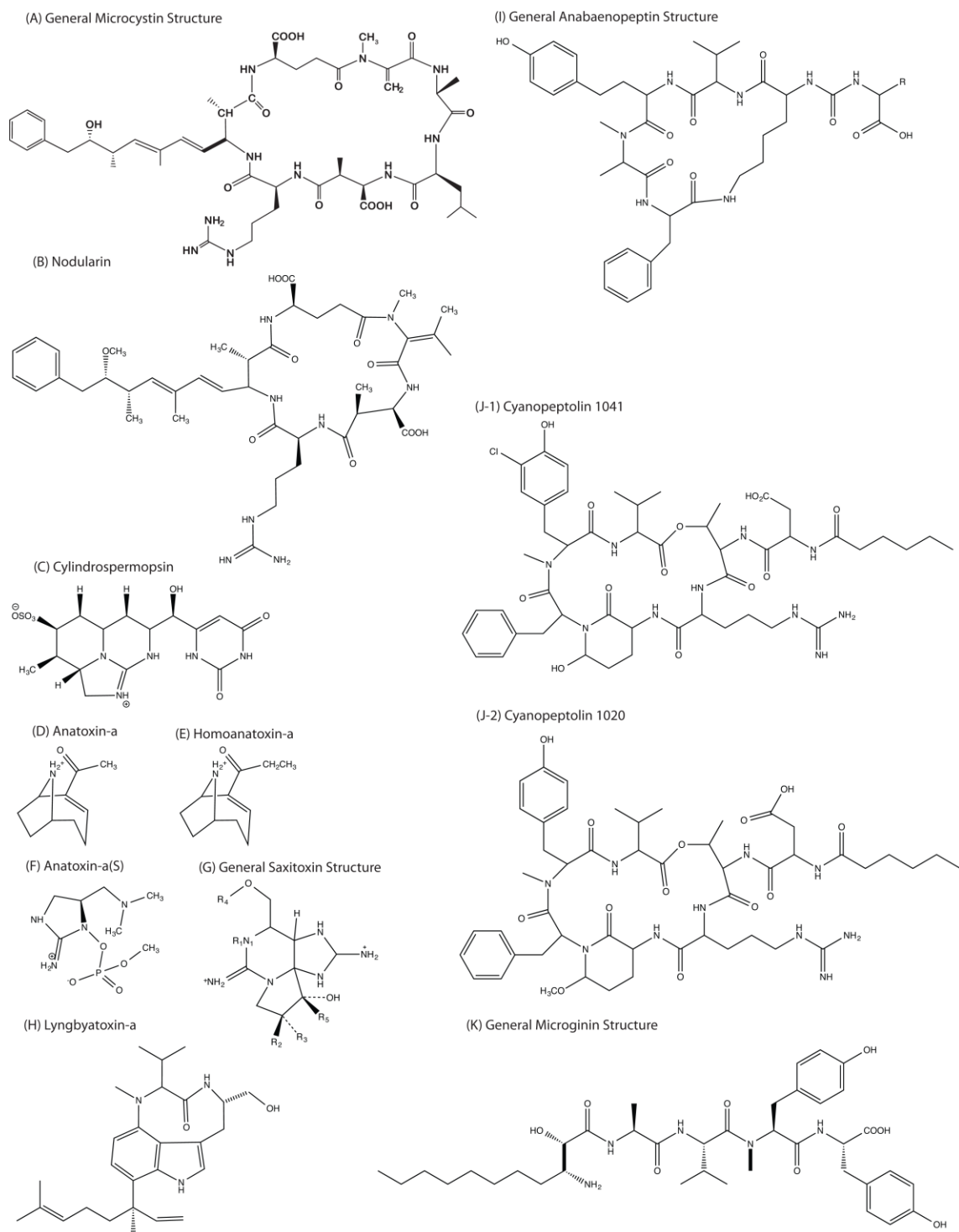
Figure 1.1: Examples of structures from cyanobacterial toxins. (A) General structure of microcystins with table showing differences in microcystin structure of microcystin-LR, microcystin-YR, microcystin-RR and microcystin-LA. (B) Nodularin. (C) Cylindrospermopsin. (D) Anatoxin-a. (E) Homoanatoxin-a. (F) Anatoxin-a(S). (G) Lyngbyatoxina-A. (H) LPS. (I) General structure of PSP.

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μg/kg bodyweight (b.w.) has been determined for microcystins based on toxicological data from mice orally administered microcystin-LR [34]. The NOAEL for microcystin-LR has been adopted for all microcystins and for nodularin because 1) microcystin-LR is the most potent and most often detected microcystin worldwide with the most information available and 2) there is a lack of sufficient toxicological data on nodularin to establish a specific NOAEL [35].

**Anatoxins and related alkaloids**

Anatoxins are the most commonly detected FHAB neurotoxins. Anatoxin-a is a bicyclic amine produced by cyanobacterial genera *Anabaena, Microcystis, Aphanizomenon* and the benthic *Oscillatoria* [69]. Anatoxin-a and its methyl derivative homoanatoxin-a (Figure 1.1D, E) mimic acetylcholine in binding to nicotinic receptors, which disrupts signaling at nervous and neuromuscular junctions, causing paralysis, possibly respiratory arrest and subsequent death [70]. Normally, a neuron's action potential causes a muscle contraction through cation influx, and then acetylcholinesterase degrades the excess acetylcholine in the neuromuscular junction to stop the muscle contraction. However, when anatoxin-a binds to the nicotinic receptors, acetylcholinesterase cannot degrade it as it normally would acetylcholine. Anatoxin-a continues to bind those receptors.

Anatoxin-a(S) is produced mainly by *Anabaena* sp. (Figure 1.1F); it is structurally unrelated to anatoxin-a, but produces the same net effect in nerve cells with significantly more potency  [71, 72]. Rather than blocking acetylcholine from interacting with its receptors, anatoxin-a(S) acts as many organophosphate pesticides in inhibiting

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acetylcholinesterase from degrading its substrate, causing continuous stimulation from the nerve cell [73]. Anatoxin-a(S) acts only on peripheral nerve junctions, so brain and eye function are kept intact even after exposure [74]. Anatoxin-a(S) has been shown to induce salivation in animals, which led to the addition of an (S) on the end of its name [71]. Otherwise, though the mechanisms of anatoxins differ in their chemical targets, common symptoms of exposure include muscle twitching, cramping, eventual fatigue and paralysis, and possible death.

**Cylindrospermopsin**

Cylindrospermopsin is a hepatotoxic alkaloid produced by several cyanobacteria including *Cylindrospermopsis raciborskii, Aphanizomenon ovalisporum, Raphidiopsis curvata,* and *Umezakia natans* (Figure 1.1C) [75-78]. Cylindrospermopsin blocks protein synthesis and acts as a general cytotoxin capable of affecting the kidney, thymus, spleen, heart and gut lining, though its primary effects are seen in the liver. Postmortem signs of cylindrospermopsin poisoning include a dark-colored liver with rib marks, pale kidneys, and necrosis of the kidneys, thymus and spleen [79-81]. Acute poisoning has caused death likely due to heart failure in mice [81]. Cylindrospermopsin-producing cyanobacteria also make two other related metabolites, deoxycylindrospermopsin and 7-epicylindrospermopsin, the latter being cytotoxic [76, 82].

**Saxitoxins**

Saxitoxins, also known as paralytic shellfish poisons (PSPs), are another group of potent neurotoxins produced by *Aphanizomenon flos-aquae, Lyngbya wollei,*

*Anabaena circinalis, Planktothrix* sp., and *Cylindrospermopsis raciborskii [83-87].* They are also well-known as being produced by marine dinoflagellates [88]. The general structure of PSPs is a tetrahydropurine with substitutions at five variable positions (Figure 1.1H). Over 20 different variants of PSPs are identified by their different structural groups, including saxitoxins - saxitoxin, decarbamoyl saxitoxin, and neosaxitoxin – which lack sulfur; gonyautoxins, having a single sulphate; C-toxins, having two sulfates; and decarbamoyl derivatives and *Lyngbya wollei* toxins [89]. All saxitoxins bind to site 1 of sodium channels at neuromuscular junctions, blocking nerve transmission and causing muscle paralysis [90, 91]. Other symptoms include loss of motor control, twitching, and convulsions. While PSPs are associated with marine and tropical freshwaters, the recent spread of *C. raciborskii* into temperate regions suggests that PSPs may soon gain a cosmopolitan distribution [92].

The European Food Safety Authority reported in 2009 that no NOAELs had been investigated for PSPs and an inability to calculate one off of previous toxicological data because of a lack of relevant data from animal-based studies. Based on epidemiological data, they determined a lowest-observed-adverse-effects level of 1.5 µg/kg b.w. [50]. From this value, they estimated a NOAEL value of 0.5 µg/kg b.w. as a reference for potential human consumption of contaminated shellfish from PSP bioaccumulation. A value of 75 µg saxitoxin equivalents/kg meat was calculated for maximum acceptable amount of saxitoxin in shellfish [50]. It is not clear whether there are any chronic diseases associated with long- term low level exposure to FHAB neurotoxins.

**Lyngbyatoxin**

*Lyngbya wollei* is a benthic cyanobacterium responsible for producing the dermatoxic lyngbyatoxins-A, -B, and -C as well as PSPs [93, 94]. Lyngbyatoxins, along with another dermatoxic alkaloid aplysiatoxin, have caused acute dermatitis, poisonings and animal death in several tropical regions [95, 96]. They act through phospholipid-dependent protein kinase C activation, causing smooth muscle contraction; symptoms include skin, eye and respiratory irritation [94]. Lyngbyatoxin A has also been shown to be a tumor promoter [97].

**Endotoxin**

Lipopolysaccharide (LPS) is a molecule in the outer cell wall of gram-negative bacteria, including cyanobacteria. Its structure consists of a sugar, commonly hexose, a lipid and fatty acid. Lipid A of LPS is the primary antigen responsible for activating an innate immune response through binding to toll-like receptors and this is present in cyanobacterial LPS [98]. Biological activity experiments performed with mice and rabbits showed that cyanobacterial endotoxins are less toxic compared with studies reported on heterotrophic gram-negative bacteria [99]. However, cyanobacterial LPS could still be considered a skin irritant after swimming or bathing in heavily algal-contaminated water bodies [3].

**Other cyanobacterial toxins**

The neurotoxic non-protein amino acid, β-methylamino-L-alanine (BMAA) is thought to be produced by nearly all cyanobacteria [100]. Several researchers have reported successfully detecting and quantifying BMAA from brain tissues of deceased

patients with amyotrophic lateral sclerosis ALS and Alzheimer's disease [101, 102].

BMAA was first detected in cycad seeds during an investigation of extremely high rates

of amyotophic lateral sclerosis/Parkinson's disease complex in local Chamorro

residents of Guam [103]. Symbiotic *Nostoc* bacteria in the roots of the cycads produce

BMAA, which then accumulates in the plant; the seeds of the plant are harvested to

make flour, and the people consume the cycad products [104, 105]. The proposed

mechanisms by which BMAA exerts its toxicity, as demonstrated in animal and human

cell studies, are excitotoxicity via N-methyl-D-aspartate receptors and production of

reactive oxygen species, leading to mitochondrial stress and DNA damage, which in

absence of sufficient repair can lead to amyotophic lateral sclerosis/Parkinson's disease

complex over time [106].

Initial studies indicating that most cyanobacteria produce BMAA, including FHAB

species, used liquid chromatography with fluorescence detection after derivatization of

BMAA with the fluorescent derivatizing agent AccQ Fluor [100]. Recent studies using

direct detection of BMAA with liquid chromatography and tandem mass spectrometry

show that most cyanobacteria do not produce BMAA or produce very low quantities

[107]. However, during these studies some cyanobacteria were shown to contain a

different amino acid neurotoxin, 2,4-diaminobutyric acid, normally associated with

*Lathyrus sativus* (grass pea) [108]. Due to a lack of sufficient toxicological data, no

NOAELs have been calculated for BMAA [53].

In addition to microcystins and nodularin, cyanobacteria produce hundreds of

other peptides [109]. These peptides have value as antibacterial, anti-malarial, anti-

tumor, and anti-viral drugs. For example, cryptophycins produced by *Nostoc* sp. are

potent cytotoxins that cause microtubule depolymerization [110]. A derivative of cryptophycin is in human trials as an antitumor drug [111]. Microginin is a pentapeptide from *M. aeruginosa* that inhibits angiotensin-converting enzyme and therefore has value in the treatment of high blood- pressure [112]. The toxicological effects of other peptides predicted by studies of cyanobacterial genomes have not been as well studied [113]. It can be concluded that in most instances, human exposure or ingestion of FHAB material likely means exposure to a wide range of compounds with varying degrees of toxicity or pharmacological activity in the human body. The molecules known as FHAB toxins discussed above are targeted for monitoring efforts because they are the constituents that have been shown to elicit the greatest toxicity in animal studies and are prevalent in the environment. However, the combination with other known or unknown compounds likely produces a complex suite of symptoms making it difficult to characterize the causative agent.

**Children are at greatest risk for FHAB toxicosis**

Children receive some of the greatest benefits from recreational waters and for the most part, in the United States a day of swimming at the local beach is likely to be a safe and enjoyable childhood experience. However, precautions should be taken to ensure the safety of recreational environments. The WHO provides guidelines for safe swimming in recreational environments that encompasses FHABs [114].

The WHO suggested a maximum acceptable concentration (MAC) for microcystin-LR in drinking water is based on the NOAEL for microcystin multiplied by an uncertainty factor of 1000 for a tolerable daily intake (TDI) of 0.04 µg/kg body weight. To

get the MAC, the TDI is multiplied by the body weight and 0.8 representing the fraction of drinking water that is ingested, divided by an assumption that an individual drinks 2 liters of water per day. The MAC often used for microcystin-LR is based on a 60 kg adult, but for children the MAC is much lower. We have calculated (Table 1.2) MACs of microcystin-LR, anatoxin-a and cylindrospermopsin for a 60-kg adult and children weighing 10, 20, or 30 kg. We assumed that children would drink half as much volume of water as an adult, at 1 liter per day. As such a 30-kg child has the same MAC as a 60-kg adult, but as the child's weight decreases, so does the MAC of toxin in ingested water. Thus, as a general guideline, it may be appropriate to use a lower MAC in order to protect children's health.

Overall, children are at a greater risk for FHAB poisoning than are adults. Compared to adults children spend more time in the water and swallow more water per body weight than adults. A study by Dufour et al. used breakdown products of chlorine stabilizers as a tracer in urine to determine that children swallow about twice as much water as adults in absolute volume (37 and 16 ml, respectively) [115]. Children as well as some adults may ignore posted warnings of FHABs or may not be aware of the hazards posed by waters containing FHAB toxins. Swimming at night is another risky behavior, which is associated with both adults and children, but is probably more prevalent among young men in their 20's [116].

Besides risky behavior and lower body weight, developmental effects must also be considered with FHAB toxins [117]. Exposure of fish embryos and larvae to microcystins causes changes in embryonic hatching, decrease in survival and growth rate, and histopathological effects (as reviewed by [118]). Zebrafish embryos exposed

Table 1.2. Maximum acceptable concentration (MAC)[1] in µg/L for common FHAB toxins.

| Bodyweight (kg) | 60 | 30 | 20 | 10 |
|---|---|---|---|---|
| Microcystin-LR | 0.96 | 0.96 | 0.64 | 0.32 |
| Anatoxin-a[2] | 12 | 12 | 8 | 4 |
| Cylindrospermopsin[3] | 0.72 | 0.72 | 0.48 | 0.24 |

1. Calculated by [TDI (µg/kg) x b.w. (kg) x drinking water fraction of diet]/L of water consumed per day = MAC (µg/L).

2. MAC for anatoxin-a was calculated based on the NOAEL established of 500 µg/kg. However, Devlin et al. reported that since the symptoms of anatoxin-a are acute, and essentially "all-or-none", that a minimum lethal dose was used in their toxicity studies instead of a 50% lethal dose.

3. MAC for cylindrospermopsin was calculated based on the conservative NOAEL of 30 µg/kg reported in Table 1.1.

to saxitoxin develop edemas of the eye, pericardium and yolk sac, curvature of the spine and sensorimotor deficits [119]. Developmental effects have also been observed for anatoxin-a and cylindrospermopsin in mouse models [1, 120]. Whether these studies translate to perturbations in human development is uncertain.

It is likely that acute FHAB toxin poisoning in the United States is rare, but the actual rate of FHAB toxin poisonings, particularly in recreational settings is unknown. This is primarily due to the lack of proven medical diagnostic tests (e.g. blood test) and a lack of formal monitoring programs to assess the rate of exposure in the U.S. population. In addition many people might not report the illness or recognize that they are ill from FHAB toxins. Contamination of drinking water at high levels causes massive illnesses, but recreational exposures are often isolated incidences involving a small number of individuals. FHAB toxin symptoms are variable between FHAB exposure events and non- specific, indicative of a number of different maladies. This makes it nearly impossible for a medical professional to identify FHAB toxins as the cause of the illness based on symptoms alone. In some heavily investigated FHAB recreational exposures a comprehensive patient history and thorough examination of patient symptoms has offered a diagnosis [121].

It is unusual for the toxin involved in FHAB toxicosis to be identified due to their short half-life, elimination or concentration in organs. For example, in the case of microcystin-LR, 67% of the toxin concentrates in the liver within 60 minutes making it difficult to detect in blood [122]. Detection of FHAB toxins in blood of recreationalists with known exposure has not been successful, but the severity of symptoms has been shown to correlate with exposure duration (i.e. time in the water) [123]. Detection of a

specific toxin involved in an illness is complicated by the fact that cyanobacteria may produce a variety of toxic or pharmacologically active metabolites all of which may be contributing to the patient's symptoms. In some patients, it has been possible to converge on a single FHAB toxin or FHAB algal species as the probable cause using the weight of several lines of evidence.

Cases of FHAB poisonings are most often confirmed by patient history, symptoms, and microscopic examinations of algal cells in stool samples as well as associated dog and cattle deaths at the time patients are exposed to FHABs [121, 124]. These criteria provide an exceptionally strong case for the involvement of FHAB toxins. However, since gastrointestinal illness is a major symptom, water- born bacterial and protozoan (e.g. *Cryptosporidium*) infection needs to be ruled out. Collecting these lines of evidence requires substantial testing, thorough examination of patient history, and adequate communication between hospitals, veterinary clinics, local health departments, sanitary district managers and departments of natural resources.

The Harmful Algal Bloom-related Illness Surveillance System (HABISS) created by the Centers for Disease Control (CDC) is a network of public health information and environmental monitoring data, with weekly web and phone reports that attempts to provide coordination among groups to deliver the kind of information that is necessary to track algal intoxications [125]. It is operated in regions that experience harmful algal bloom events including the Great Lakes region for monitoring FHABs, Gulf of Mexico, Gulf of Maine, Hawaii, and Washington State where toxic marine dinoflagellates and diatoms are problematic. A variety of other harmful algal bloom monitoring programs are in place, but most are for coastal ocean environments and not freshwater [126].

The majority of information about FHAB poisonings in recreational settings comes from media and case reports. Recent FHAB intoxications reflect the impact of FHABs on children, teens and young adults in recreational settings. Here we review a few cases to illustrate the conditions under which children and young adults have become ill due to FHAB toxins and scenarios under which FHAB toxins were implicated.

Dillenberg and Dehnel [121] report dog/cattle deaths and human illnesses surrounding various lakes during the summer of 1959 in Saskatchewan, Canada. Most of the lakes during this period were producing intense FHAB events. Two adults in separate incidences and ten children at a camp were reported ill with vomiting and gastrointestinal distress after swimming in the lakes. Evidence that FHAB toxins were the cause included detection of algal cells in stool samples, co-occurrence of dog/cattle deaths after the animals drank from the same lake water, and onset of symptoms in patients following exposure to the lake water, as well as anecdotal reports from farmers and other citizens about the appearance of the water on the day of exposure. Water samples were investigated several days after the children became ill, but the FHAB had dissipated. This is not surprising since wind events, viral infections of cyanobacteria, and bloom senescence can quickly disperse accumulations of algae [127, 128]. In addition, cyanobacteria have the ability to migrate vertically up and down in the water column using gas vacuoles [129]. Cells can distribute throughout the water column, or concentrate in surface waters within hours [130]. This can make it exceedingly difficult to investigate FHAB related illnesses after exposure and is why regular monitoring programs are needed.

The only known death attributed to a FHAB toxin during recreational exposure in the United States occurred in Dane County, Wisconsin in 2002 after several teenage boys swam in a scum covered golf course pond. At least one became ill with severe diarrhea and abdominal pain while the other suffered a seizure and died of heart failure overnight [131]. A year later the coroner was able to identify the underlying cause of death as intoxication with anatoxin-a. The toxin and *Anabaena flos-aque* were apparently detected in stool and stomach contents. There is still some debate about whether this was the cause of death since anatoxin-a, known as "very fast death factor," generally elicits its effects within minutes in animal studies [19].

In 2008, several teens swam late at night (2:30 am) in Lake Mendota, WI. One female became ill with severe joint pain, rash, headache, fatigue, and upset stomach consistent with microcystin poisoning, although the exact toxin(s) involved is uncertain [132]. Scums were present in the swimming area during the day, but went unnoticed at night and the female swimmer noted the possibility of swallowing water by mistake.

In Monroe County, Pennsylvania 12 teens and one adult became ill after exposure to blooms of *Anabaena* species followed by several other reports of illness associated with swimming in three other lakes nearby [133]. Symptoms were gastrointestinal distress and allergy-like symptoms. In this case, the presence of algae in the water was not enough to deter the children from swimming in the area.

An informative case study of microcystin-LR intoxication in a teen was reported recently from Argentina [36]. A 19-year-old male accidentally fell into waters supporting an intense cyanobacterial bloom of *M. aeruginosa* while riding his jet ski. Concentrations of microcystin-LR in water samples collected afterwards were nearly 50

µg/L, a level similar to that reported in other eutrophic lakes during the peak of FHAB events during summer [134]. Hours after exposure the individual experienced dyspnea, nausea, abdominal pain and fever which became worse over a four day period. Medical tests showed respiratory distress, hypoxemia, renal failure, decreasing platelets, increasing leukocytes and an increase in liver enzymes aspartate transaminase, alanine transaminase, and γ-glutamyltransferase. The patient was mechanically ventilated for respiratory distress and although tests ruled out several infectious diseases, the patient was administered antibiotics. A full recovery was observed after 20 days.

Stewart et al. [5] conducted a systematic review of reports of confirmed FHAB toxin illnesses over the past several decades. Of all events identified as clearly due to FHAB toxins 80% involved, or exclusively involved children. At least half involved pre-adolescent children and where adults were involved child victims always out numbered the adults. These case reports of recreational illness due to FHABs in children suggest that children, teens, and young adults either ignored or were unaware of the dangers of blue-green discolored surface waters. Education and signs warning of the possible presence of FHAB toxins are likely to be effective in reducing child FHAB related illnesses in recreational settings.

**Exposure to FHABs through drinking water**

FHAB toxins have been detected in municipal drinking water in many developed and undeveloped or economically emerging countries including the United States, Canada, Argentina, Germany, China, Portugal, Spain, Poland, and Thailand among others [135]. In a recent survey of finished drinking water supplies from 24 plants in the

United States, 75% of samples tested positive for MC-LR and some samples contained concentrations unacceptable for human consumption [136]. This suggests that treatment strategies are not always effective at removing FHAB toxins and occasionally fail to reduce concentrations to below the WHO advisory level. Studies investigating the efficacy of treatment processes have focused primarily on microcystins, while little is known about other FHAB toxins. This is problematic since future scenarios of climate and land use change suggest the intensity and frequency of cyanobacterial blooms will increase [26, 137]. The most commonly employed drinking water treatment processes in the United States include sedimentation (i.e. flocculation and clarification), sand filtration, ozonation, activated/granulated carbon filtration, and disinfection with chlorine or other oxidizing agents.

The ability of drinking water treatment plant (DWTP) processes to remove FHAB toxins depends upon the processes employed, cyanotoxin load and environmental conditions of the source waters (reviewed in [135]). Sedimentation alone does not remove dissolved FHAB toxins or toxin-containing cells due to cyanobacterial positive buoyancy. Sand filtration does not remove dissolved toxins, but may remove toxin containing cells, or cause cell lysis and toxin release. Chlorination is the most widely used process that has been shown to destroy some, but not all FHAB toxins. Removal efficiency is dependent upon a large number of factors. For example, microcystin-LR reacts with HOCl approximately 20 times faster than with OCl$^-$ and degradation rates are negligible at pH values above 7.5 since decreasing pH favors the formation of hypochlorous acid (HOCl) [136]. Similar results have been found for cylindrospermopsin [28]. Interestingly, chlorination of saxitoxin shows an opposite trend with pH (i.e. higher

oxidation in alkaline conditions) and chlorination is not effective in oxidizing anatoxin-a [138, 139]. As would be expected the chlorine oxidation rate of FHAB toxins is linear with chlorine dose and temperature where at least 0.5 mg/L residual chlorine is required for 30 min. contact time (reviewed in [138]). Chlorination is the primary process facilitating cell lysis thereby causing toxin release [140]. However, this process is species dependent. For example, in a Belgian drinking water plant toxic *Microcystis* was removed by 40-80%, and *Anabaena* by 90-100%, but *Planktothrix* only by 30% [28]. Chlorination of FHAB toxins is in direct competition with naturally occurring dissolved organic matter. Evaluation of methods for cyanobacterial cell lysis and toxin (microcystin-LR) [141]. Pre-chlorination of raw water before sedimentation and filtration may improve degradation of microcystins during chlorination in the final stage of treatment by oxidizing organic matter and causing cell lysis. However, chlorination may not destroy all FHAB toxins (e.g. anatoxin-a) and chlorination by-products of microcystins may be produced [142]. Furthermore, increasing the chlorine dose or frequency increases the probability of forming toxic trihalomethanes such as bromodichloromethane and chloroform from organic matter in early stages of treatment [143].

Ozonation appears to be slightly more effective than chlorination in removing FHAB toxins, but the rate of degradation is also dependent on a range of factors. As for chlorination these factors are ultimately tied to the characteristics of the incoming raw water. For example, ozonation has been shown to remove anatoxin-a and to a lesser extent saxitoxin, but this process is inhibited by relatively small amounts of dissolved organic matter and is highly temperature dependent [144]. In a recent study

cyanobacterial extracts containing 135-220 pg/L microcystin-LR required 1.0 mg/L ozone over 5 min. for complete toxin destruction [145]. As with chlorination, there is the possibility for ozonation to transform FHAB toxins or other organic matter to toxic by-products [146, 147].

Granulated activated carbon (GAC) filtration or powdered activated carbon (PAC) is probably the most effective for removal of many FHAB toxins. It has shown to be effective in the removal of microcystin-LR and some, but not all saxitoxins using either absorption or biodegradation (i.e. microbial growth on GAC) removal mechanisms and in conjunction with ozonation [144]. A study of four drinking water treatment plants in Wisconsin showed an average 61% reduction in microcystins after the pretreatment stage which involved use of powdered activated carbon [148]. It is not clear if GAC and PAC are effective in binding/degrading other toxins such as anatoxin-a and BMAA. In fact, little to no information exists on the effect of any treatment strategy for the removal of BMAA or similar neurotoxic amino acids. As with other treatment strategies, GAC/PAC filtration is dependent upon a mixture of physical and chemical factors as well as biological factors in the case of GAC/PAC filtration using biodegradation [149].

As opposed to recreational exposures, cases of FHAB related illnesses associated with drinking water consumption have affected a larger number of individuals and as with recreational exposures, children are often disproportionately affected. In a case now well- investigated over the past 20 years, >100 children and 10 adults on Palm Island, northern Queensland, Australia became ill with gastroenteritis after the water supply for the island had been treated with copper sulfate to control odor and taste issues. It was later discovered through ecological studies that the drinking water

reservoir supported regular blooms of cylindrospermopsin-producing *C. raciborskii* [150, 151]. Treatment with copper sulfate caused algal cell lysis and release of the toxin into the dissolved phase, which may have allowed the toxin to pass through treatment processes, such as primary clarification or sedimentation.  For example, treating fish ponds with copper sulfate to control algal blooms has resulted in massive fish die-offs due to toxin release [152].

Lake Taihu is the largest freshwater lake in China bordering Jiangsu and Zhejiang provinces. The lake supplies drinking water to an estimated 10 million people [153]. Unfortunately it is also a shallow, eutrophic lake that supports massive FHABs of microcystin– producing *Microcystis, Anabaena,* and *Aphanizomenon* species. A large number of studies have been conducted in this region recently prompted by ecological and human health crises associated with poor air and water quality due to FHABs [154-161]. In 2007 the drinking water supply to Wuxi, on the coast of Taihu, was choked by massive agglomerates of decaying cyanobacterial scums [153]. In this case, local residents avoided the municipal water supply due to its offensive odor. However, over 2 million people were without a potable drinking water supply for one week. Massive engineering projects have helped to improve lake water quality and the safety of drinking water, but substantial improvements in Taihu water quality will require watershed wide reductions in agricultural runoff and wastewater nutrient loading [156].

The United States has had few outbreaks of FHAB related illness associated with drinking water. In August of 1975, 62% of the town of Sewickley, PA was afflicted with gastrointestinal illness [162]. Unfortunately little is known about the progression of symptoms in the population. In such wide-spread instances drinking water is a likely

source of the disease- causing agent. It was found that the finished drinking water reservoirs contained large growths of algae including *Lyngbya* sp. At that time the reservoirs were not covered and recommendations were made that they be enclosed. Since this outbreak occurred, surveys of open, finished drinking water reservoirs show that these systems allow chlorine disinfection residuals to dissipate, organic carbon to increase as a result of excess algal growth, redisinfection, and contamination [163]. They have also been shown to have high endotoxin concentrations due to excess algal growth [164]. Many cities in the United States are still serviced by open finished drinking water reservoirs.

While outbreaks of acute FHAB related illness due to contaminated drinking water is rare, long- term consumption of drinking water containing low levels of FHAB toxins, particularly microcystins has been linked to chronic diseases. This is a concern for children since they are most sensitive to FHAB toxins and have a longer time of exposure to develop chronic diseases. A recent study of 1,322 children ages 7 – 15 in the Three Gorges Reservoir Region, China showed that children who receive their drinking water from sources containing microcystins and other FHAB toxins have higher levels of liver enzymes in their blood suggesting liver damage, than children who receive drinking water with low or no FHAB toxins [6]. FHAB toxins are but one potential cause of liver problems. A high rate of primary liver cancer in certain areas of China may be due to the combined effects of long-term (i.e. over a life time) consumption of microcystins in drinking water, aflatoxin in stored grains, and high rates of hepatitis B infection [165, 166].

Other studies have found weak associations between consumption of waters containing microcystins and cancer. Zhou et al. [9] found a higher incidence of colorectal cancer in citizens of Haining City of Zhejiang Province, China who obtained their drinking water from river and pond water compared to those obtaining drinking water from wells or tap water. It is suggested that microcystins may be involved since concentrations of microcystin were significantly higher in pond and river water. Similarly, in Serbia, Svircev et al. [8, 167] detected up to 650 μg/L mcirocystin-LR in reservoirs that are used for purifying drinking water and 2.5 μg/L in the tap water, well over the WHO recommended drinking water limit. The authors also observed associations between high incidences of death due to primary liver cancer in regions where FHABs were intensified.  In the United States, a study by Fleming et al. [168] showed higher incidence of primary liver cancer in people within the service area of a municipal drinking water plant in Florida drawing from surface waters, compared to people living nearby. However, it was not significant compared to random sampling of Florida residences or background levels of liver cancer in Florida. These studies are relatively new and while only associations, move a step closer in determining what impact long-term exposure to FHABs may have on the development of chronic diseases.

**FHAB toxins in food**

Microcystins, nodularin, cylindrospermopsin, saxitoxins and anatoxin-a have been detected in aquatic organisms including invertebrates, such as mollusks and crustaceans, and both planktivorous and carnivorous groups of fish [169, 170]. Microcystins and nodularin have been detected in animals at all trophic levels within

water bodies in both experimental and field studies, including invertebrates [171-175], fish [170, 176-181], and water birds [6], as well as in mouse studies [182]. Microcystins accumulate in the liver, as well as kidneys, and gut. They have been detected in muscle tissue of perch, walleye and other fish, but may not bioaccumulate in this tissue to unacceptable levels [183]. On the other hand, risk analyses have suggested that if fish consumption is high enough then consumption can exceed TDI levels [184]. Therefore, bioaccumulation may not be a necessary prerequisite for the toxin to become a health hazard, if concentrations reach high enough levels in muscle tissues. Much less data is available for bioaccumulation of cylindrospermopsin and FHAB neurotoxins in aquatic organisms [169]. However, cylindrospermopsin and PSPs were recently shown to bioaccumulate in finfish from Lake Catemaco, Mexico [185]. In addition to fish, FHAB toxins have been detected in dietary supplements including microcystins and anatoxin-a [186, 187].


**Detecting algal toxins in clinical settings**

A variety of methods have been established for detecting FHAB toxins in environmental water samples and biological tissues. Typical laboratory analysis of FHAB toxins involves a chemical extraction procedure to purify the toxin away from other contaminants followed by liquid chromatography (LC) and one of several detection methods including photodiode array, ultraviolet-visible (UV-Vis), and mass spectrometry. UV-Vis detection is based on a compound's absorption of a specific wavelength of light. Photodiode array (PDA) detection allows for wavelength scans producing an absorbance spectrum that may be characteristic of the target analyte.

Both UV-Vis and PDA have been used to detect FHAB toxins in environmental water samples and in biological tissues [188, 189]. Since many compounds can have similar absorption spectra these methods must rely upon adequate LC separation of compounds in samples. In some cases this is not possible and more sophisticated methods are required. This is particularly true for biological materials. Thus, UV-Vis and PDA are unlikely to be used in a clinical setting to detect FHAB toxins.

Several forms of mass spectrometry combined with LC separation have been used to measure FHAB toxins in water and biological tissues. Single quadrupole mass spectrometers detect compounds based on their native mass while tandem mass spectrometers detect both the native mass and the transition from native or parent mass to several daughter ions as the molecule is fragmented by an electron beam. The tandem mass spectrometer is generally more sensitive and specific since many compounds have the same mass, but possess a unique fragmentation pattern. A variety of forms of ionization are available for detecting a wider array of compounds including atmospheric pressure, electrospray, and desorption electrospray ionization. Tandem mass spectrometers provide the best option for quantification of known compounds and therefore have been used extensively for the analysis of common FHAB toxins in water, drinking water and biological tissues where it is important to understand whether levels have exceeded guideline values (e.g. MAC) or NOAELs [176, 190, 191] [51, 192]. Time of flight mass spectrometers and ion trap mass spectrometers have proven useful in rapid presence/absence detection or semi- quantitative analysis of FHAB toxins in biological samples such as fish tissue, or algal colonies. These techniques are also considered "exact mass" methods, which allow discriminating between closely related

structural variants. Matrix-assisted laser desorption/ionization mass spectrometry may provide the most rapid and sensitive method for clinical analysis of FHAB toxins where qualitative (presence/absence) detection is needed.

Enzyme-linked immunosorbent assays (ELISAs) are an efficient, inexpensive method to detect broad groups of toxins. ELISAs are based on antibodies binding to a target molecule, which is then bound by anti-IgG tagged with a reporter molecule (e.g. horseradish peroxidase) and quantified by a spectrophotometric assay. In the case of microcystins, antibodies bind the Adda chain that is part of the structure for all microcystin variants (see Figure 1); this means that it is generally nonspecific due to cross-reactivity among microcystins (and possibly nodularin) and cannot provide any information about the relative toxicity of the water sample. ELISA kits have previously been used to detect cylindrospermopsin, microcystins, saxitoxin (and other PSPs) [193, 194]. An ELISA to detect BMAA is also commercially available from Abraxis [195]. In addition to the ELISA, microcystins have been detected based on their ability to inhibit protein phosphatases [196].

Attempts have been made to apply the above methods for the detection of FHAB toxins in exposed individuals. Serum and blood analyses of FHAB toxins are difficult because the toxins either breakdown quickly or rapidly accumulate within organs. Nonetheless, a few epidemiologic studies have detected microcystins in human blood. Chen et al. reported finding maximum concentrations of 0.685 µg/L microcystin-RR, 0.316 µg/L microcystin-YR and 1.221 µg/L microcystin-LR in the sera of fishermen of Lake Chaohu, Anhui, China [197]. Li et al. also detected levels of 0.4 µg/L and 1.3 µg/L microcystins in low exposed and high exposed school children when examining sources

of exposure in food and drinking water in relation to childhood liver damage in the Three Gorges Reservoir Region of China [6].

An alternative approach is to identify biomarkers of exposure to FHAB toxins. One potential biomarker for microcystin exposure is serum liver enzyme concentrations. Giannuzzi et al. measured alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyltransferase (γ-GT) and alkaline phosphatase (ALP) levels in the serum of a patient who had an acute recreational exposure to cyanobacteria [36]. The same enzymes were measured in epidemiological study of school-age children in China assessing liver damage from microcystin exposure and in investigating liver damage in mice exposed to microcystin-LR [6, 198]. All of these enzymes are expected to be at increased levels in the event of liver damage. Creatinine and urea levels were also measured in serum to assess kidney damage caused by microcystins [36]. When combined with patient history and symptoms these tests may be conclusive for microcystin exposure.

As reviewed by Paskerová, Hilscherová and Bláha, intracellular detoxification enzymes may be sufficient biomarkers of exposure to microcystins and cyanobacteria [199]. The authors noted that effects of microcystins or cyanobacteria on seven discussed biomarkers varied, depending on exposure times of greater than (chronic) or less than (acute) seven days. Similarly, other content of the cyanobacterial bloom may alter concentrations of the markers, such as LPS. Lipopolysaccharide is capable of inhibiting glutathione-S-transferases [200], enzymes that conjugate glutathiones to xenobiotic substances such as algal toxins, essentially inhibiting portions of cellular detoxification systems. The biomarkers suggested for monitoring included catalase,

glutathione peroxidase, glutathione reductase, glutathione, glutathione-S-transferase, lipid peroxidation, superoxide dismutase [199]. The enzymes are responsible for preparing xenobiotic substances for elimination from the body via chemical transformation in the form of oxidation, reduction, or hydrolysis (Phase I) and then conjugation with compounds such as glutathione or glycine (Phase II). Lipid peroxidation is a measurement of phospholipid peroxidation from the cell membrane, reflecting cell damage from ROS, hyperphosphorylation, etc. Proteomic analyses have identified differentially expressed proteins in fish early after exposure to microcystins including phenylalanine hydroxylase (PAH) and keratin 18 type I [201]. It is possible that bioassays could be developed to detect expressed proteins in human sera indicative microcystin exposure. Such assays would provide the means to conclusively identify individuals that have been exposed to a FHAB toxin with a single test.

**Conclusions**

FHABs are increasing in the U.S. and elsewhere due to increased nutrient enrichment of waterways from agricultural and residential non-point source runoff. Decades of intense management efforts will be required to reduce this problem. In the meantime, FHAB-related illnesses are commonly reported and the majority involve children, teens, and young adults. Children are more sensitive to FHAB toxins and engage in risky behaviors making them more likely to be exposed to FHAB toxins. FHAB-related illnesses are caused by ingestion of one or more toxins produced by cyanobacteria. While acute poisonings are likely rare events, the actual rate of FHAB poisonings is not known. Patient history, symptoms, and environmental monitoring data

provide the best evidence for diagnosing FHAB-related illnesses. Readily detectable biomarkers of exposure are needed in the clinical setting. Contamination of drinking water with FHAB toxins has resulted in massive acute poisonings while long- term exposure to low levels of some FHAB toxins in drinking water is associated with chronic diseases. The increase in duration, intensity and frequency of FHAB events in surface waters is placing stress on drinking water treatment processes such that costly upgrades to treatment plants will likely be required. Detecting human illnesses associated with FHAB toxins will require adequate communication among medical professionals, research scientists, and government agencies. In recreational settings, educating young adults, parents and children about the dangers of FHAB toxins in will likely go a long way in preventing FHAB-related illnesses.

**CHAPTER 2:**

**Factors Related to Interannual Variability in Cyanobacterial Community Composition and Total Biovolume over Two Decades in a Eutrophic Lake**

**Introduction**

Large accumulations of cyanobacteria (i.e. cyanobacterial harmful algal blooms, cyanoHABs) regularly occur in many lakes. CyanoHABs have the potential to disturb ecosystems, affect animal and human health, impact recreational tourism and lakeside property values, and exacerbate the expense of producing clean, odor-free, potable water. Economically, these threats to ecosystem and human health are estimated to cost over $2.2 billion per year in the U.S. [202]. Recent reviews of climate or hydrological models and studies of climate effects on cyanobacterial production point to climate change favoring cyanobacterial dominance in lakes [203-205].

High light intensity [206], warm water temperatures [206, 207], thermal stratification [208], high nutrient loading [209, 210], reduced grazing, and pH buffering above neutrality [211, 212] are factors commonly recognized as supporting cyanoHAB occurrence. Based on these environmental factors, predicted changes in air temperature, carbon dioxide concentrations, and weather events will likely influence lake mixing regimes, nutrient inputs, water temperatures, primary productivity, and food webs, thus possibly exacerbating cyanoHAB occurrence [205, 213].

Recent research using long-term data has demonstrated increasing air and lake surface water temperatures and decreases in wind speeds. Summer (July-September) surface water temperatures have increased from 1985-2009 at an average global rate of 0.34 °C per decade [214]. Analysis of 150 years of data from 39 Northern

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Hemisphere lakes showed ice breaking up 6.5 days per 100 years earlier and developing 5.8 days per 100 years later [215]. A review of global wind speeds found that annual average wind speeds decreased by 20% in Central Asia and by 10% in regions of other northern latitudes from 1979 to 2008 [216]. Such long-term changes in wind speeds could potentially impact overall lake physics. These two factors could affect cyanobacterial productivity due to warming water temperatures and changes in the extent of lake mixing.

Some models suggest that warmer water temperatures may extend the cyanobacterial growing season (CGS) and increase the duration and intensity of cyanoHAB events [217]. A long-term study of three large Swedish lakes identified years with milder winter/spring temperatures having earlier detection of cyanobacteria [218]. Adrian et al. suggested that winter and spring phytoplankton inocula can affect timing of spring algal blooms and species composition for the rest of the year [219]; therefore, the success of under-ice phytoplankton may limit the succession of spring diatom and summer cyanoHABs timing and species composition. During summer cyanoHABs, surface cyanobacterial colonies can increase water temperature by absorbing light, thus promoting growth conditions and increases in cyanobacterial biomass [220]. Changes in water temperatures may also impact intra-CGS succession of cyanobacterial species based on optimal growth temperatures. For example in Lake Mendota, Wisconsin, USA, of the two dominant bloom-formers, *Aphanizomenon* has historically bloomed earlier during the CGS than *Microcystis* [221]. In culture experiments, *Aphanizomenon* and *Anabaena* isolates from Mendota grew slowly, yet steadily, at 10° and 15°C, whereas *Microcystis* could only grow above 15° C (and below 37° C) [207], indicating that

*Aphanizomenon spp.* in Mendota could have a higher tolerance for growth in a colder water column in early summer or late fall. The impact of such phenomena on multi-year variability in cyanobacterial production is hampered by a lack of long-term monitoring datasets. During summer cyanoHABs, surface cyanobacterial colonies can increase water temperature by absorbing light, thus promoting growth conditions and increases in cyanobacterial biomass [220].

Nutrient loading (phosphorus, P, and nitrogen, N) is thought to be the key driver of cyanobacterial production [222]. Increasing $CO_2$ levels have demonstrated conflicting cyanobacterial responses to increased dissolved inorganic C (reviewed in [203]). Since N and C are readily available through fixation processes, P is often most limiting [223]. Short- and long-term climate changes may impact nutrient availability for cyanobacterial productivity. Strong stratification resulting from increased water temperatures could decrease available P in the water column, favoring N-fixing cyanobacteria and those containing gas vesicles, which allow buoyancy regulation to gain access to nutrients in the hypolimnion [203, 224, 225].

Increased precipitation is responsible for exogenous nutrient input into lakes, which may promote excess algal production. Various analyses have shown climate change may affect nutrient loading to lakes. It was predicted there could be a 3.3 to 16.5% increase in P loading in Danish waters over the next 100 years due increased winter rainfall [226]. Recent floods in the Midwestern United States resulted in substantial transport of both N and P to local waters. Over 16 days of heavy rains in June of 2008, four tributaries of the Mississippi River in Iowa received significantly larger inputs of N and P than median loads in June between 1979 and 2007 [227].

However, Robertson et al. (2016) showed loading to Lake Michigan, USA, may either increase or decrease depending on how precipitation changes in the future [228]. An increase in precipitation of over 8% is needed to compensate for the increase in evaporation caused by warmer air temperatures.

In addition to climate change, interannual variations in air temperature, strength of lake stratification, and nutrient availability may impact presence or dominance of particular cyanobacterial species in different years. The dominant bloom-forming cyanobacterial species in temperate zones include *Microcystis*, *Aphanizomenon*, *Anabaena*, and *Planktothrix* among others. Each of these species has unique optimal growth temperatures, seasonal growth trends, and varying capabilities for N fixation and toxin production, the role(s) of which in cyanobacterial homeostasis and growth is unknown. The environmental factors described above could impact these physiological processes, potentially causing changes in cyanobacterial community composition (CCC). An example of changes in overall phytoplankton composition and shifts in cyanobacterial dominance is evident in long-term data from Lake Champlain [229]. Reductions in both N and P concentrations, increases in lake temperature and stratification, and reduction of predation coincided with shifts from abundance of *Microcystis* and diatoms to that of *Aphanothece*, *Aphanizomenon*, and *Anabaena* across three different time periods (1970-1974, 1991-1992, 2006-2009) [229]. It was recently determined that while nutrient concentrations influenced extents of cyanobacterial biomass, physical factors such as temperature, light, and water stability or residence time are responsible for changes in CCC [230].

In this study, we use a long-term dataset (20 years from 1995 to 2014) from the North Temperate Lakes (NTL) site of Long Term Ecological Research (LTER) program to identify climatological, physical, and chemical factors affecting CCC and annual mean cyanobacterial biovolume (BV) in Lake Mendota, WI, a eutrophic lake. One goal of LTER is to observe ecological phenomena occurring over extended temporal and spatial scales. The goal of this study was to identify environmental factors that may drive interannual variations in total cyanobacterial abundance, individual cyanobacterial genera, and CCC.

## Materials and Methods

### Study site

Lake Mendota is the northern-most of four eutrophic lakes along the Yahara River with an area of 39.98 km$^2$. The lake is surrounded by agricultural and urban lands [231], and the lake is popular for recreational activities. The NTL-LTER program has monitored Lake Mendota's Deep Hole (max depth = 25.3 m; 43.06337, -89.36086) on a biweekly basis since 1995, gathering biological, chemical and physical limnological data. The lake is dimictic, thermally stratified between April/May and October/November, with annual mixing patterns remaining consistent from 1995 to 2010 [232, 233]. This is reinforced using data through 2014 in Appendix A, Figure A1. Mean depths of the epilimnion (metaT) and top of the hypolimnion (bottom of metalimnion, metaB) during the CGS (June through October) from 1995-2014 were 10.62±1.57 and 14.15±1.09 m, respectively (Table 2.2). In comparison, mean CGS photic zone depth receiving 1% photosynthetically active radiation (PAR) was 4.45±1.59 m. Mendota's

phytoplankton community is characteristically dominated by diatoms during spring and fall (>80% March through May phytoplankton abundance, >50% abundance September through November) and cyanobacterial dominance (>90% relative abundance) during the summer (mid-May through mid-September) [233]. All datasets used in this analysis from Lake Mendota are described in Appendix A, Table A1.

**Phytoplankton data**

Integrated (0-8 m) water samples for phytoplankton identification and measurement were collected once every two weeks, and preserved with 0.25 – 1% gluteraldehyde. Samples were kept in the dark and refrigerated until shipping for microscopic analysis by Phycotech (St. Joseph, MI). Three slides were made from each sample using a resin of 2-hydroxypropyl methacrylate and azo-bis-iso-butyronitrile. Samples were enumerated to a minimum of 400 microorganisms using an Olympus BX60 compound microscope. BV was estimated by measuring the greatest axial dimension and length, width, and depth of each colony or cell; taxonomic identification and measurements were confirmed using the ASA software database. Average cell BV measurements were multiplied by cell density of the species to calculate total BV for each species. For analysis of cyanobacteria, BV measurements were summed by genus per sample date. Total *Cyanophyta* BV was calculated by summing the BV of all cyanobacterial genera per sample date. Cyanobacterial genera that contributed on average greater than 1% overall BV across all years are shown in Figure 2.1A.

Figure 2.1 Average biovolume of cyanobacterial taxa from 1995-2014
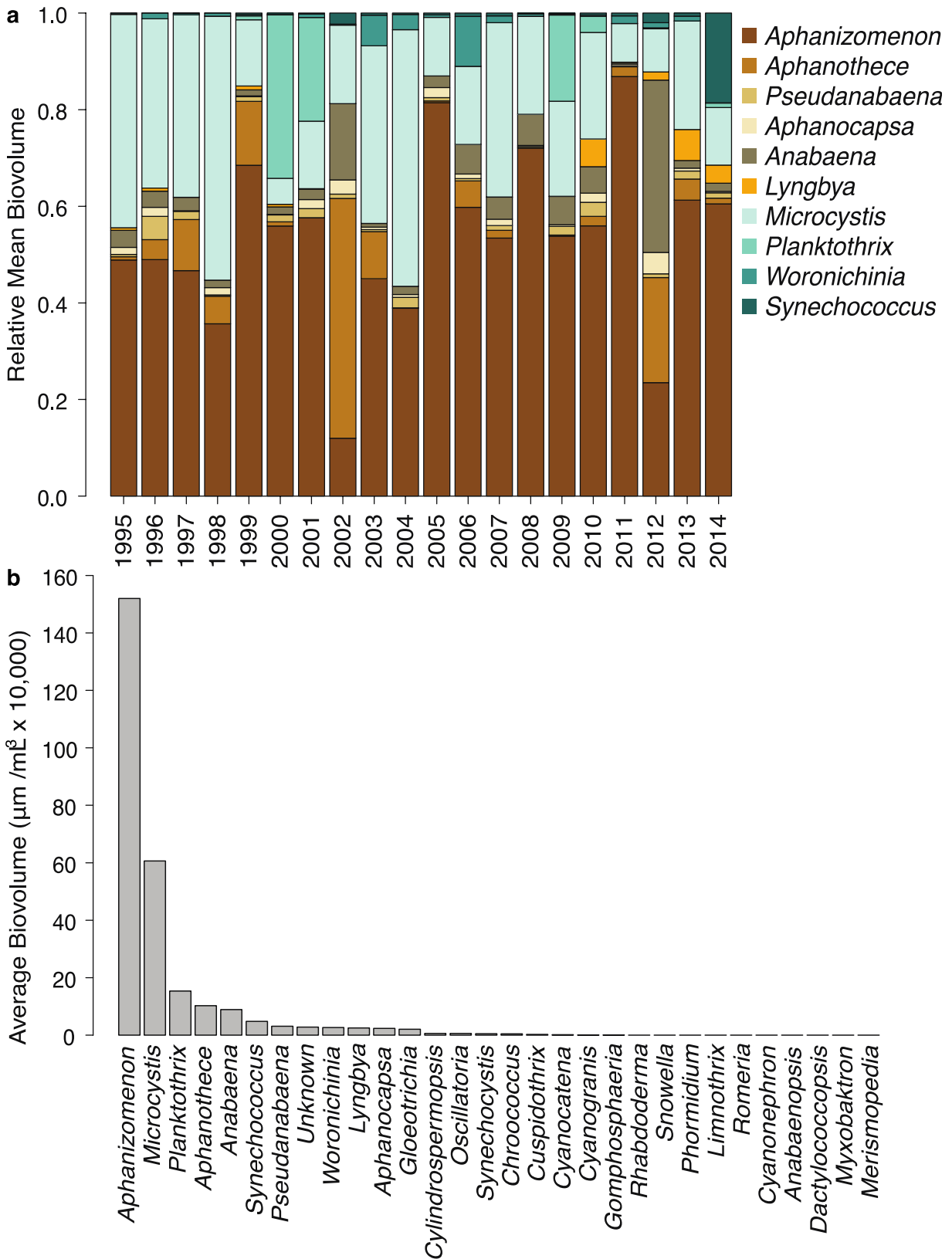
Figure 2.1A. Annual mean relative BV for genera contributing >1% relative BV:  Relative

BV is measured as a fraction of 1, equal to total BV. This figure represents relative

abundance of these taxa throughout all months each year. Potentially N-fixing

cyanobacteria are identified in shades of brown, while non-N-fixing cyanobacterial taxa

are identified in shades of teal; 2.1B. Average BV for all cyanobacterial taxa from 1995-

2014: *Aphanizomenon*, *Microcystis*, *Planktothrix*, *Aphanothece*, and *Anabaena*

consistently dominated Lake Mendota's CCC in terms of average BV ($\mu$m$^3$/mL x

10,000).

**Chemical data**

Surface water samples for nutrient analysis were collected every two weeks during May through September from 1995 to 2014, and preserved with 1% HCl. Nitrate plus nitrite ($NO^-_3NO^-_2$), ammonium ($NH^+_4$), and dissolved reactive P (DRP) were measured in filtered samples, and Kjeldahl N (KJN) and total P (TP) measured in unfiltered samples [232]. Total N (TN) was calculated as the sum of $NO^-_3NO^-_2$ plus KJN. Winter nutrient concentrations were not included in our analyses because sampling and nutrient processing outside the open water season in Lake Mendota was sparse in some years.

**Physical limnology and climatic data**

Water temperature was measured every two weeks by depth every meter from 1995 to 2014. Water temperatures measured from the surface to 8 meters were averaged (wtemp) to compare with 0-8 m composite phytoplankton samples. Cumulative annual and cumulative spring/early summer (April – June) rainfall data was collected from the Dane County Regional-Truax Airport weather station in Madison (KMSN), Wisconsin. Daily  wind speed from 1995 to 2014 for Lake Mendota were extracted from the North American Land Data Assimilation System ([234]). Spatial subsetting of the dataset was performed using the *geoknife* package in R (accessed May 1, 2016; [235, 236], which accesses processing capabilities from the USGS Geo Data Portal [237]. Maximum daily average daily wind speed was estimated. Lake bathymetry and water temperature data were used to calculate daily Schmidt Stability (St) and metalimnion depth (metaT,) for sample dates, using the rLakeAnalyzer

package [238]. Lake Number [239, 240] is a quantitative indicator of lake mixing and

was calculated with the addition of wind speed data also using rLakeAnalyzer. Photic

zone depth to 1% PAR was estimated by multiplying secchi depth (m) by 1.6 [241]. The

depth of the photic zone was divided by metaT (PAR:Epi) to create a variable

representing the proportion of the epilimnion receiving PAR.

The Wisconsin Climatology Office provides ice-on/off data for Lake Mendota

[242]. Several metrics were calculated to describe ice cover and/or ice-free duration in

each year relative to the CGS. The number of open water days was calculated by

subtracting ice cover duration in days from 365 for each year. To examine the impact of

ice cover prior to the CGS of a given year we summed the total number of days

between ice-on and ice-off during the winter prior to each CGS. To examine the effect of

an earlier or later date of ice formation, the number of days since October 31st (the end

of the CGS) to the date of ice-on was calculated. Finally, the duration of ice cover since

January 1st of each year to the date of ice-off was calculated to examine the effect of

the timing of ice-off relative to the CGS of that year.


**Statistical analyses**

All statistical analyses and figures were designed in R [235]. Unconstrained

Detrended Correspondence Analysis (DCA) was performed using the *vegan* package

[243]. To produce a community data matrix as input data, the BV of each cyanobacterial

genus was summed for each year and then relativized by taking the summed BV of

each genus and dividing it by total *Cyanophyta* BV for that year. Correlations between

environmental variables and CCC were calculated using the *envfit* function in the *vegan*

package. For this analysis, environmental variables were log-transformed. Cluster analysis was performed using the *hclust* function using a Euclidean distance matrix of log-transformed average annual cyanobacterial BV. K-means cluster analysis was performed using the *kmeans* function in the stats package.

Pair-wise correlation tests were performed to test for relations between the abundance of total *Cyanophyta*, *Microcystis*, or *Aphanizomenon* BV and environmental variables listed above using Kendall's Tau-b statistic (τ; *kendall*) [244, 245]. The maximum value of nutrient concentrations in April was selected as an estimate of spring nutrient loading; this is consistent with other studies of nutrient loading on Mendota [246, 247]. Because data from LTER nutrient analyses started in May 1995 (i.e. missing April), correlations between cyanobacterial BV and spring (April max) nutrients were only assessed for 1996-2014. Mean CGS BV, wind speed, wtemp, St, Lake Number, photic zone depth, metaT, PAR:Epi ratio were calculated using data from June through October. To account for variable numbers of samples taken per month and year, monthly means were calculated prior to calculating annual means or means of variables from June through October. The strength of interannual correlations over 19-20 years was measured using the *kendall* function with the Kendall's tau-b statistic ($p < 0.05$; *kendall*). All data used for assessing strength and statistical significant of pair-wise correlations are presented in Appendix A, Table A2; summary statistics for each variable indicating interannual variability and annual means are included in Tables 1-3.

Since the growth of microorganisms is often non-linear with optimal levels of environmental variables (e.g. optimal growth temperature), we first determined whether changes in total *Cyanophyta*, *Microcystis,* or *Aphanizomenon* BV followed exponential,

Table 2.1. Average Biovolume of Cyanobacterial Genera Detected More Than Five

Years in Lake Mendota from 1995-2014

| Cyanobacterial BV ($\mu m^3$/mL) | Mean | SD |
|---|---|---|
| Total *Cyanophyta* | 1481719.169 | 2722814.028 |
| *Aphanizomenon* | 903714.96 | 2288457.38 |
| *Microcystis* | 348053.27 | 836255.77 |
| *Planktothrix* | 86923.28 | 680588.15 |
| *Aphanothece* | 58504.26 | 194787.53 |
| *Anabaena* | 58481.12 | 224234.38 |
| *Synechococcus* | 28131.61 | 399231.12 |
| *Pseudanabaena* | 17923.93 | 47368.18 |
| Unknown | 16871.21 | 195596.01 |
| *Woronichinia* | 15168.15 | 52392.16 |
| *Lyngbya* | 14098.46 | 80665.28 |
| *Aphanocapsa* | 13445.19 | 34753.68 |
| *Gloeotrichia* | 11554.09 | 138071.63 |
| *Synechocystis* | 3874.12 | 15571.82 |
| *Cylindrospermopsis* | 3403.62 | 29806.38 |
| *Oscillatoria* | 3362.79 | 44965.22 |
| *Chroococcus* | 2588.36 | 16091.08 |
| *Cyanogranis* | 286.76 | 1276.82 |
| *Gomphosphaeria* | 152.50 | 1066.32 |
| *Rhabdoderma* | 90.84 | 608.55 |

Table 2.1 provides descriptive statistics (average, standard deviation (SD)) for BV of

each cyanobacterial taxon counted in the LTER dataset in five years or more from

1995-2014. The mean and standard deviaion were calculated over all months in all

years of the dataset, rather than being limited to BVs measured during the CGS.

Table 2.2. Variables Used for Analysis of Long Term (1995-2014) Effects of Climate,

and Physical Limnology on Cyanobacterial Biovolume and CCC in Lake Mendota

| Variable | Mean | SD | $C_V$ |
|---|---|---|---|
| Rainfall (mm) | 1028.72 | 597.79 | 0.55 |
| Apr-Jun Rainfall (mm) | 356.17 | 138.46 | 0.39 |
| Mean CGS Lake Number | 5.79 | 1.89 | 0.33 |
| Number of Days Ice Cover in Winter Prior | 83.8 | 26.6 | 0.32 |
| Number of Days Since Jan. 1 to Ice-off | 81.7 | 21.1 | 0.26 |
| Mean CGS PAR:Epi (m) | 0.44 | 0.09 | 0.20 |
| Number of Days Since Oct. 31 to Ice-on | 59.5 | 12.19 | 0.20 |
| Mean CGS MetaT (m) | 9.24 | 1.73 | 0.19 |
| Mean CGS Photic Zone (m) | 4.54 | 0.82 | 0.18 |
| Mean CGS St (J/m$^2$) | 435 | 74.04 | 0.17 |
| Mean CGS Secchi (m) | 2.80 | 0.46 | 0.16 |
| Mean CGS MetaT (m) | 10.62 | 1.57 | 0.15 |
| Mean CGS Wind speed (m/s) | 3.90 | 0.48 | 0.12 |
| Number of Open Water Days | 284.35 | 23.03 | 0.08 |
| Mean CGS Wtemp (°C) | 20.2 | 1.15 | 0.06 |

Table 2.2 provides descriptive statistics (average, standard deviation (SD), coefficient of

variation ($C_v$)) for variables created for analyzing annual changes in climate and

physical limnology, which may have an impact on cyanobacterial BV.

Table 2.3. Water Quality Variables Used for Analysis of Long Term (1995-2014) Effects of Nutrient Availability on Cyanobacterial Biovolume and CCC in Lake Mendota

| Variable | Spring (April) | | | Mean CGS (Jun – Oct) | | |
|---|---|---|---|---|---|---|
| | Mean | SD | $C_V$ | Mean | SD | $C_V$ |
| TN (mg/L) | 1.58 | 0.29 | 0.19 | 1.00 | 0.18 | 0.18 |
| KJN (mg/L) | 0.87 | 0.13 | 0.14 | 0.85 | 0.13 | 0.15 |
| $NO_3^- NO_2^-$ (mg/L) | 0.73 | 0.30 | 0.41 | 0.15 | 0.08 | 0.53 |
| $NH_4^+$ (mg/L) | 0.15 | 0.11 | 0.72 | 0.1 | 0.04 | 0.43 |
| TP (mg/L) | 0.11 | 0.03 | 0.28 | 0.06 | 0.02 | 0.32 |
| DRP (mg/L) | 0.07 | 0.04 | 0.58 | 0.03 | 0.01 | 0.52 |
| TN:TP | 19.59 | 7.88 | 0.40 | 21.67 | 4.76 | 0.22 |

Table 2.3 provides descriptive statistics (average, standard deviation (SD), coefficient of variation ($C_v$)) for variables created for analyzing annual changes in lake nutrients, which may have an impact on cyanobacterial BV. The SD and $C_v$ represent interannual variability in nutrient concentrations rather than seasonal nutrient fluctuations. Spring descriptive statistics represent 1996-2014 because no nutrient concentrations were measured in April 1995.

binomial, or sigmoidal relationships with the environmental variables. To test for exponential, binomial, or sigmoidal relationships, each variable was subjected to a log or polynomial transformation to the second or third degrees, respectively. Only non-linear correlations with a lower p-value than a linear correlation are reported. A total of 24 environmental variables were tested for linear, exponential, binomial, or sigmoidal correlation with mean *Cyanophyta*, *Microcystis*, or *Aphanizomenon* BV. P-values less than α=0.002 are considered statistically significant according to Bonferroni. Correction (alpha 0.05 divided by the number of variables tested).

**Results**

We defined the CGS as occurring between June and October. Mean monthly cyanobacterial BV reached a maximum in July each year (Appendix A, Figure A2). Mean monthly *Aphanizomenon* BV tended to also peak in July, while mean monthly *Microcystis* peaked in August. *Aphanizomenon* and total cyanobacterial BV started to decline in August, and *Microcystis* followed with declines in September.

*Aphanizomenon* and *Microcystis* have dominated CCC in Lake Mendota over the past two decades. Average BVs of *Aphanizomenon* and *Microcystis* were over seven and three times higher, respectively, than the next three most abundant genera, *Planktothrix*, *Aphanothece*, and *Anabaena* (Figure 2.1B). *Aphanizomenon, Aphanothece, Pseudanabaena, Aphanocapsa, Anabaena, Microcystis, Planktothrix, Woronichinia*, and *Synechococcus* contributed on average at least 1% total cyanobacterial BV from 1995 to 2014 (Figure 2.1A). Genera considered possible N-fixing species (brown bars in Figure 2.1A) contributed at least 40% relative BV from

51

1995 to 2014, and this appears to have increased over time. For example, in the first decade (1995 – 2004) possible N fixers contributed on average 58% of the BV, while in the second decade (2005 – 2014) they contributed almost 76% of the BV, a significant difference (t-test with p=0.01).  In most cases these increases were due to the abundance of *Anabaena*, *Aphanothece*, and/or *Aphanizomenon*. *Aphanizomenon*, *Microcystis*, *Planktothrix*, *Aphanothece*, and *Anabaena* consistently had the highest average year-round BV in Lake Mendota from 1995-2014 (Figure 2.1B).

Figure 2.2 shows that genera frequently occurring at consistently moderate-to-high relative BV cluster on the right side of the plot. Less abundant genera or genera that do not consistently occur in all years cluster on the left side of the plot. A K-means cluster analysis for two groups splits the CCC into two groups, which are not significantly correlated with one another (Mantel test with p=0.35). Some of the less abundant genera occur sporadically at relatively moderate-to-high levels in certain years including *Lyngbya*, *Planktothrix*, *Cylindrospermopsis*, and *Oscillatoria*.

In 2000, 2001, and 2009 *Planktothrix* accounted for nearly 36%, 38%, and 17% of the total *Cyanophyta* BV, whereas this genus was not detected in 11 years of the dataset and accounted for less than 5% of total *Cyanophyta* BV in all other years. The greatest mean annual abundance of *Planktothrix* coincided with peak average annual KJN in 2009 (Appendix A, Figure A3), and of all environmental variables measured, the mean annual abundance of *Planktothrix* was significantly correlated with average annual and CGS mean KJN (R=0.3 - 0.4, p<0.05).

Figure 2.2 Dendrogram heat map of annual average BV for all cyanobacterial taxa

counted in Lake Mendota
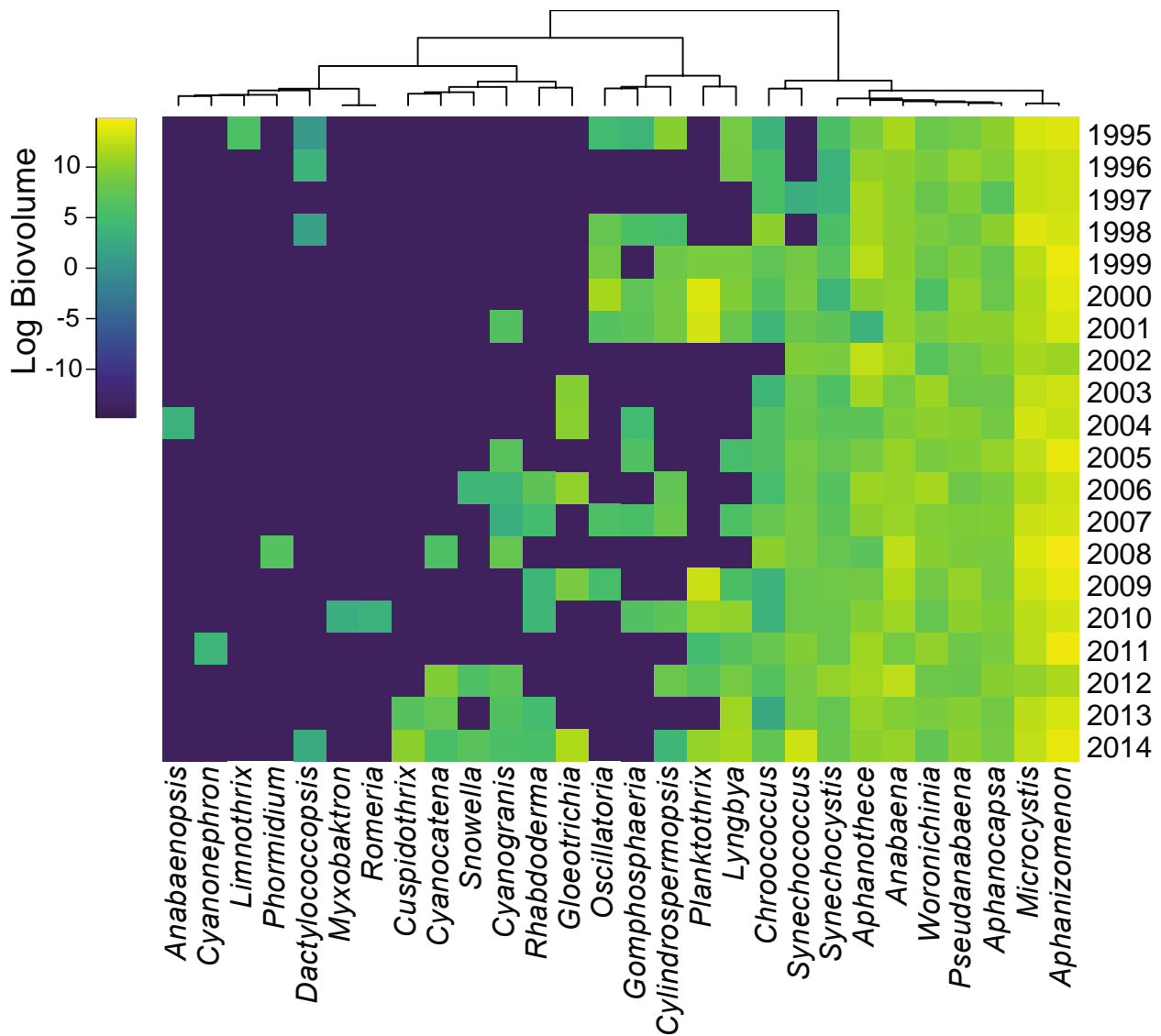


Each rectangle in Figure 2.2 represents BV for a given cyanobacterial genus (columns)

in a given year (rows). The color of each rectangle represents a genus's abundance (log

BV); purple and blue squares indicate years of less abundance, whereas green and

yellow squares indicate years of high abundance. A dendrogram identifies clusters of

taxa frequently or sporadically occurring among years, as well as those taxa co-

occurring within Lake Mendota's CCC.

Similarly, *Synechococcus* was most abundant in 2002, 2009, and 2014 when it accounted for 16, 8.2, and 9.2% of CCC, whereas it was not detectable or less than 1% of CCC in all other years (Appendix A, Figure A3). Its average annual abundance was most significantly correlated with open water duration (R=0.35, p=0.03). Thus, *Planktothrix* and *Synechococcus* abundance drive the CCC in outlier samples and their average annual abundance is associated with KJN or ice cover.

Since rare taxa contributed disproportionately to CCC, a DCA was used to investigate interannual changes in CCC. DCA removes non-linear relations and allows for down-weighting of rare taxa [248]. A DCA was performed on average annual BV of all genera to identify correlates of interannual changes in CCC with annual environmental driver variables (Figure 2.3). The DCA shows that CCC is relatively homogeneous from year to year after rare taxa are down-weighted. The axis of the DCA ordination is the standard deviation of sample scores, which for the majority of samples is <1. Of all nutrient variables tested, spring $NO^-_3NO^-_2$ (R=0.35, p=0.045), mean CGS TP (R=0.54, p=0.006), CGS TN (R=0.064, p=0.005), CGS DRP (R=0.046, p=0.016), CGS KJN (R=0.068, p=0.004), and mean CGS TN:TP (R=0.038, p=0.032) correlated significantly with interannual changes in CCC. In addition, number of days of ice cover in the winter prior (R=0.044, p=0.016), number of open water days in a calendar year (R=0.042, p=0.017), number of days from January 1 to ice-off (R=0.044, p=0.022), and Apr-Jun Rainfall (R=0.034, p=0.043) were the climate variables significantly correlated with CCC. Finally, mean CGS Lake Number (R=0.040, p=0.032) and mean CGS MetaT (R=0.040, p=0.024) were the only lake physics variables correlated with changes in CCC.

Figure 2.3 Detrended correspondence analysis correlating annual average cyanobacterial genera biovolumes and environmental driver variables
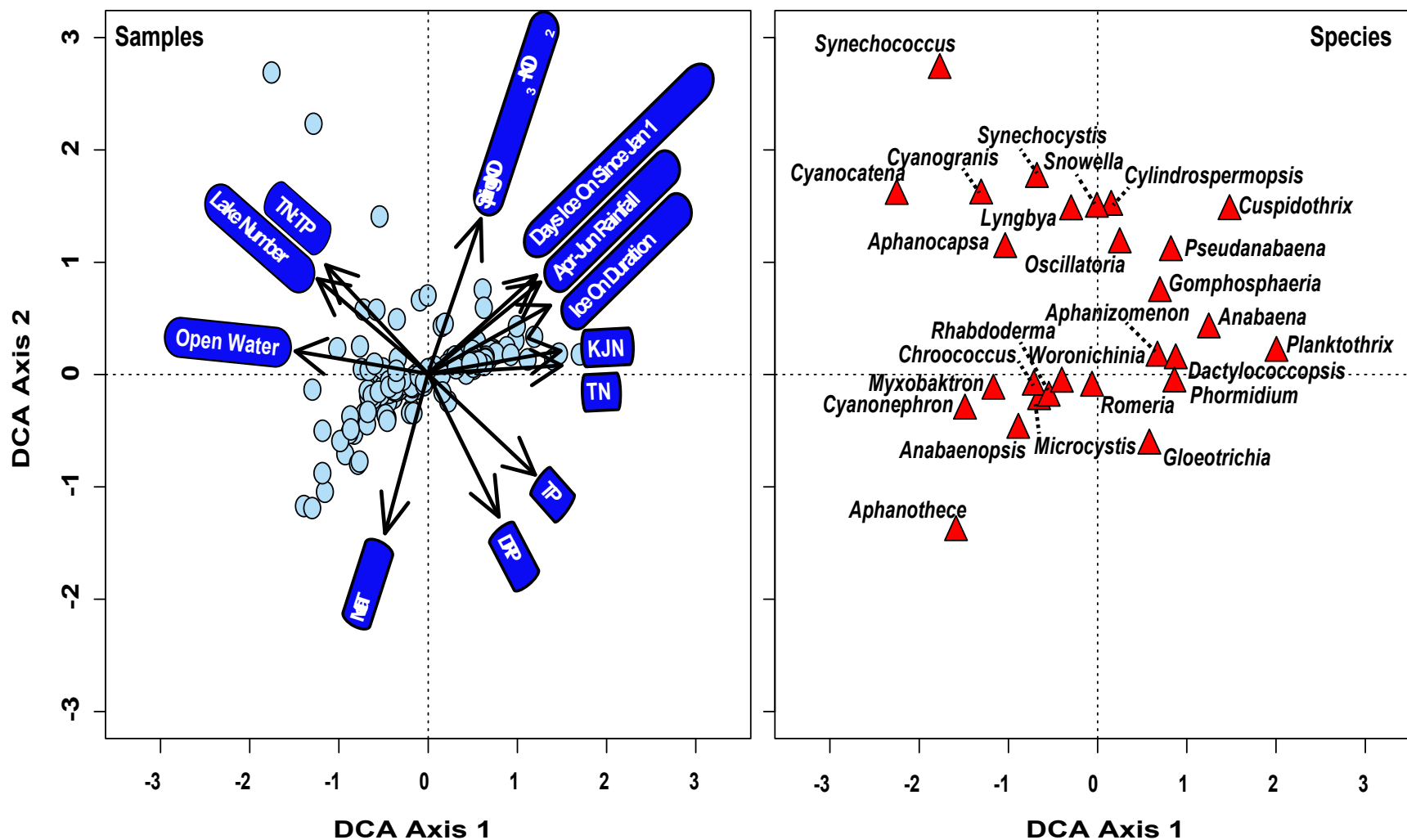
Figure 2.3 presents a detrended correspondence analysis (DCA) in which rare cyanobacterial taxa (identified by an initial

correspondence analysis) were downweighted to reduce an inherent arch effect. The axes of the DCA ordination are the

standard deviations of sample scores, <1 for the majority of samples. Red circles represent each individual genus

identified in Lake Mendota, and blue triangles represent each year from 1995-2014. Environmental variables significantly

correlated with interannual changes in cyanobacterial community composition (CCC) are highlighted in green. A barplot to

the right of the ordination illustrates the % contribution of six cyanobacterial genera impacting interannual CCC. The red

line along the barplot x-axis represents the potential contribution if all species contributions were equal.

Since *Microcystis* and *Aphanizomenon* are the dominant genera in Lake

Mendota and important cyanoHAB species, we then attempted to determine which

environmental variables are most closely associated with interannual variations in their

abundance, as well as the abundance of total *Cyanophyta*. Kendall's tau-b was used to

determine the strength and direction of these relationships over the past 20 years, as

well as if these patterns were linear or non-linear in nature. The nine environmental

parameters that were significantly correlated ($p<0.05$) with *Cyanophyta*, *Microcystis*,

and/or *Aphanizomenon* BV are tabulated in Table 2.4 and shown in Figure 2.4. CGS

KJN and TN were most strongly correlated ($\tau>0.5$) with the three measures of

cyanobacterial BV (Figure 2.4).

Mean CGS KJN and CGS TN were correlated positively with mean *Cyanophyta*

($\tau=0.568$, $p<0.0006$; $\tau=0.529$, $p<0.002$, respectively; Figure 2.4e & 2.4f). Mean CGS TP

also correlated with mean *Cyanophyta* ($\tau=0.413$, $p<0.02$; Figure 2.4g), while CGS

TN:TP was negatively correlated with mean *Cyanophyta* ($\tau=-0.36$, $p<0.05$; Figure 2.4h).

Mean St was the only physical limnological variable negatively correlated with total

*Cyanophyta* ($\tau=-0.411$, $p<0.02$; Figure 2.4i) in a binomial pattern, suggesting that

strength of lake mixing could having varying effects on cyanobacterial growth. Mean

*Cyanophyta* correlated positively with Apr-Jun rainfall ($\tau=0.326$, $p<0.05$; Figure 2.4b).

Mean *Aphanizomenon* was most strongly correlated with CGS TN and spring

KJN ($\tau=-0.531$, $p<0.002$). Apr-Jun rainfall also correlated with mean *Aphanizomenon* in

a binomial pattern ($\tau=0.38$, $p<0.05$; Figure 2.4b); in some years these two variables

followed the same trends (e.g. 1996, 1999, 2000, 2007, 2008, 2012), but in other years,

Table 2.4. Significant Interannual Correlations Between Cyanobacterial Biovolume and

Environmental Water Quality Variables

| Mean Cyanobacterial BV | Environmental | τ | p | Relationship |
|---|---|---|---|---|
| | Mean CGS KJN* | 0.568 | <0.0006 | Linear |
| | Mean CGS TN* | 0.529 | <0.002 | Linear |
| | Mean CGS TP | 0.413 | <0.02 | Linear |
| Cyanophyta | Mean St | -0.411 | <0.02 | Binomial |
| | Spring KJN | -0.389 | <0.03 | Sigmoidal |
| | Mean CGS TNTP | -0.361 | <0.04 | Linear |
| | Apr-Jun Rainfall | 0.439 | <0.01 | Linear |
| | Mean CGS TN* | 0.402 | <0.002 | Linear |
| | Spring KJN* | -0.531 | <0.002 | Sigmoidal |
| | Mean CGS KJN | 0.442 | <0.008 | Linear |
| Aphanizomenon | Mean St | -0.434 | <0.02 | Binomial |
| | Mean CGS TNTP | 0.433 | <0.02 | Sigmoidal |
| | Apr-Jun Rainfall | 0.345 | <0.05 | Binomial |
| | Mean CGS TP | 0.328 | <0.05 | Linear |
| | Spring KJN | 0.472 | <0.006 | Linear |
| | Rainfall | 0.421 | <0.02 | Linear |
| Microcystis | Spring TP | 0.411 | <0.02 | Linear |
| | Apr-Jun Rainfall | 0.347 | <0.04 | Linear |

*Environmental variables significantly correlated with cyanobacterial BV after Bonferroni

Correction with α<0.002.

Table 2.4 presents Kendall's tau-b statistics calculated to evaluate interannual

relationships between cyanobacterial BV and environmental variables by cyanobacterial

grouping and ordered by most significant p-values. All correlations presented were

statistically significant at p<0.05; p-values were rounded to the first two significant

figures.

Figure 2.4 Annual trends of environmental factors significantly correlated with

cyanobacterial biovolume
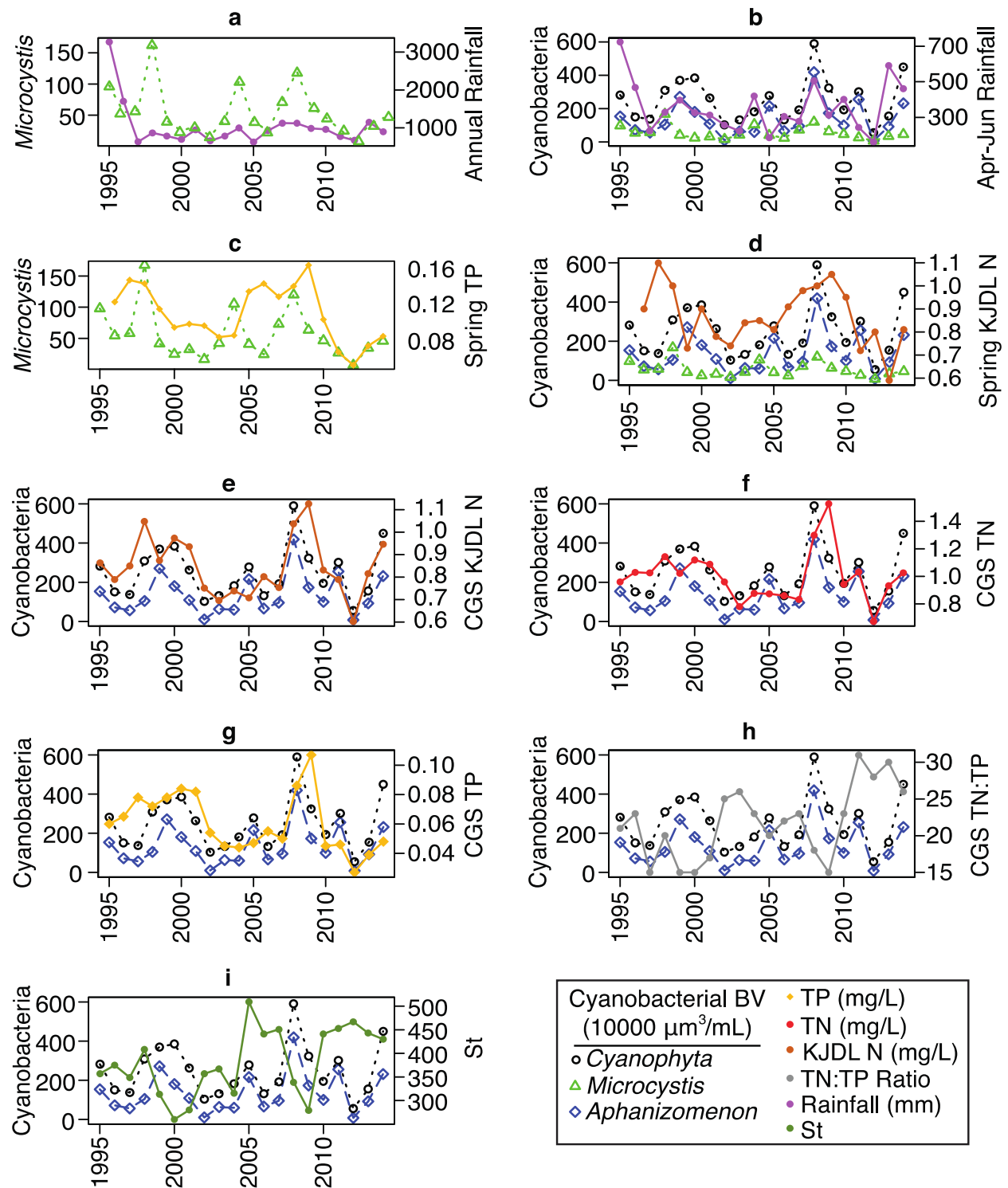
All variables presented are significant at α<0.05. a. Mean CGS *Microcystis* BV (10,000 μm$^3$/mL) and annual rainfall (mm); b. Mean CGS *Cyanophyta, Microcystis*, and *Aphanizomenon* BV (10,000 μm$^3$/mL) and Apr-Jun rainfall (mm); c. Mean *Microcystis* BV (10,000 μm$^3$/mL) and spring (April) TP (mg/L); d. Mean CGS *Cyanophyta, Microcystis*, and *Aphanizomenon* BV (10,000 μm$^3$/mL) and spring (April) KJN (mg/L); e. Mean CGS *Cyanophyta* and *Aphanizomenon* BV (10,000 μm$^3$/mL) and mean CGS KJN (mg/L); f. Mean CGS *Cyanophyta* and *Aphanizomenon* BV (10,000 μm$^3$/mL) and mean CGS TN (mg/L); g. Mean CGS *Cyanophyta* and *Aphanizomenon* BV (10,000 μm$^3$/mL) and mean CGS TP (mg/L); h. Mean CGS *Cyanophyta* and *Aphanizomenon* BV (10,000 μm$^3$/mL) and mean CGS TN:TP ratio; i. Mean CGS *Cyanophyta* and *Aphanizomenon* BV (10,000 μm$^3$/mL) and mean Schmidt Stability (St)

either variable's strength of decrease was not as steep as the other's (e.g. 1997, 2002) or decreased when Apr-Jun rainfall increased (e.g. 2005, 2010).

In almost half the years in this dataset spring organic N, plus $NH_4^+$ trended in opposite years of *Aphanizomenon* BV including 1996, 1999, 2005, 2006, 2009, 2011, 2012, and 2013 (Figure 2.4d). Mean CGS TN and mean CGS TP correlated positively with mean *Aphanizomenon* (TN: τ=0.402, p<0.002; TP: τ=0.328, p<0.05; Figure 2.4f & 2.4g). Having a slightly stronger correlation with TN than with TP, *Aphanizomenon* BV demonstrated a positive sigmoidal relationship with mean CGS TN:TP (τ=0.43, p<0.05; Figure 2.4h), following patterns similar to mean *Cyanophyta*. As with total *Cyanophyta*, mean St was negatively correlated with mean *Aphanizomenon* BV (τ=-0.434, p<0.05; Figure 2.4i) in a binomial relationship.

Mean *Microcystis* correlated with the least number of environmental variables among the three groups: spring TP (τ=0.411, p<0.02; Figure 2.4c) and spring KJN (τ=0.472, p<0.006; Figure 2.4d) correlated most strongly with Microcystis. In addition, cumulative annual rainfall correlated linearly with mean *Microcystis* (τ=0.42, p<0.02; Figure 2.4a). The amount of rainfall measured in 1995 is a clear outlier driving this correlation (Appendix A, Table A2). Mean *Microcystis* also correlated positively with rainfall summed from April through June (τ=0.347, p<0.04; Figure 2.4b).

We then evaluated whether *Microcystis*, *Aphanizomenon,* total *Cyanophyta*, and any of the environmental variables have significantly changed from 1995 to 2014. The average mean CGS TN:TP ratio has increased linearly (τ=0.38, p=0.024) concomitant with a decrease in mean CGS DRP (τ=-0.40, p=0.015). In comparison, spring $NH_4^+$ decreased linearly over the past 20 years (τ=-0.35, p<0.05). The mean photic zone

depth has also decreased, suggesting an increase in turbidity (e.g. microbial growth)

during the CGS (τ=-0.54, p=0.0011). However, neither total *Cyanophyta* nor either of

the major bloom-formers *Microcystis* or *Aphanizomenon* has changed significantly over

the past 20 years.


**Discussion**

**Factors Affecting Cyanobacterial Biovolume and Composition**

Our analyses identified factors that are correlated with changes in Lake

Mendota's CCC (*Cyanophyta* composition, total cyanobacterial abundance, and

abundance of major bloom-forming species) and identified long-term changes in the

CCC and a few of their correlated environmental factors. It should be noted that the

correlations found here do not necessarily represent causal relationships. Nonetheless,

an analysis of this long-term dataset identifies relationships that may be explored further

in future experimental studies. It also highlights some potentially important links

between cyanobacterial growth and indicators of meteorological variability.

In our analysis, interannual variations in mean *Aphanizomenon* and *Cyanophyta*

BV were significantly related to variations in mean CGS TN, KJN, and TP. Since both

TN and KJN include particulate and/or cell-bound forms of N, it is possible these

variables are direct indicators of biomass; this would also pertain to the relation between

KJN and CCC variability. *Aphanizomenon* is a potentially diazotrophic genus

responsible for input of new N into lakes, which is usually released in the form of

organic N or amino acids. As such, some portion of variability in CGS KJN with

*Aphanizomenon* BV may be due to its N fixation. Cyanobacteria are also able to take up

and store amino acids in phycocyanin and cyanophycin, then use those compounds later during periods of N starvation [249, 250].

It is possible that *Aphanizomenon,* which occurred early in the summer, provided a source of N to other cyanobacterial species present during the same months or in the latter part of the CGS once *Aphanizomenon* started declining. This is supported by early studies by Torrey and Lee who estimated that 7% of TN produced by N fixation in Lake Mendota could support summer algal growth [251]. A scenario where N fixing cyanobacteria supplement the growth of other *Cyanophyta* species potentially explains the succession of *Microcystis* dominance after *Aphanizomenon* in Lake Mendota. Beversdorf et al. found that N fixation in Mendota peaked during *Aphanizomenon* blooms and was followed by toxic blooms of *Microcystis* [233]. The same anti-correlative relationship between these two genera has been observed in other lakes over multiple years [252, 253]. Another explanation for this selective growth of cyanobacterial genera at different times is that these species thrive at optimal growth temperatures in addition to variations in light and water stability [207, 221, 230, 253]. As such, the decline and cell lysis of *Aphanizomenon* beyond its maximal growth temperatures may provide the nutrient availability for the later success of *Microcystis.*

In temperate climates, spring nutrient runoff from snow melt and rainfall is often the greatest source of nutrients to lakes and often drives their trophic status. Spring N and P have commonly been found to correlate with phytoplankton densities and chlorophyll across spatial gradients. In this study, measures of spring N and P were also correlated with interannual changes in cyanobacterial BV where N species, particularly DON. There are various possible mechanisms for how spring N and P can drive

cyanoHABs that reach peak BV several months later during the CGS. In one such mechanism demonstrated in other systems, heterotrophic bacteria are responsible for recycling of organic N and P that was delivered to the upper mixed layer during spring and thus providing a source of available inorganic N and P for phytoplankton growth later in the summer [254].

Spring TP was correlated with summer *Microcystis* BV, and spring KJN was correlated with summer total *Cyanophyta*, *Microcystis*, and *Aphanizomenon* BVs, providing potential early predictors for cyanoHABs in Lake Mendota before the CGS in a given year. These observations agree somewhat with those of others. Lathrop, Carpenter, and Robertson found that Lake Mendota's April TP concentrations were inversely related to water clarity (i.e. Secchi disk depth) between June and August, which includes a portion of the CGS [247]. This is significant as highest average *Microcystis* BV occurred in August each year in Lake Mendota (Figure 3). Spring TP has also been used to predict summer cyanobacterial bloom densities and chlorophyll [247, 255]. Dillon and Rigler found that measuring TP as well as KJN at spring turnover would allow prediction of chlorophyll concentrations during summer stratification [255]. In our analyses, spring $NH^+_4$ and $NO^-_2$ $NO^-_3$ did not correlate with measures of cyanobacterial BV, pointing to organic and particulate N as the important components of spring KJN for *Microcystis* growth during the CGS.

There are several possible reasons why spring KJN and not other measures of the N pool area an indicator for *Microcystis* BV months later during the CGS. It could be that KJN is an indicator of diatom biomass and the influence of other factors including spring TP on diatom growth. Indeed, interannual variation in spring TP is correlated with

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spring KJN ($\tau$=0.598, p<0.0005). However, spring inorganic N measures ($NH^+_4$ and $NO^-_2$ $NO^-_3$) were not significantly correlated with cyanobacterial BV, suggesting that spring dissolved and/particulate organic N are the important indicators of cyanobacteria abundance during the CGS. It could be that snow melt washed urea as a source of organic N from fertilizers into the lake, increasing spring KJN. Urea has been shown to account for over half of N fertilizer ingredients used [256]. Previous culture studies of *Aphanizomenon*, *Microcystis*, or total environmental cyanobacterial populations showed cyanobacteria were able to grow at intermediate or maximum rates using urea over other organic N and inorganic N sources in enriched media [257, 258]. Prior to these bottle experiments, Berman demonstrated that *Aphanizomenon ovalisporum* isolated from Lake Kinneret was able to grow with increases in dissolved organic N and not due to N fixation, both within the lake and supplemented bottle experiments [259]. However, another *Microcystis* culture study showed that growth was inhibited in media enriched with either urea or glutamic acid [260]. The specific component(s) of spring organic N that influenced *Microcysti*s growth later in the summer in Lake Mendota are not known. It is possible that P and N storage mechanisms [250, 261-263] allowed cyanobacterial stores of available spring nutrients while still in low BV concentrations during the early CGS until water temperatures increased.

Previous studies have demonstrated *Microcystis* growth being related to increased surface water temperatures, increased mixing and re-suspension, and changes in stratification [264, 265]. In this study, we found a negative binomial correlation between St and total *Cyanophyta* and *Aphanizomenon*, suggesting increased mixing was related to algal blooms; however, no relations were found with

wind or surface water temperatures. In previous studies, reduced mixing and the onset of thermal stratification have previously been associated with the appearance of cyanoHABs and surface scums in lakes, primarily of *Microcystis* species [213, 266], while in other studies no significant trends were detected between scum formation and St [214, 267]. The lack of correlations with wind speed and mixing may be have been caused by the complex relations between mixing and cyanobacteria blooms. Initially strong winds are needed to cause mixing and entrainment of nutrient-rich metalimnetic water. Then a calm period is needed to provide conditions appropriate for cyanobacterial growth. Therefore the non-linear (binomial) negative correlation between St and *Aphanizomenon* and *Cyanophyta* BV in our study is reasonably explained and we would not expect any single seasonal indicator of mixing and stratification to relate to linearly to cyanobacteria BV.

Another reason for the lack of consistent relations with wind, mixing, and turbidity is that some forms of cyanobacteria, such as *Aphanizomenon*, can control their buoyancy and thus gain access to a nutrient-rich hypolimnion during periods of limited mixing. In addition, during times of high turbidity, buoyant forms of cyanobacteria can form surface scums to avoid light limitation and still gain access to nutrients from sufficient lake mixing. Within species it is also possible to have different responses to the same conditions, depending on colony size. A previous study on Taihu Lake found that larger colonies were less able to move in response to light intensity during calm conditions compared to smaller colonies; these larger colonies generally concentrated in the upper layer in both calm and windy conditions [268]. This process is likely mediated by regulating carbohydrate ballast [269].

**Potential Effects of Climate Change**

The results of this study suggest that changes in Lake Mendota's CCC occurred as a result of changes in ice duration (changes in the open water period). This appears to have instigated the sporadic occurrence of normally less abundant genera including *Planktothrix*, *Oscillatoria*, *Synechocystis*, and *Cyanocatena*. It is unclear why these genera benefited from a longer open water period. An indirect environmental change from climate warming correlating with CCC but not major cyanobacterial producers suggests that blooms of historically less dominant cyanobacterial species may become less sporadic in the future, as temperatures continue to increase.

Annual mean surface water temperatures are expected to increase by 3.3 °C due to carbon dioxide emissions [270]. Indeed, summer surface water temperatures in Great Lake Superior, North America, have increased approximately 3.5 °C in the last century [271]. Using an environmental dataset from over 1000 lakes in the United States, Beaulieu et al. estimated that cyanobacterial biomass would double, accordingly with changes in carbon dioxide and surface water temperature [272]. However, this analysis of the U.S. EPA National Lakes Assessment associated single measurements of environmental variables with each other, rather than considering events across several months that affect cyanoHAB occurrence, including ice cover and rainfall.

Though many scientists agree cyanoHABs will increase with climate changes, predictions of how cyanobacterial genera will respond in lakes have been limited [203]. Several studies have implicated a more widespread appearance of typically sub-tropical cyanobacterial species (e.g. *Cylindrospermopsis*) in temperate climates [273]. In our study, *Cylindrospermopsis spp.* were counted intermittently in nine years between 1995

and 2014 in lake Mendota. Elliott et al. used the Regional Climate Model and Phytoplankton Responses To Environmental Change (PROTECH) model to predict results of increased cyanobacterial dominance led by *Planktothrix spp.* as well as an earlier end to the CGS due to increased spring growth and resulting early nutrient limitation [274]. In a second study, these models also predicted increased annual mean phytoplankton biomass with increased temperature and nutrient loading, consistent with the results of our study; cyanobacteria increased in dominance with warmer water temperatures [275]. However, even at similar latitudes, lakes have been warming inconsistently across seasons. For example, Central European lakes have been warming primarily in spring months [276]. The differences in timing of increased water temperatures among regions may thus impact events such as ice cover and break-up, affecting changes in CCC.

Regional differences in intra-annual temperature increases may also show sporadic increases in historically non-dominant cyanobacterial genera based on optimal growth temperatures, such as the annual succession of *Aphanizomenon* and *Microcystis spp.* Giordanino et al. were able to show that a 5°C increase without a change in irradiance was beneficial for photosynthesis in *Nostoc* and *Anabaena spp.*, while it hindered photosynthetic processes in *Microcystis* and *Arthospira spp.* [277]. Alterations in the typical seasonal temperatures may therefore increase blooms of *Planktothrix, Oscillatoria, Synechocystis*, and *Cyanocatena* observed in Lake Mendota based on their optimal growth temperatures and future seasonal patterns. Thought PAR in the epilimnion, St, and Lake Number did not correlate with individual cyanobacterial

genera or CCC in our study, it likely co-mediates success of cyanobacterial growth with changes in wtemp.

In our study, *Microcystis* was positively correlated with Apr-Jun rainfall and annual rainfall, while mean *Cyanophyta* and *Aphanizomenon* were positively correlated with just Apr–Jun rainfall. If Apr-Jun rainfall and runoff provided an initial source of nutrients for *Aphanizomenon* growth, the dominant component of total *Cyanophyta*, enough nutrients may have been available to provide a source of N (Table 2.3) until the energy-demanding process of N fixation was necessary, which corresponds to increases in BV in May and June (Figure 2.3). Historically, rainfall has been an important driver for cyanobacteria blooms in Lake Mendota. In 1993, extremely heavy rains and resulting high TP in Mendota led to cyanobacterial BV approximately double that measured in the summer prior [278]. Rainfall may also have been important during specific years in contributing nutrients from the urban and agricultural land usage surrounding Lake Mendota [231]. Highest spring TP, TN, and $NO^-_3NO^-_2$ measurements in our dataset occurred in 2009, one year after a 100-year flood event in the Midwestern United States. Similarly, highest KJN measurements occurred in 2009, as did an outlier BV for *Planktothrix* species.

It is possible that changes in salinity due to storm events and drought cycles, in addition to road salt applications, may also provide certain cyanobacterial genera with higher salt tolerances at a competitive advantage among other phytoplankton [203]. A recent study of long-term chloride trends in North America lakes found that chloride levels are increasing in freshwater ecosystems [279]. It has previously been suggested that *Microcystis spp.* are salt-tolerant based on culture experiments [280]. Linear trends

in rainfall were evident with corresponding changes in *Microcystis* BV, and with Apr-Jun

rainfall and total *Cyanophyta* and *Microcystis.* However, a binomial relationship between

Apr-Jun rainfall and mean *Aphanizomenon* was also apparent, consistent with other

studies suggesting changes in rainfall can have inconsistent effects on cyanoHAB

development [281], or that rainfall has the greatest effect on cyanobacterial growth at

some optimal level. The impact of Apr-Jun rainfall on cyanobacterial success likely

varies, as interannual changes in CCC were significantly correlated with Apr-Jun

rainfall.

Non-linear correlations between environmental factors related to climate change

and genus-specific adaptations to such changes make responses of cyanoHABs and

CCC to long-term trends in climate somewhat difficult overall to predict. Many scientists

have suggested overall increases in BV and a longer CGS due to slowly increasing air

temperatures. It is possible that as the deviations in carbon dioxide, air temperature,

and water temperatures become more apparent over time that correlations from long-

term datasets including cyanobacterial monitoring will more explicitly define the

relationships of environmental variables on cyanoHAB success and diversity.

**Conclusions**

We have identified factors related to interannual trends in cyanobacterial BV and

CCC in a highly studied eutrophic lake. Mean CGS and spring TP, spring KJN, TN:TP

ratio, Apr-Jun and annual rainfall, and St were significantly correlated with interannual

changes in *Aphanizomenon, Microcystis*, or total *Cyanophyta* BV in Lake Mendota.

However, other species under the orders of *Oscillatoriales* and *Synechococcales* also

achieved dominance of the CCC in some years. Detrended Correspondence Analysis

showed that longer ice duration, KJN, and spring $NO^-_3NO^-_2$ were associated with

deviations in bloom dominance or changes in CCC. Further analyses could investigate

how these interacting correlative variables may affect cyanoHAB severity and the

dominant bloom formers in Lake Mendota under various future climate change

scenarios, which will be helped by continual long-term monitoring of cyanobacteria, in

addition to changes in climatology and nutrient assessments.

**CHAPTER 3:**

**Large-Scale Spatial Perspective on Cyanotoxin Occurrence in Freshwater Lakes**

**Introduction**

Toxic cyanobacteria are a concern gaining awareness for freshwater lakes and reservoirs used for recreation and drinking water worldwide. Freshwater harmful algal blooms (FHABs) are known for unsightly scums, taste and odor compounds in recreational and drinking waters, and human and animal illness or death, related to their biomass and toxin production. Much of the growing cyanotoxin research published up to 2012 has focused on hepatotoxic microcystins (MCs) [282]. However, FHABs are potentially capable of producing a variety of compounds with different toxic and bioactive properties.

Cyanobacteria are capable of producing generally three toxin types, which have a variety of toxic mechanisms and effects: hepatotoxins (MCs, nodularin, cylindrospermopsin), neurotoxins (paralytic shellfish poisons, anatoxins, β-methyl-amino-L-alanine (BMAA)), and dermatoxins (lyngbyatoxin, lipopolysaccharide). However, with the variety of cyanobacterial secondary metabolites observed, some identified compounds do not fit into these categories and/or are capable of multiple toxic effects. The current study is focused on cyclic toxic and biologically active (or "bioactive") cyanopeptides. Possibly the most well-known of these are MCs and nodularin (NOD). These seven- and five-amino acid cyclic cyanopeptides, respectively, bind protein phosphatases (PP) [56, 59], causing liver hemorrhaging and potentially liver and colorectal cancers [8, 283-285] ENREF 4, based on the locations of MC-transporting, organic anion transporting polypeptides [286].

In addition to toxins, cyanobacteria produce several other cyclic peptide secondary metabolites that exert biological activity, mainly via inhibition of digestive proteases (e.g. serine/threonine proteases), which could potentially serve as therapeutic agents in medicinal purposes. Such bioactive peptides include cyanopeptolins (Cpts), anabaenopeptins (Apts), and microginins (Mcgns). Production of MCs and bioactive peptides is through non-ribosomal peptide synthases and polyketide enzymes, but not much is known about how expression of these pathways is regulated [287, 288]. Apts, Cpts, and Mcgns have been co-detected with MCs under varying culture conditions and in field analyses [289-291]. Recent studies have observed spatial and temporal trends of MCs in freshwaters, but similar patterns of occurrence or ecological influence have yet to be elucidated for other bioactive compounds produced by cyanobacteria.

Over 120 Cpts have been identified (reviewed in [292]); Cpts are cyclic hexadepsipeptides with their defining feature being 3-amino-6-hydroxy-2-piperidone ("Ahp") moiety in the second amino acid position ([293]; reviewed in [294]). Ahp and residue 1 (Arg) are responsible for protease specificity of these inhibitors, as well as blocking water molecules for the normal hydrolysis that takes places at the catalytic site of trypsin. Residue 6 (Thr) is responsible for binding the trypsin enzyme at subsites S2-S4 [295]. Though this activity initially indicated as bioactive inhibition, recent studies of Cpt 1020 have identified the compound as neurotoxic to crustaceans and zebrafish [296, 297], though this has yet to be shown for other Cpts.

Apts are also cyclic, but are pentapeptides with a characteristic ureido moiety and ring formation and side chain attachment through Lys at residue 1 [298]. At least 29 Apts have been identified (reviewed in [292]), and most inhibit exopeptidases, such as

carboxypeptidases [299] or protein phosphatases [300], depending on the isoform. Apt inhibition of carboxypeptidase U is potentially important for treatment of cardiovascular diseases in which blood does not clot, since the enzymes are involved in fibrinolysis [301, 302]. Structural similarity of carboxypeptidase to other metalloproteases makes Apts potential inhibitors of angiotensin-converting enzyme (ACE) [292, 303]. Mcgns have similarly been identified as inhibiting ACE [304, 305], or otherwise leucine aminopeptidase [306, 307]. ACE is responsible for converting angiotensin I to angiotensin II for vasoconstriction of blood vessels. Therefore, compounds such as Mcgns and Apts could potentially treat hypertension and congestive heart failure by inhibiting this enzyme as a drug target [292, 308]. Mcgns are, however, structurally different from other cyanopeptides in that they are linear with a range of four to six amino acids [305, 307, 309].

Few studies of cyanotoxins have included broad geographic areas with more than one sample to indicate temporal patterns for multiple lakes. For example, the US EPA 2007 National Lakes Assessment recorded MC and cylindrospermopsin concentrations among other environmental variables in 1028 U.S. lakes [310], but analysis of this dataset is limited by lack of a temporal scale and no measurements of other cyanotoxins. Other studies in Portugal, Germany, Poland, and specific regions of the United States have focused on detecting a specific toxin or group of toxins (e.g. MCs, anatoxin-a, cylindrospermopsin) in lakes and streams [47, 311-314] ENREF 16, but have geographical and temporal limitations in terms of identifying patterns generalizable to toxin production in lakes worldwide.

Despite a growing number of detection methods, analytical capabilities are still limited for cyanotoxins. Cyanotoxin production genes are strain-specific, but multiple toxic cyanobacterial species and cyanobacteria with several toxin-producing genes [298, 315] can be present in a FHAB [316]. Strains of the same species have been documented as toxic or non-toxic, which is sometimes demonstrated in seasonal FHAB compositions [38, 55, 317]. In addition, "toxic" cyanobacteria do not necessarily produce cyanotoxins at a constitutive rate [318-320], creating an important temporal aspect of toxin monitoring. Analytical tests available for cyanotoxin monitoring also vary in the data they can provide and their accuracy, making it potentially difficult to compare results among analyses conducted in different laboratories. Some secondary metabolites co-produced by cyanobacteria can also interfere with some tests for others. For example, in addition to other nontoxic effects, Apts have been implicated in biological inhibition of PP [300], similar to the toxic activity of MCs, an important consideration for using the PP1/2A inhibition assay as a MC screening method. Finally, reference materials are not available for all the known (and unknown) toxic or bioactive metabolites produced by cyanobacteria. This adds further complexity to the most accurate type of analysis, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), which relies on purified reference standards of each congener for analysis.

Given the spatial and temporal variability of toxin occurrence, the variety of toxins and analysis methods, and the importance of each of these elements in successful monitoring, the goal of this study is to characterize a broad range of cyanopeptides in lakes distributed in a near global fashion using the same extraction methods and

analytical techniques. This study illustrates the cyanotoxin profiles of 32 lakes from seven countries on three continents – including detection of five MCs, NOD, three Cpts, three Apts, and Mcgn 690 using LC-MS/MS – and identifies challenges and potential solutions for preserving samples for cross-continental shipping and collaboration.

## Methods

### Sample Collection

Whole lake water samples were requested for cyanotoxin analysis through the Global Lakes Ecological Observatory Network (GLEON; www.gleon.org) and via collegial contacts. Samples were received from a total of 39 lakes from North America, South America, Europe and Africa (Table 3.1). The number of samples per lake varied from two to 68, collected between 2008 and 2014. In order to have similar content from all lakes, some samples were not included in our final analysis: samples collected at depths below 1 m; integrated samples rather than surface samples (Lake Stechlin); filters processing large volumes of biomass and the resulting filtrate (Lough Neagh, Lough Erne, Upper Lough Erne, Lough Corbet, Lough Kernan). Other samples were also received and analyzed that were from multiple sampling sites within the same lake or at discrete or integrated depths other than the surface, which were excluded from further analysis. The final sample set included samples collected between 2012 and 2014.

All samples included in the analysis were collected at 0-1 m. Due to the nature of international shipping and capabilities of collaborators in other countries, varied preservation methods were required prior to shipping and analysis. All U.S. locations

shipped samples frozen overnight, which in all cases arrived frozen. Not all researchers were capable of sending samples frozen overnight or on dry ice in a timely fashion. As such, more convenient preservation methods appropriate for cross-continental shipping distances were explored. Testing showed that appropriate methods included dilution and storage with ethanol (EtOH) to 50% or lyophilization. We have included spike-recovery experiments for using EtOH as a cyanotoxin preservative, a method used by participants for shipping samples from six lakes in this study. All water samples were cataloged upon arrival and stored at -20°C until analysis.

Some intricacies with international shipping and transport proved this process extremely difficult. For example, researchers' water samples are inevitably valued as "priceless"; marking them as such with a high value flagged some samples to be held in U.S. Customs, delaying arrival and requiring fees for removal from Customs withholding. Furthermore, communications with Customs regarding travel to the U.S. with frozen water samples was challenging.

**Reagents and Materials**

Standards for microcystin-RR (MC-RR), Cpts 1020, 1041 and 1007, Apts B and F, and Mcgn 690 were obtained from MARBIONC Development Group, LLC (Marine Biotechnology in North Carolina, Wilmington, USA). Certified reference standards of MC-LR, 7-[Dha$^7$]-MC-LR (dmMC-LR) and NOD in 50% methanol (MeOH):water were obtained as Certified Reference Materials (National Research Council, Halifax, Nova Scotia, Canada).

Table 3.1. Characteristics of Samples Collected

| Lake | Country | Samples Analyzed | Samples Included | Physical State | Frequency | Depths (m) |
|---|---|---|---|---|---|---|
| Auburn Lake | USA | 32 | 14[1] | Frozen | Weekly | 0 |
| Falling Creek Reservoir | | 32 | 22[2] | Frozen | Weekly | 0 |
| Fish Lake | | 10 | 10 | Frozen | Bi-weekly | 0 |
| Lake Giles | | 7 | 7 | Frozen | Bi-weekly | 0 |
| Lake Koshkonong | | 10 | 10 | Frozen | Bi-weekly | 0 |
| Lacawac | | 7 | 7 | Frozen | Bi-weekly | 0 |
| Lake Lillinonah | | 131 | 18[3] | Frozen | Weekly | 0, 1 |
| Lake Mendota | | 9 | 9 | Frozen | Bi-weekly | 0 |
| Lake Monona | | 10 | 10 | Frozen | Bi-weekly | 0 |
| Panther Pond | | 23 | 7[1] | Frozen | Weekly | 0 |
| Lake Wallenpaupack | | 12 | 6[4] | Frozen | Monthly | 0 |
| Lake Waynewood | | 3 | 0[5] | Frozen | Monthly | 0 |
| Lake Wingra | | 10 | 10 | Frozen | Bi-weekly | 0 |
| Lake Winnebago | | 10 | 10 | Frozen | Weekly | 0 |
| Bright Lake | Canada | 7 | 7 | Frozen | Monthly | 0 |
| Caribou Lake | | 11 | 11 | Frozen | Bi-weekly | 0 |
| Desbarats Lake | | 13 | 13 | Frozen | Bi-weekly | 0 |
| Little Basswood Lake | | 8 | 8 | Frozen | Monthly | 0 |
| Laguna el Carpincho | Argentina | 2 | 0[5] | EtOH | Monthly | 0 |
| Laguna de Chascomús | | 4 | 4 | EtOH | Monthly | 0 |
| Laguna Gomez | | 2 | 0[5] | EtOH | Monthly | 0 |
| Laguna Grande | | 15 | 15 | EtOH | Monthly | 0 |
| Peri Lake | Brazil | 20 | 20 | Frozen | Monthly | 0 |
| Ahijarv | Estonia | 6 | 0[5] | EtOH | Monthly | 0.3, 4 |
| Kaiavere | | 13 | 13 | EtOH | Bi-weekly | 0.3 |
| Maekula | | 3 | 0[5] | EtOH | Monthly | 0.3 |
| Puhajarv | | 6 | 0[5] | EtOH | Monthly | 0.3, 5 |
| Tundre | | 8 | 0[5] | EtOH | Monthly | 0.3, 3, 4, 7 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Uljaste | | 6 | $0^5$ | EtOH | Monthly | 0.3, 3 |
| Vanamoisa | | 2 | $0^5$ | EtOH | Sub-weekly | 0.3 |
| Parentis Lake | France | 37 | 12 | EtOH | Monthly | 0, Secchi, Photic Zone |
| Lake Stechlin | Germany | 19 | $17^6$ | Lyophilized /Dried | Biweekly | 0-25, 5-10, 5-15 |
| Lough Corbet | Northern Ireland | 35 | $0^7$ | Lyophilized /Filters | Monthly | 0 |
| Lough Erne | | 94 | $0^7$ | Lyophilized /Filters | Bi-weekly | 0 |
| Upper Lough Erne | | 66 | $0^7$ | Lyophilized /Filters | Bi-weekly | 0 |
| Lough Kernan | | 9 | $0^7$ | Lyophilized /Filters | Monthly | 0 |
| Lough Neagh | | 92 | $0^7$ | Lyophilized /Filters | Bi-weekly | 0 |

1. First sample shipment ended with broken shipping cooler and lost samples recovered after days thawed. Second shipment sent for analysis (included).

2. Other sample sites included in samples submitted.

3. Samples from Lillinonah were collected over multiple years at multiple sites. A single site was selected from a buoy location so samples would be more comparable to those from other lakes, rather than shoreline samples. Experimental samples were also included in this sample set.

4. Samples selected from one of two sample sites provided for lake. Cyanopeptide detection from other site described in Appendix B.

5. Samples excluded due to low number of samples at surface depth. Data for other depths is included in Appendix B text and Tables B3, B4.

6. Samples excluded because they were collected from integrated depths, rather than the lake surface. Data is presented from Lake Stechlin in Appendix B, Table B2.

7. Samples excluded because sample volumes exceeded that of all other lakes. Additionally, samples were provided as filters and filtrate without any whole water comparison made available.

MC-YR, and MC-LA were obtained from Sigma-Aldrich Co. (St. Louis, Missouri, USA). Algal toxin stocks were stored in amber glass vials and stored at -20°C, resuspended in 100% MeOH if necessary. High performance LC (HPLC)-grade acetonitrile (ACN), water, and formic acid were obtained from Fisher Scientific (Fair Lawn, NJ, USA). HPLC-grade MeOH was obtained from Alfa Aesar (Ward Hill, MA, USA). Ammonium acetate (purity 99.15%) was obtained from Amresco, LLC (Solon, OH, USA) and diluted to 1M in HPLC-grade water for addition to the mobile phase.

**Extraction of Cyanobacterial Peptides from Frozen Water Samples**

Solvent extraction methods for MCs, NOD, and bioactive peptides from frozen water samples submitted to this project have been described previously [321]. Briefly, toxins were extracted from these samples using lyophilization, three freeze-thaws in 0.1% formic acid in water (e.g. cell lysis), and extraction of MCs and bioactive peptides with ~67% MeOH. Freeze-thaws have been identified as the most efficient methods of lysing cells for release of MCs [322]. Previously lyophilized samples were extracted in shipped containers with the same methods described above. To estimate cyanotoxin recovery from lyophilized samples, 5 µL of the reference standard mixture was spiked into 13 samples collected from one of our sample sites (Lake Lillinonah, Connecticut, USA). These samples were extracted using lyophilization, freeze-thaws, and a 2:1 MeOH:0.1% formic acidic water solvent extraction, as described.

As an alternative to shipping samples frozen or lyophilized, participants were encouraged to send samples diluted to 50% with EtOH as a preservative. Prior to extraction, samples were portioned into glass scintillation vials and dried using heat

(45°C) and N$_2$ gas. The resulting residue was re-suspended in 0.1% formic acid in water and immersed in a sonicating water bath at 45°C for 10 minutes to ensure suspension into solution. From this point, toxin extractions continued with freeze-thaws as described above. To test this preservation method for shipping, either *Cylindrospermopsis raciborskii* (University of Texas Culture Collection LB 2897) or *Microcystis aeruginosa* (University of Texas Culture Collection 2385) in modified BG11 (recipe in Appendix C, Tables C2-C8) were substituted as sample matrix. Equal parts of culture and EtOH were mixed in glass scintillation vials in triplicate. Each sample was spiked at 1 µg/L with toxin reference standards; a separate vial contained culture and EtOH without a spike. EtOH samples were stored for three days at room temperature in the dark to simulate shipping conditions and toxins extracted as described above.

**Algal Toxin Detection Using Liquid Chromatographic Separation Coupled with Tandem Mass Spectrometry**

Toxin extracts were measured using LC (Shimadzu UPLC) coupled with tandem MS/MS (AB Sciex 4000 Qtrap). Five MCs (-LR, -RR, -YR, -LA, dmMC-LR), NOD, three Apts (A, B, F), three Cpts (1020, 1041, 1007) and Mcgn 690 were separated using a C18 column and included in a scheduled multiple reaction monitoring method as previously described ([321]). Diagnostic daughter ions of *m/z* 135 [323] were included for all MCs (Table A1, Appendix A). The limit of quantification for all analytes was 0.5 µg/L; this was the lowest standard detected with a signal-to-noise ratio of 3:1. depending on the sample volume extracted, the limit of quantitation was determined and

toxin detects below the limit of quantitation were then removed from the dataset as "below detection limit" (Table B1, Appendix B).

**Study Areas**

Between four and 22 surface samples were included for analysis from each lake (average=11±5). After excluding lakes with sample matrices incomparable to most other samples and those taken at different sample depths, 248 samples (Table 3.1) from 22 lakes were included for toxin profile analysis. The majority of toxin extractions were from frozen, whole water samples; samples from Argentinian and Estonian lakes and Lac Biscarrosse de Parentis (Parentis Lake, France) were preserved with EtOH, some samples from Northern Ireland lakes and all Lake Stechlin were lyophilized, and some Northern Ireland lakes and Lagoa de Peri (Peri Lake) samples were filtered. Only results from whole water and EtOH-preserved samples were considered for analysis of toxin occurrence.

Figure 3.1 illustrates the diversity of lakes in regards to surface area, volume, maximum depth, and trophic status. The largest lake (Lake Winnebago, WI USA) is an outlier in terms of surface area and volume (Figure 3.1A). Table 3.2 includes further descriptive information about each lake. Of the lakes included for statistical analysis, five are oligotrophic or oligo-mesotrophic, five are mesotrophic or meso-eutrophic, nine are eutrophic, and three are hypereutrophic. Trophic status was based on historical observations by the scientists who donated samples to be analyzed by this project. The average mean depth among all lakes is 6.1 m, and max depths range from 1.9 m to 36 m.

Table 3.2. Descriptive Characteristics of Lakes Comparing Toxin Profiles

| Lake | Longitude | Latitude | Mean (Max) Depths (m) | Area (km$^2$) | Elevation (m) | Trophic Status | Volume (10$^6$ m$^3$) | Dreissenids Present | Land Types |
|------|-----------|----------|----------------------|---------------|---------------|----------------|----------------------|---------------------|------------|
| Parentis | -1.16 | 44.36 | 6.7 (22) | 31.9 | 21 | E | 213.79 | NC | F, L, A |
| Lillinonah | -89.4 | 43.07 | 11.9 (34) | 76.9 | 60 | M-E | 915.11 | C | F, U, A |
| Falling Creek | -79.84 | 37.31 | 4 (9.3) | 0.1 | 509 | E | 0.48 | NC | F |
| Mendota | -89.42 | 43.11 | 12.8 (25.3) | 40 | 259 | E | 511.74 | C | U, A |
| Monona | -89.36 | 43.07 | 8.2 (22.5) | 13.6 | 258 | E | 111.74 | NC | U, A |
| Wingra | -89.42 | 43.05 | 2.4 (4.3) | 1.4 | 258 | E | 3.26 | C | U |
| Fish | -89.65 | 43.29 | 6.6 (18.9) | 0.8 | 261 | M-E | 5.32 | NC | A |
| Koshkonong | -88.96 | 42.87 | 1.5 (2.1) | 42.9 | 236 | HE | 65.34 | C | A |
| Winnebago | -88.5 | 44.03 | 4.7 (6.4) | 533.9 | 227 | HE | 2510.09 | C | U, A |
| Grande | -60.41 | -32.59 | 0.75 (1.2) | 1.6 | 1 | E | 1.02 | NC | V |
| Chascomús | -58.03 | -35.59 | 1.5 (1.9) | 28.7 | 7 | HE | 45.15 | NC | U |
| Kaiavere | 26.67 | 58.61 | 2.8 (5) | 2.5 | 51 | E | 7.00 | C | A, F |
| Bright | -83.30 | 46.27 | 4.91 (19) | 12.3 | 181 | M | 60.49 | NC | F, W, A |
| Little Basswood | -83.42 | 46.31 | 6.2 (12) | 0.4 | 201 | O | 2.41 | NC | A, V |
| Caribou | -79.78 | 47.06 | 3.7 (6.15) | 93.1 | 222 | M | 344.47 | NC | F, W, V |
| Desbarats | -83.93 | 46.39 | 6.7 (10) | 3.6 | 182 | E | 24.12 | NC | F, A |
| Auburn | -70.25 | 44.15 | 12.2 (36) | 9.1 | 79 | O-M | 111.58 | NC | F |
| Panther | -70.46 | 43.93 | 7.9 (21) | 5.8 | 84 | O-M | 46.15 | NC | F |
| Wallenpaupack | -75.23 | 41.42 | 9.1 (18) | 23 | 361 | E | 209.3 | NC | F |
| Lacawac | -75.29 | 41.38 | 5.2 (13) | 0.2 | 446 | M | 1.04 | NC | F |
| Giles | -75.09 | 41.38 | 10.1 (24.1) | 0.5 | 428 | O | 5.05 | NC | F |
| Peri | -48.53 | -27.73 | 4.2 (11) | 5.7 | 3 | O-M | 23.94 | NC | F |

Abbreviations: E=Eutrophic; HE=Hypereutrophic; M=Mesotrophic; M-E=Meso-eutrophic; O=Oligotrophic; O-M=Oligo-mesotrophic; C=Colonized by dreissenid mussels; NC=Not colonized by dreissenid mussels; F=Forest; L=Lakes; U=Urban; A=Agriculture; W=Wetland; V=Vegetation

Figure 3.1. Area, volume, trophic status, and max depth of lakes
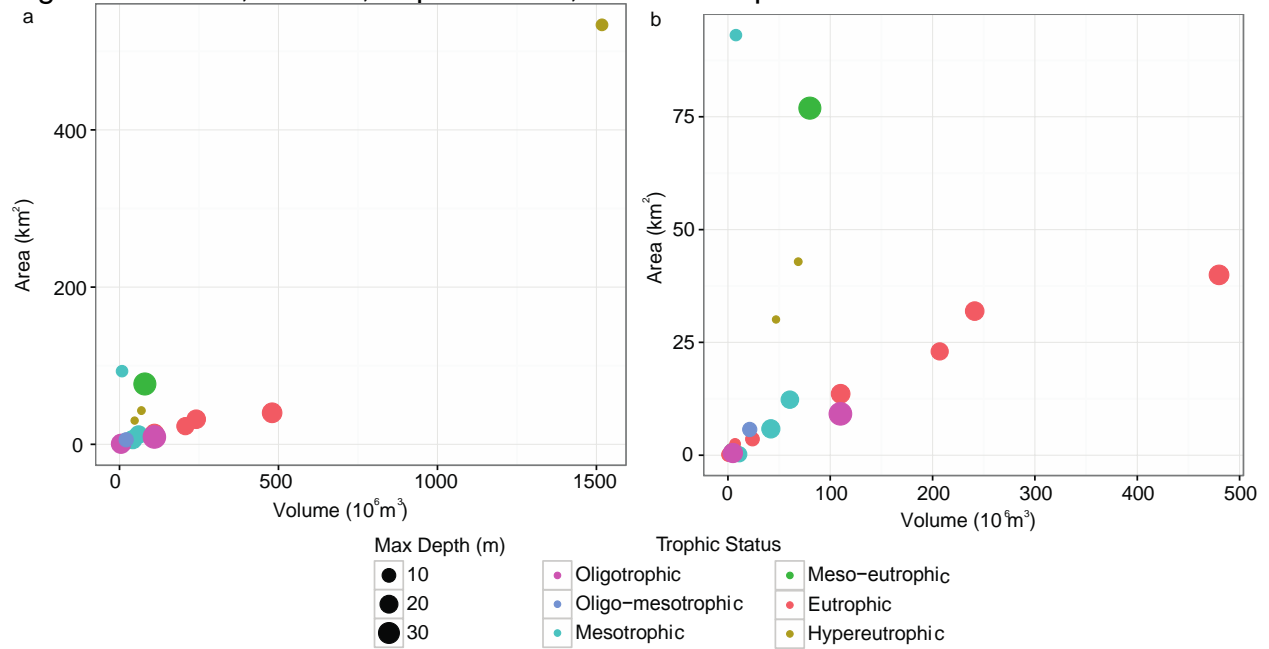


Figure 3.1 A) Lake surface area (km$^2$), volume ($10^6$ m$^3$), max depth (m), and trophic status for each lake. Each circle represents a single lake; the size and color of each circle represents the max depth and trophic status, respectively. B) Same as A, but with Lake Winnebago excluded

**Statistical analyses**

Analysis of extraction efficiency was calculated from the spike recovery experiments using Lillinonah lake water, *C. raciborskii*, or *M. aeruginosa* cultures in BG11. For each cyanopeptide measured, the expected concentration in the final extract was calculated based on the spiked liquid volume concentrated during extraction. The percent recovery of each toxin for each extraction method was calculated by dividing the concentration calculated in test extract by the expected concentration and multiplied by 100.

For comparing toxin profiles among lakes, we only included samples that were collected from the center of each lake in lakes with four or more water samples collected at depths between 0-1 m. The mean, standard deviation, and frequency of detection for each cyanopeptide were calculated from all samples. Cyanopeptide variance among different lakes was then related to environmental characteristics of each lake. Environmental characteristics used to consider related to differences in toxin concentrations included trophic status? presence of dreissenid mussels, surface elevation, surface area, lake volume, mean/max depth, dominant land use, latitude, mixing pattern, and country. Lake volume for each lake was estimated by multiplying mean depth (m) by surface area ($km^2$). Land usage categories included forest, urban, agriculture, and vegetation; "vegetation" refers broadly to areas consisting of grasses, shrubbery, and few trees.

Statistical significance of differences among cyanopeptides concentrations among mesotrophic lakes and eutrophic/hypereutrophic lakes was tested using the Kruskal-Wallis Test in R (*kruskal.test*). The purpose of this test was to investigate

effects of environmental characteristics on cyanopeptides occurrence within lakes of the same trophic status. Lakes described as "meso-eutrophic" were included with those described as "mesotrophic" for this test.

## Results

### Spike-recovery analysis

Average recoveries for each cyanopeptide and extraction method are presented in Table 3.3. We expected variations in recovery of MCs and other cyanopeptides based on variations in hydrophobicity. Cyanopeptides were recovered from a range of 56.7% (NOD) to 99.7% (MC-RR) from spiked extractions of Lake Lillinonah samples. MCs' recovery averaged 79.54%, and similarly, total cyanopeptide recovery averaged 76.2% (Table 3.3).

Similar to extractions from frozen lake water samples, recovery of cyanopeptides preserved with EtOH for three days and extracted with 70% MeOH ranged from 60.5% (Mcgn690) to 93% (MC-RR), with an average of 75.23% recovery of all cyanopeptides in EtOH mixed with *C. raciborskii*. MCs alone averaged 80.1% recovery with this preservation and extraction technique (Table 3.3). When this testing of preservation and extraction was repeated with *M. aeruginosa* for three days, recovery ranged from 57.8% (Apt A) to 85.5% (NOD). EtOH preserving spiked *M. aeruginosa* for seven days demonstrated higher recoveries between 79.4% (MC-YR) and 94.8% (Cpt 1020) (Table 3.3). MC-LR, dmMC-LR, Cpt 1007, Cpt 1041, and Mcgn 690 recovery could not be analyzed with *M. aeruginosa* UTEX strain 2385 because these peptides were produced

Table 3.3. Percent Recovery of Cyanobacterial Peptides from Tested Extraction

Methods

| Cyanopeptide | Lyophilization + 70% MeOH | 50% EtOH Preservation *C. raciborskii* | 50% EtOH Preservation (3 d) *M. aeruginosa* | 50% EtOH Preservation (7 d) *M. aeruginosa* |
|---|---|---|---|---|
| MC-LR | 81.2 | 76.3 | NA | NA |
| MC-RR | 99.7 | 93.0 | 78.6 | 86.7 |
| MC-YR | 71.1 | 70.1 | 64.3 | 79.4 |
| MC-LA | 70.9 | 79.4 | 81.9 | 90 |
| dmMC-LR | 74.8 | 81.7 | NA | NA |
| NOD | 56.7 | 71.3 | 85.5 | 91.9 |
| Apt A | NA | NA | 57.8 | 91.5 |
| Apt B | 85.2 | 67.7 | 70 | 88.5 |
| Apt F | 73.4 | 78.6 | 75.7 | 90.4 |
| Cpt 1007 | 84.1 | 75.1 | NA | NA |
| Cpt 1020 | 63.8 | 75.8 | 85.4 | 94.8 |
| Cpt 1041 | 77.8 | 73.2 | NA | NA |
| Mcgn 690 | 75.9 | 60.5 | NA | NA |

Abbreviations: MC=microcystin; L=leucine; R=arginine; Y=tyrosine; A=alanine;

dm=desmethyl ([Dha$^7$]); Apt=anabaenopeptin; Cpt=cyanopeptolin; Mcgn=microginin;

NOD=nodularin; d=days; MeOH=methanol; EtOH=ethanol

by the culture at the time of experiment (Appendix B, Figure B1). Regardless of sample matrix, all extractions resulted in a range of recovery from 50-80%.

**Cyanopeptide occurrence**

Figure 3.2 displays the median and range of individual cyanopeptide concentrations detected from all samples; the mean, standard deviation, maximum concentration, and frequency of detection of each cyanopeptide in lake water samples are presented in Table 3.4. Cyanopeptides were not detected in Peri Lake, Little Basswood Lake, Auburn Lake, Lake Giles, Lacawac Reservoir, or Panther Pond. Apts were the most frequently detected cyanopeptides in 33.1% (Apt B) and 27% (Apt A) of samples. MC-LR was the most frequently detected MC in 19.8% of samples, followed by MC-RR in 12.1%. Cpt 1041 was detected in 12.9% of samples. MC-YR, dmMC-LR, NOD, and Cpt 1020 were detected in less than 5% of samples.

Cyanopeptide profiles for each lake are illustrated in Figures 3.3 and 3.4. Of all U.S. lakes, Falling Creek Reservoir in Virginia had the lowest concentrations of cyanopeptides measured (Figure 3.3B). The highest cyanopeptide concentration was detected in a sample from Lake Winnebago, Wisconsin at 38.25 µg/L (MC-LR; Figure 3.3I); this sample also had the highest concentration of total cyanopeptides at 61.53 µg/L. The lake in which the highest variety of cyanopeptides was detected was Lake Winnebago, Wisconsin, USA; 10 of 13 peptides were detected in three samples. Six of nine U.S. lakes are located in Wisconsin (Figure 3.3D-I). Cpt 1020 was the only cyanopeptide measured that was not detected in Wisconsin lakes. Apts B and F were the primary peptides detected in Lake Lillinonah, Connecticut (Figure 3.3A) and Lake

Figure 3.2. Boxplot of cyanopeptides detected in all analyzed samples



Figure 3.2 shows the distribution of cyanopeptide concentrations among all samples.

Circles represent outliers. Abbreviations: MC=microcystin; L=leucine; R=arginine;

Y=tyrosine; A=alanine; dm=desmethyl ($[Dha^7]$); Apt=anabaenopeptin;

Cpt=cyanopeptolin; Mcgn=microginin; NOD=nodularin.

Table 3.4. Mean, Standard Deviation, and Frequency of Cyanopeptide Detection

| Cyanopeptide | Mean (± SD) (µg/L) | Max (µg/L) | % Frequency |
|---|---|---|---|
| Apt B | 1.94±3.52 | 24.10 | 33.1 |
| Apt A | 1.01±2.13 | 12.66 | 27.0 |
| MC-LR | 3.80±7.16 | 38.25 | 19.8 |
| AptF | 0.54±0.61 | 3.40 | 15.3 |
| Cpt 1041 | 0.89±1.34 | 7.54 | 12.9 |
| MC-RR | 1.06±1.15 | 5.14 | 12.1 |
| Cpt 1007 | 0.62±1.23 | 6.38 | 10.5 |
| MC-LA | 0.38±0.35 | 1.11 | 8.9 |
| Mcgn 690 | 0.40±0.69 | 2.21 | 8.1 |
| MC-YR | 0.19±0.19 | 0.53 | 4.4 |
| NOD | 0.38±0.12 | 0.54 | 1.2 |
| dmMC-LR | 0.17±0.11 | 0.29 | 0.8 |
| Cpt 1020 | 0.07±0 | 0.07 | 0.04 |

Percent frequency was calculated by dividing the number of samples in which a compound was detected by the total number of samples included for statistical analysis. Abbreviations: MC=microcystin; L=leucine; R=arginine; Y=tyrosine; A=alanine; dm=desmethyl ([Dha$^7$]); Apt=anabaenopeptin; Cpt=cyanopeptolin; Mcgn=microginin; NOD=nodularin.

Figure 3.3. Cyanopeptide detection in lakes in the United States
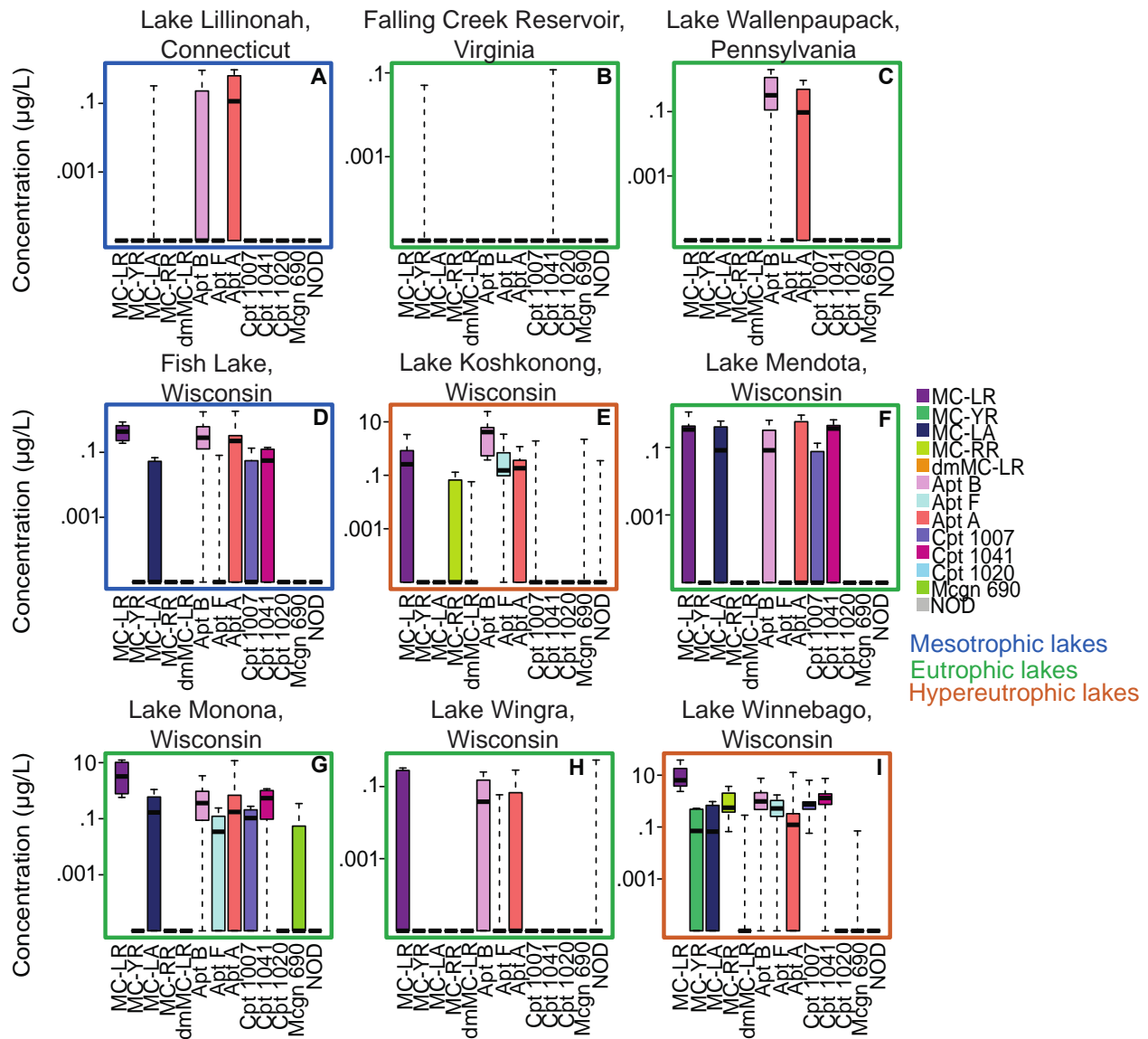


Figure 3.3 demonstrates the variety of, median, and maximum concentrations of cyanopeptides detected in samples from each lake in the United States. Whiskers extend to the max concentrations. Abbreviations: MC=microcystin; L=leucine; R=arginine; Y=tyrosine; A=alanine; dm=desmethyl ([Dha$^7$]); Apt=anabaenopeptin; Cpt=cyanopeptolin; Mcgn=microginin; NOD=nodularin

Figure 3.4. Cyanopeptide detection in lakes in Europe, Canada, Argentina
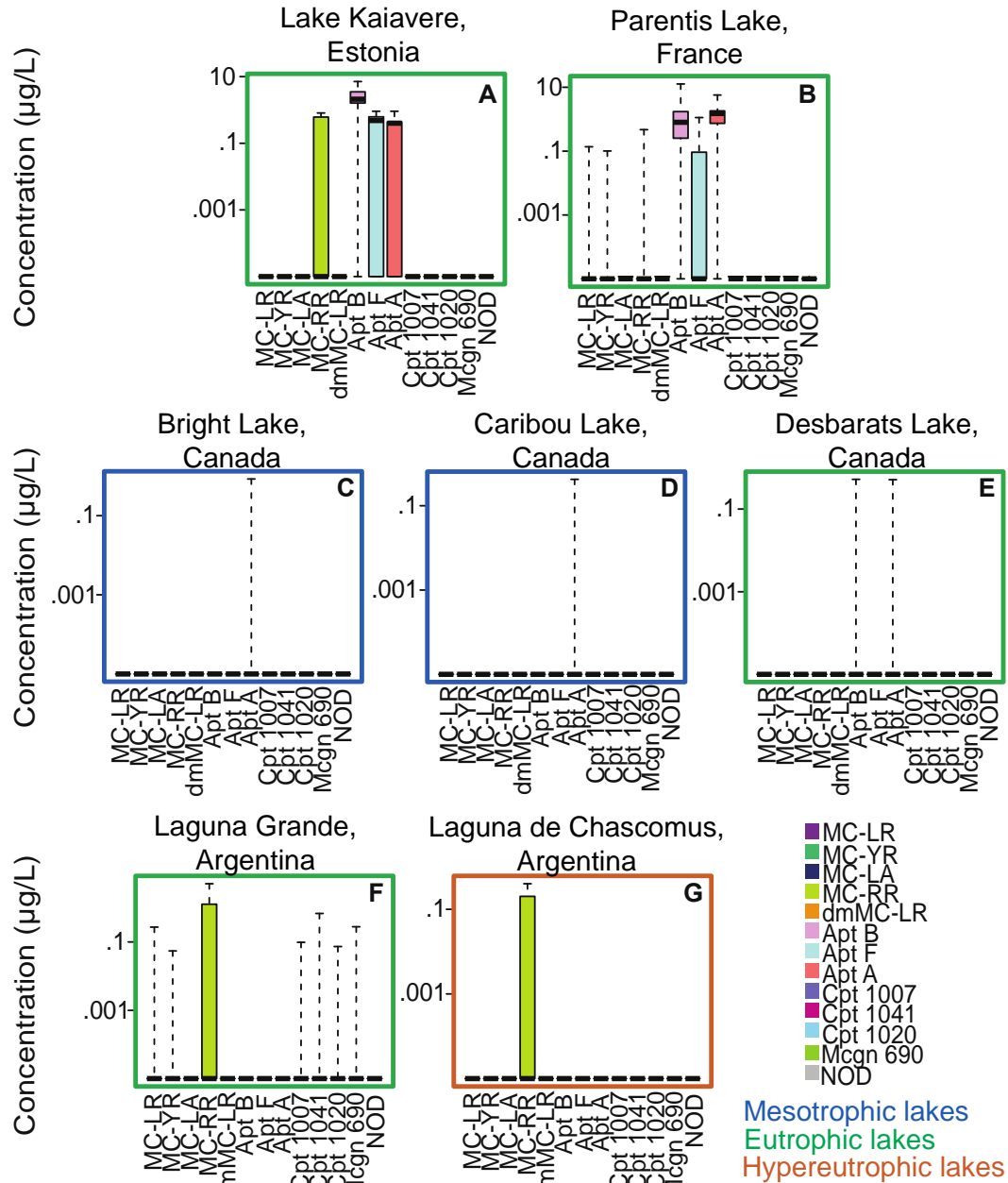
Figure 3.4 demonstrates the variety of, median, and maximum concentrations of cyanopeptides detected in samples from each lake in the United States. Whiskers extend to the max concentrations. Abbreviations: MC=microcystin; L=leucine; R=arginine; Y=tyrosine; A=alanine; dm=desmethyl ([Dha[7]]); Apt=anabaenopeptin; Cpt=cyanopeptolin; Mcgn=microginin; NOD=nodularin.

Wallenpaupack, Pennsylvania (Figure 3.3C); otherwise, only MC-LA was detected in one sample from Lake Lillinonah.

Only Apts A and B at low concentrations were detected in three lakes in Ontario, Canada (Figure 3.4C-E). Similarly, only MC-RR was detected in Laguna de Chascomús, Argentina. The lakes outside the U.S. exhibiting the most variety in cyanopeptides were Parentis Lake, France (Figure 3.4B) and Laguna Grande Argentina (Figure 3.4F). Samples from Lake Kaiavere, Estonia and Parentis Lake, France compared similar levels of MC-RR and Apts A, B, and F (Figure 3.4A-B).

**Cyanopeptides by land usage and mussel invasion status**

Overall, MC-LR, MC-RR, Cpt 1007, Cpt 1041, and Cpt 1020 were detected at similar levels among water bodies of varying trophic status. This indicates that cyanobacteria are producing toxic and bioactive cyanopeptides in mesotrophic lakes that may not be as regularly monitored as eutrophic and hypereutrophic lakes due to relatively less impairment. Trophic status of lakes fluctuates over time and is impacted by several factors, some of which are compared here among lakes with cyanopeptides occurrence, including surrounding land usage and presence of mussels.

Due to an inability to accurately capture all subsets of the land usage surrounding lakes from all countries, the majority use of land surrounding each site (not the watershed) was considered as a factor potentially affecting cyanopeptide occurrence. Figure 3.5B displays average cyanopeptides concentrations distributed by four land usage categories surrounding these lakes. Highest average MC-YR, Apt A, and Apt F levels were detected in lakes surrounded by agricultural land. Sites

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surrounded by urban shoreline had higher average levels of dmMC-LR, Apt B, Cpt 1007, and Cpt 1041. Forested sites had the highest levels of Mcgn 690; 12 out of 22 sites had predominantly forested shorelines. The differences in the dominant category of land use surrounding lakes correlated with occurrence of MC-LR, MC-LA, Cpt1041, Cpt1007, Apt B in lakes of mesotrophic to hypereutrophic status, and for MC-RR, MC-YR, and Apt F in eutrophic/hypereutrophic lakes according to a Kruskal-Wallis test (Tables 3.5 and 3.6). Not surprisingly, Kruskal-Wallis also determined that average total amounts of MCs, Apts, and Cpts significantly varied by dominant land usage.

Dreissena spp. mussels (i.e. *Dreissena polymorpha* or *Dreissena bugensis*) have colonized less than one third (n=6) of the sample sites in this study (Table 3.2). Most of the sites colonized with mussels are located within the U.S., but Lake Kaiavere in Estonia is also colonized. Qualitatively, MC-YR, MC-LA, dmMC-LR, and all Apts measured (A, B, F) were detected at higher average concentrations in sites colonized with *Dreissena* mussels (Figure 3.5C). Only MC-RR and Cpt 1041 had higher average concentrations in lakes not colonized by these invasives. *Dreissena* mussel colonization was statistically correlated only to differences in Apt B and Apt F profiles in eutrophic/hypereutrophic lakes (Table 3.6).

**Cyanopeptides, surface elevation, and mixing patterns**

When comparing toxins in lakes by surface elevation (Figure 3.6A), MC-YR, MC-LA, MC-RR and Apt B had higher average concentrations in lakes elevated <100 m above sea level, accounting for about one third of lakes (n=7). Lakes elevated between 100 and 200 m above sea level had higher concentrations of Apts A, F, Mcgn 690, and

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Table 3.5. Kruskal-Wallis results comparing cyanopeptides among mesotrophic lake characteristics

| Cyanopeptide | Characteristic | H | Degrees of Freedom | p-value (<0.05) |
|---|---|---|---|---|
| Microcystin-LR | Dominant Land Use | 45 | 10 | .000002175 |
| | Mixing Pattern | 27 | 10 | .002604 |
| | Location | 26.403 | 10 | .003234 |
| | Surface Area | 25.167 | 10 | .005037 |
| | Lake Volume | 25.167 | 10 | .005037 |
| | Surface Elevation | 25.167 | 10 | .005037 |
| Microcystin-LA | Dominant Land Use | 11.458 | 4 | .02187 |
| Anabaenopeptin B | Dominant Land Use | 39.442 | 16 | .0009388 |
| | Location | 28.218 | 16 | .02978 |
| | Lake Volume | 26.897 | 16 | .04264 |
| | Surface Elevation | 26.897 | 16 | .04264 |
| | Mixing Pattern | 23.021 | 9 | .006149 |
| Anabaenopeptin A | Dominant Land Use | 29.598 | 18 | .04154 |
| | Mixing Pattern | 16.389 | 9 | .05918 |
| Cyanopeptolin 1007 | Dominant Land Use | 19.756 | 5 | .001388 |
| Cyanopeptolin 1041 | Dominant Land Use | 15.429 | 4 | .00389 |
| | Location | 11.592 | 5 | .04083 |
| Total Microcystins | Mixing Pattern | 45 | 11 | $4.852\ e^{-6}$ |
| | Dominant Land Use | 45 | 11 | $4.852\ e^{-6}$ |
| | Location | 26.934 | 11 | .004701 |
| | Surface Elevation | 25.674 | 11 | .007256 |
| | Lake Volume | 25.674 | 11 | .007256 |
| | Surface Area | 25.192 | 11 | .008544 |
| Total Anabaenopeptins | Mixing Pattern | 45 | 22 | .002654 |
| | Dominant Land Use | 45 | 22 | .002654 |
| | Location | 33.98 | 22 | .04936 |
| Total Cyanopeptolins | Mixing Pattern | 24.3 | 6 | .0004599 |
| | Dominant Land Use | 24.3 | 6 | .0004599 |
| | Location | 14.258 | 6 | .02689 |
| | Surface Area | 13.59 | 6 | .03456 |
| | Surface Elevation | 13.59 | 6 | .03456 |
| | Lake Volume | 13.59 | 6 | .03456 |

Table 3.6. Cyanopeptides in eutrophic and hypereutrophic lakes by environmental

characteristics according to a Kruskal-Wallis test

| Cyanopeptide | Characteristic | H | Degrees of Freedom | p-value (<0.05) |
|---|---|---|---|---|
| Microcystin-LR | Dominant Land Use | 65.812 | 37 | .002455 |
| | Surface Area | 60.359 | 39 | .01568 |
| | Lake Volume | 58.295 | 39 | .02412 |
| Microcystin-RR | Dominant Land Use | 35.09 | 21 | .0276 |
| Microcystin-YR | Volume | 24.926 | 10 | .005487 |
| | Surface Area | 23.987 | 10 | .007634 |
| Microcystin-LA | Dominant Land Use | 32.108 | 18 | .02135 |
| | Lake Volume | 31.597 | 18 | .02453 |
| Anabaenopeptin B | Mussel Invasion | 93.568 | 63 | .007469 |
| | Dominant Land Use | 89.492 | 63 | .01578 |
| | Surface Area | 83.451 | 37 | .04719 |
| Anabaenopeptin F | Dominant Land Use | 63.765 | 37 | .004049 |
| | Mussel Invasion | 56.974 | 37 | .01899 |
| | Surface Area | 52.494 | 37 | .04719 |
| Cyanopeptolin 1041 | Lake Volume | 46.364 | 24 | .004001 |
| | Dominant Land Use | 40.951 | 22 | .008352 |
| Cyanopeptolin 1007 | Lake Volume | 40.727 | 22 | .008878 |
| | Dominant Land Use | 37.955 | 21 | .01305 |
| Total Microcystins | Location | 77.982 | 54 | .01803 |
| | Dominant Land Use | 74.391 | 45 | .003802 |
| Total Anabaenopeptins | Mussel Invasion | 96.732 | 68 | .01259 |
| | Dominant Land Use | 92.15 | 68 | .02728 |
| | Surface Area | 90.593 | 68 | .03496 |
| Total Cyanopeptolins | Lake Volume | 50.606 | 29 | .007764 |
| | Dominant Land Use | 44.685 | 26 | .01274 |
| | Surface Area | 43.887 | 29 | .03761 |

Figure 3.5. Average cyanopeptide concentrations by trophic status, land usage, and mussel colonization
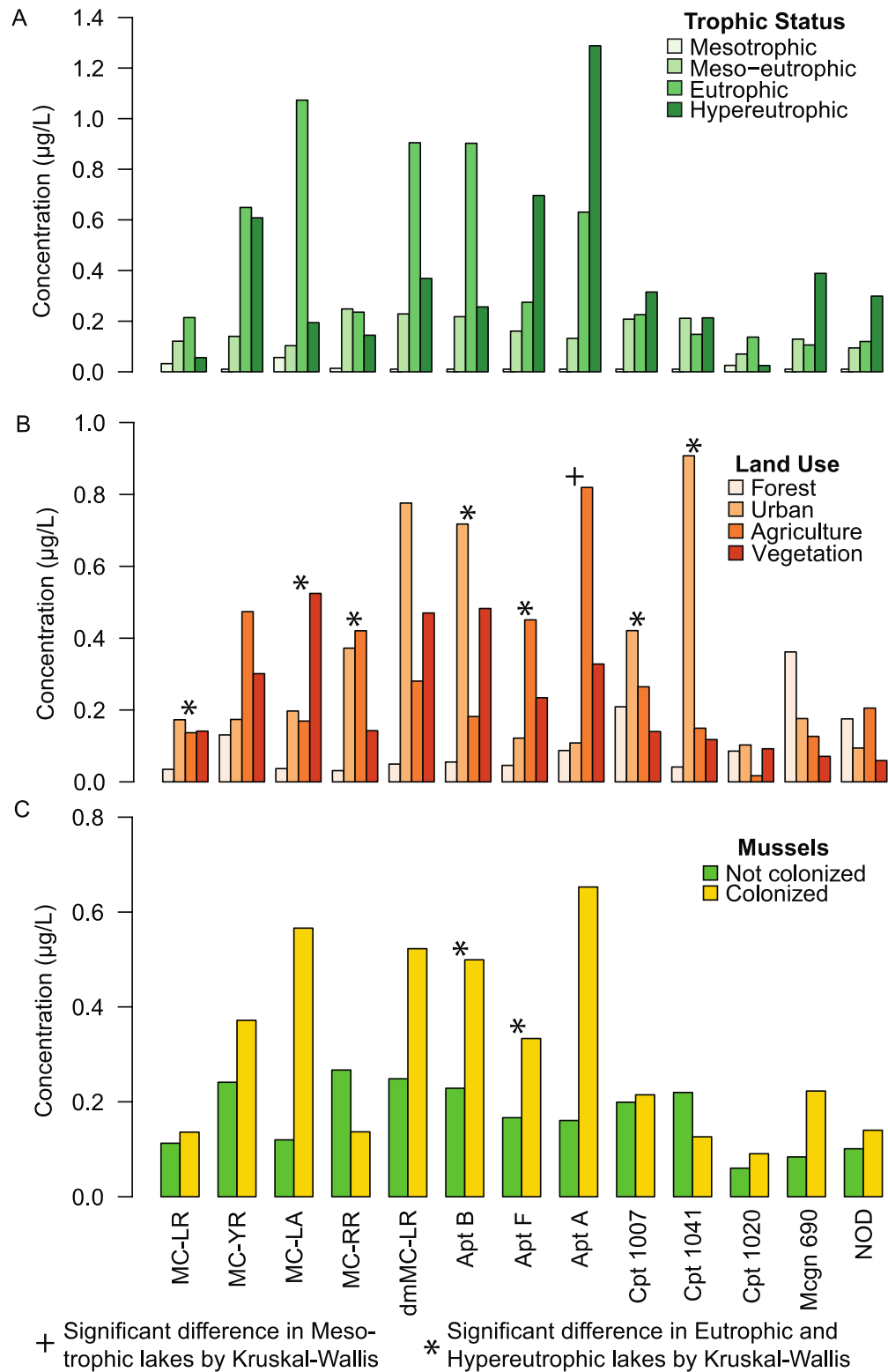
Figure 3.5 illustrates mean concentrations (µg/L) of each cyanopeptide by lake characteristics of A) trophic status, B) primary land usage surrounding each lake, and C) colonization or lack of colonization by *Dreissena* sp. mussels. One or two stars above bars for an individual toxin refer to significant differences in occurrence of that cyanopeptides according to a Kruskal-Wallis in lakes of either mesotrophic or eutrophic/hypereutrophic lakes, respectively. Abbreviations: MC=microcystin; L=leucine; R=arginine; Y=tyrosine; A=alanine; dm=desmethyl ([Dha$^7$]); Apt=anabaenopeptin; Cpt=cyanopeptolin; Mcgn=microginin; NOD=nodularin.

Figure 3.6. Average cyanopeptide concentrations by elevation, lake mixing, and surface area



+ Significant difference in Meso-
trophic lakes by Kruskal-Wallis

* Significant difference in Eutrophic and
Hypereutrophic lakes by Kruskal-Wallis
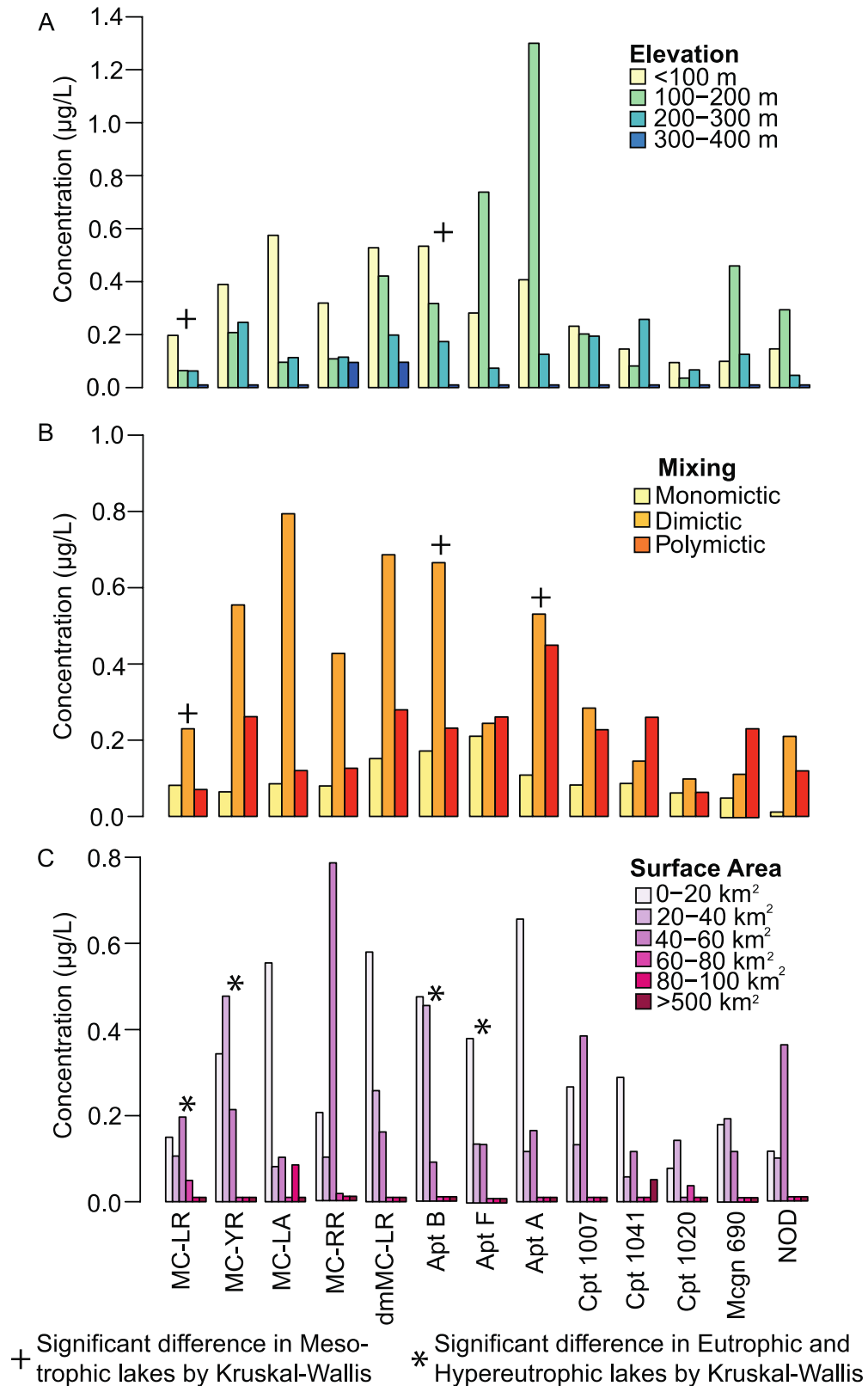
Figure 3.6 illustrates mean concentrations (µg/L) of each cyanopeptide by lake characteristics of A) surface elevation (m), B) lake mixing pattern, and C) surface area (km$^2$). One or two stars above bars for an individual toxin refer to significant differences in occurrence of that cyanopeptides according to a Kruskal-Wallis in lakes of either mesotrophic or eutrophic/hypereutrophic lakes, respectively. Abbreviations: MC=microcystin; L=leucine; R=arginine; Y=tyrosine; A=alanine; dm=desmethyl ([Dha$^7$]); Apt=anabaenopeptin; Cpt=cyanopeptolin; Mcgn=microginin; NOD=nodularin.

NOD. The only instances in which Kruskal-Wallis determined surface elevation was related to significant differences in toxins were concentrations of MC-LR and Apt B among mesotrophic lakes (Table 3.5).

Because different toxin-producing cyanobacteria prefer different water column mixing conditions, we investigated potential differences in cyanopeptide concentrations by lake mixing status (Figure 3.6B). Average concentrations of MC-YR, MC-LA, MC-RR, dmMC-LR, and Apt B were highest among dimictic lakes. Dimictic and polymictic lakes tended to have higher average concentrations of cyanopeptides compared to monomictic lakes. A Kruskall-Wallis test determined that MC-LR, Apt B, and Apt A concentrations were significantly different among mesotrophic lakes, but not eutrophic or hypereutrophic lakes.

**Cyanopeptides and lake size (volume, surface area) and morphology (max, mean depths)**

The largest site is also the largest inland lake in Wisconsin, USA – Lake Winnebago. The second and third largest sites are also in North America – Lake Lillinonah in Connecticut, USA and Caribou Lake in Ontario, Canada (Table 3.2). Some of the highest mean toxin concentrations trended based on site surface area and volume. The highest MC-LA, dmMC-LR, Apt A, Apt F and Cpt 1020 concentrations were detected in the smallest lakes, less than 20 $km^2$ (Figure 3.6C). Highest mean MC-RR, Cpt 1007, and NOD concentrations were detected in lakes between 40 and 60 $km^2$ in area. According to Kruskall-Wallis, in eutrophic/hypereutrophic lakes, differences in MC-LR, MC-YR, Apt B, and Apt F were significant among lakes with differing surface area.

Differences in mean total MC concentrations were significant in mesotrophic lakes, differences in total Apts significant in eutrophic/hypereutrophic lakes, and differences in mean total Cpt concentrations were significant in lakes of all three trophic statuses.

When considering lake volume, incorporating mean depth, mean Apt A and F concentrations were highest in Lake Winnebago, the only site with volume greater than $1000 \times 10^6$ m$^3$ (Figure 3.7A). Lakes with volumes ranging between $100\text{-}500 \times 10^6$ m$^3$ had higher mean concentrations of MC-YR and MC-LA, and the lake with the largest volume, Lake Winnebago, had mean concentrations of dmMC-LR and Apt B, similar to these smaller lakes. Several mean cyanopeptide concentrations were significantly different when comparing by lake volume. MC-LR concentrations were significantly different among mesotrophic, eutrophic, and hypereutrophic lakes. Average MC-YR, MC-LA, Cpt 1007, and Cpt1041 were significantly different among lakes of higher trophic status, whereas mean Apt B concentrations were significantly different among mesotrophic lakes (Tables 3.5, 3.6). Lake volume was related to significant differences in total Cpt concentrations in mesotrophic, eutrophic, and hypereutrophic lakes, whereas differences in total MCs were only related to volume in mesotrophic lakes.

The deepest sample site is located in Maine, USA (Auburn Lake, max depth=36 m); the shallowest sample site is in Argentina (Laguna Grande, max depth=1.2 m). Almost half (n=9) of the lakes had max depths <10 m (Table 3.2). Highest average MC-RR, Cpt 1007, and NOD detects were detected in sites with a max depth of 18-24 m. Cpt 1020 was detected at the lowest mean concentrations across lakes, regardless of max depth. When considering mean depth as a factor affecting toxin occurrence (Figure 3.7C), MC-LR, MC-RR, Apt F, Cpt 1007, Cpt 1041, Cpt 1020, Mcgn 690, and NOD were

Figure 3.7. Average cyanopeptide concentrations by lake volume and depth

+ Significant difference in Meso-trophic lakes by Kruskal-Wallis

* Significant difference in Eutrophic and Hypereutrophic lakes by Kruskal-Wallis
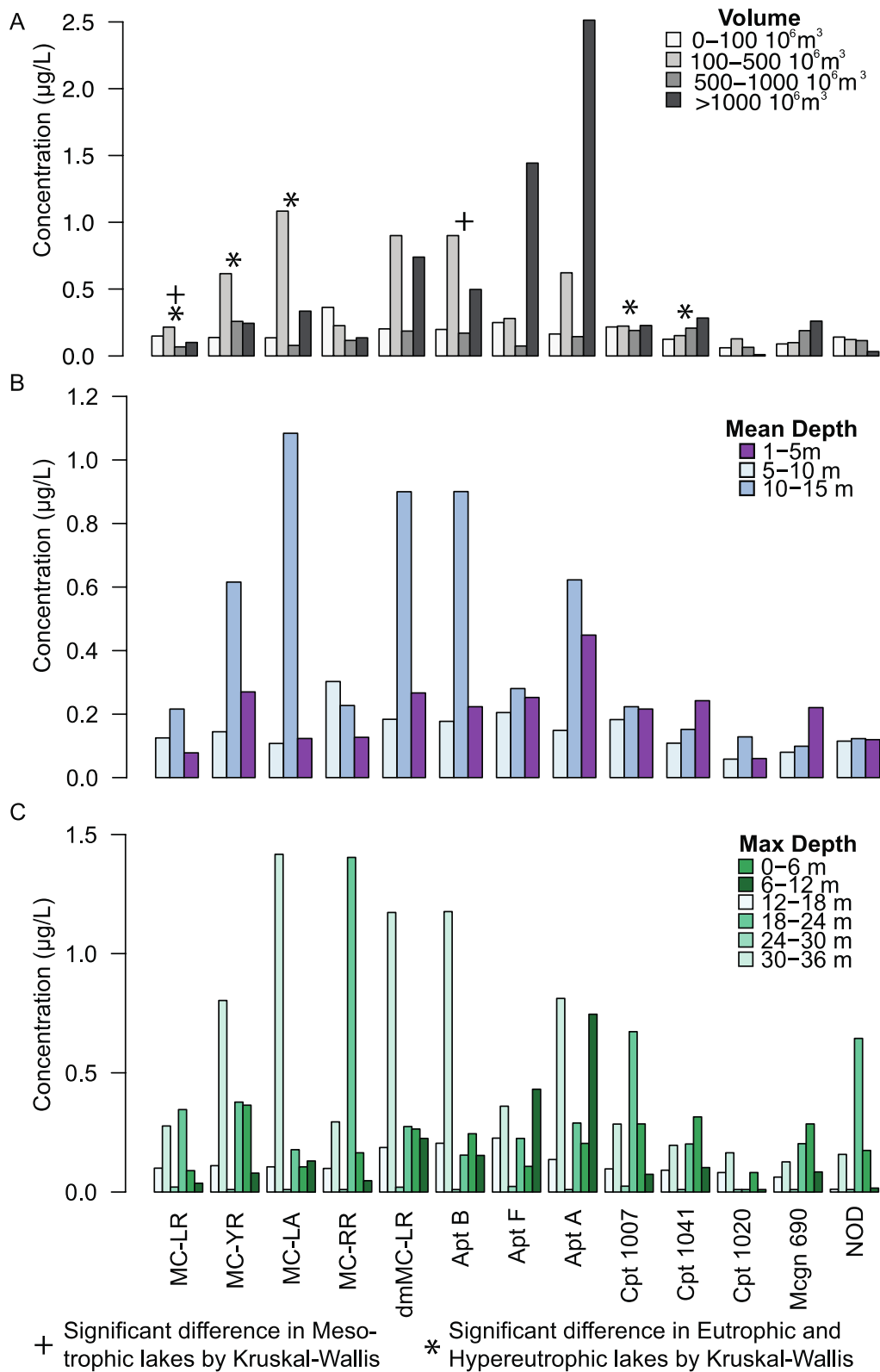
Figure 3.7 illustrates mean concentrations (µg/L) of each cyanopeptide by lake characteristics of A) lake volume ($10^6$ m$^3$), B) mean lake depth (m), and C) max lake depth (m). One or two stars above bars for an individual toxin refer to significant differences in occurrence of that cyanopeptides according to a Kruskal-Wallis in lakes of either mesotrophic or eutrophic/hypereutrophic lakes, respectively. Three stars means that there is a significant difference in peptide occurrence in both mesotrophic, eutrophic, and hypereutrophic lakes. Abbreviations: MC=microcystin; L=leucine; R=arginine; Y=tyrosine; A=alanine; dm=desmethyl ([Dha$^7$]); Apt=anabaenopeptin; Cpt=cyanopeptolin; Mcgn=microginin; NOD=nodularin.

detected at similar levels among lakes, regardless of mean depth. Highest levels of MC-YR, MC-LA, dmMC-LR, and Apt B were detected in lakes with average depths between 10-15 m (Figure 3.7B). A Kruskal-Wallis test did not determine any differences among cyanopeptides concentrations significant in mesotrophic or eutrophic/hypereutrophic lakes.

## Discussion

### Extraction Recovery

This study provides novel evidence of differences in cyanopeptide congener recovery from multiple sample matrices. Our extraction procedures resulted in >56% recovery among all analytes, sample matrices, and lengths of preservation time tested. Use of 70% MeOH has also been shown to be optimal for extraction previously for MCs and Apts [324]. Potential losses in extraction recovery could result from more hydrophobic cyanopeptides binding to storage bottles [325] and being lost during supernatant/solution transfer from containers throughout the extraction processes (from storage to lyophilization; from storage to drying vial; from drying vial to resuspension and transfer to small centrifuge tube, etc.). We chose not to correct reported cyanotoxin concentrations for loss of recovery because isotope dilution quantification was not possible due to a lack of isotopically labeled surrogate standards. This means that the concentrations reported are an underestimate of actual concentrations; therefore, there are potentially higher toxin levels present in surface waters of these lakes..

**Cyanopeptide Preservation in Field Samples**

A potential limitation of shipping samples intercontinentally is the inability to ship samples overnight and in a frozen state; freezing samples until further analysis is standard procedure for water quality analyses. If samples cannot be easily shipped frozen, adding EtOH to 50% provides an opportunity for low-cost shipping. A sample can either be shipped with EtOH included in solution as a preservative, assuring ~50-95% recovery of various cyanobacterial metabolites based on our extraction methods, or a sample could be dried to a residue with the addition of EtOH potentially making the extraction process faster without impacting recovery. EtOH preservation could also be useful for public health researchers or managers when engaging in lake or drinking water monitoring efforts with non-local laboratories and/or needing to confirm tests suggested positive by enzyme-linked immunosorbent assay (ELISA). Our results show that preservation with EtOH to 50% is acceptable for at least three to seven days; extended storage times *in lieu* of freezing should be evaluated by further spike-recovery experiments.

**Cyanopeptide Occurrence and Relations to Lake Characteristics**

This study has completed a cross-continental multi-lake analysis of cyanopeptides using the same analytical instrumentation and validated preservation and extraction methods. We were able to analyze cyanopeptides from 22 lakes, which is smaller than some other studies, but wider in geographic spread. Other studies have primarily focused on individual classes of peptides or cyanotoxins. For example studies of MCs were conducted in 55 German lakes [47], 77 or 39 Michigan (USA) lakes [314,

326]; 80 lakes were sampled for ATX analysis in Germany [47]; and 36 lakes were sampled in Poland for cylindrospermopsin analysis [312]. In comparison, samples were collected from seven Greek lakes and analyzed for MCs, Apts, and anabaenopeptinilides [327]. Not only does the present study expand across three continents, different latitudes and longitudes, it also characterizes occurrence of congeners from five classes of cyanopeptides. Apts, Cpts, and Mcgn are only recently being included in studies on temporal and spatial patterns of cyanopeptides.

I sought to describe cyanopeptide profiles in as many lakes in as large of a geographic range as possible, utilizing newly developed preservation and extraction methods and LC-MS/MS analysis conducted in a single laboratory. Most samples received were from freshwater lakes and reservoirs in North America, which is the primary demographic of scientists involved in GLEON. This is significant, as the geographical range of this study is still limited. Despite this locale majority, we believe this is a spatiotemporal analysis of multiple cyanopeptides and their congeners described using the same storage, extraction, and LC-MS/MS detection techniques to date. By incorporating four or more samples from each lake site, we were able to identify average cyanopeptide levels seasonally at each site.

Some lakes did not have any cyanopeptides detected. It is important to note that other cyanotoxins and biologically active cyanobacterial secondary metabolites may still have been being produced by cyanobacteria in these lakes, but were not at the times these samples were collected. It is also possible that the congeners used for analysis were not those being produced in these lakes. This is the trade-off between high specificity of LC-MS/MS analysis and a lack of available reference standards for all

known and unknown congeners. Other analyses, such as the conversion of MCs to 3-methoxy-2-methyl-4-phenylbutyric acid (MMPB) and measurement via LC-MS/MS are promising for specifically detecting total MCs [328], rather than use of an ELISA which may lack specificity in antibody binding.

Associations were drawn between lake characteristics and cyanopeptide profiles. Variations in MC and Apt concentrations were increased in lakes of hypereutrophic and mesotrophic lakes, respectively, according to Figure 3.5A and determined significant by a Kruskall-Wallis test. Eutrophic or hypereutrophic status is usually regarded as hallmark of FHAB occurrence and resulting cyanotoxin detection in lakes. However, recent studies have detected MCs in oligotrophic or mesotrophic lakes, including lakes in this study [329-331]. However, other factors are interactive with trophic status; though nutrient availability *may* impact likelihood of toxin production or the toxin congeners produced, higher trophic state is not indicative of toxic cyanoHABs.

Two studies in Michigan suggested that dreissenid mussel could be an indicator for MCs in lakes. A survey of 39 oligotrophic or mesotrophic lakes, those colonized by zebra mussels (*D. polymorpha*) had 3.3 times greater MC concentrations and 3.6 times great *Microcystis aeruginosa* biomass compared to lakes without; in other lakes without *D. polymorpha* colonization, *Anabaena* spp. biomass was 4.6 times higher [326]. A larger analysis of 77 lakes showed that MCs in the euphotic zone were 8 times higher in colonized lakes with total P ranging from 5-10 µg/L [314]. Our results are in agreement with these studies, as cyanopeptide concentrations were significantly different based on colonization status by dreissenid mussels; graphical comparison of these differences in Figure 3.5C shows higher MC concentrations measured in colonized lakes. The

presence of mussels in a lake affected by FHABs could be an indicator for higher MC

concentrations and a need for monitoring focus.

The nature of dominant land use surrounding lakes, surface area, and lake

volume all were significantly related to differences in multiple cyanopeptides classes on

individual and total (sum of multiple congeners) levels, both when considering

mesotrophic lakes and eutrophic/hypereutrophic lakes. Though data from the US EPA

National Lakes Assessment were collected from single timepoints and are thus different

from the current study, an analysis of MC concentrations above and below 1.0 μg/L

found that 59.4% and 16.6% of sample concentrations were >1.0 μg/L in lakes

surrounded by agriculture or forested land, respectively [332]. For lesser MC

concentrations <1.0 μg/L, 32.8% and 39.4% of these lakes were surrounded by

agricultural or forested land, respectively. Land use patterns can determine sources of

different types and amounts of nutrients flushing into lakes, which may relate to

cyanobacterial productivity of these types of cyanopeptides.

Surface area and volume would then have an impact on the areas where

cyanobacteria are retrieving these nutrients. Though depth did not seem to be a

statistically relevant determinant of cyanopeptide occurrence among lakes, its impact on

lake shape and resulting lake volume can determine access to nutrients in the

hypolimnion, which can also be dependent on the mixing pattern of a given lake (i.e.

whether a lake is polymictic), and if present cyanobacteria can control their buoyancy.

The impact of lake surface area on cyanopeptide production is more difficult to

determine, given a single sampling point at the center of the lake. It could be suggested

that the highest cyanotoxin concentrations may be detected at the edges of the water body, where cyanobacteria may become more concentrated at shallower depths.

Personnel, time, materials and solvents, and equipment resources ultimately determine the number of lakes in which water quality monitoring and algal toxin analysis can be attained. Some monitoring programs can be made successful using an organized volunteer monitoring program, such as at Lake Lillinonah or others [314]. Access to LC-MS/MS instrumentation and reference materials is also costly, which limits the use of this technology for cyanotoxin analysis. Through this work, we were able to provide cyanopeptides data to 14 colleagues who otherwise may not have received specific LC-MS/MS quantification of these compounds for their water quality studies. In addition, we tested novel storage and extraction techniques to utilize for measuring cyanopeptides from >600 total samples in the same laboratory. Such single-laboratory analysis of 13 cyanopeptides from 36 lakes is unique and has been beneficial for expanding resources for algal toxin analysis to intercontinental colleagues. Additionally, associations between lake characteristics and cyanopeptide concentrations may be used for increased monitoring of specific lakes, provided these associations can be repeated in other lake measurements.

**CHAPTER 4:**

**Cyanopeptides in Four Wisconsin Drinking Water Treatment Plants**

**Introduction**

Freshwater harmful algal bloom (FHAB) development is a concern for freshwater lakes, especially those providing a source of drinking water. Several studies in the last two decades have reviewed successful treatment options for removing cyanotoxins from affected surface waters [1-11]. However, these reviews contain information on some primary research, which tested removal efficiency that may have overestimated cyanotoxin removal and/or used detection methods having high detection limits and low specificity. For example, HPLC with photodiode array detection had limits of detection of 0.2-0.5 ug/L for paralytic shellfish poisons, cylindrospermopsin (CYN), and microcystin (MC) congeners [12, 13], missing low-dose toxin concentrations. Overall, very little is known about the "dosing" of cyanotoxins through drinking water, or how or if these toxins in long-term, low-dose exposures affect public health.

FHABs present other problems to drinking water systems, including preventing floc formation, producing taste and odor compounds [14], and fouling of treatment systems [15]. Their biomass can also be transformed into toxic disinfection byproducts (DBPs) from certain processes [16, 17], adding complexity to treating FHAB-affected surface waters for potable water [18].

Little has been published regarding cyanobacterial toxin occurrence in modern drinking water treatment plants (DWTPs). MCs, liver toxins, have been detected at concentrations of 0.002-7.79 µg/L in finished water from DWTPs in the United States, Canada, Egypt, Serbia, Australia, Czech Republic, Finland, France, Poland,

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Switzerland, Germany and Taiwan in the past two decades [2, 19-32]. However, many of these studies used enzyme-linked immunosorbent assays (ELISAs) for MC detection, which have demonstrated potential for false positive detects in drinking water samples [33]. CYN, another hepatotoxin, was also detected as high as 1.3 and 8.6 µg/L in finished drinking water from two Taiwanese DWTPs and 0.7-2.2 µg/L from distribution system taps in 2007 [23].

Common drinking water treatment processes have demonstrated differences in their efficiency of removal of cyanobacteria and their metabolites from source water. Flocculation, coagulation, and settling treatments remove cyanobacterial cells, but may increase dissolved cyanotoxins after treatment due to cell lysis and inability to remove dissolved toxins [12]. Ultimately, multi-barrier processes using ozonation and hydroxyl radicals (reviewed by [34]) are the most effective processes for removing MCs, anatoxin-a (ATX), and CYN, though these processes, cannot remove saxitoxin (STX) [35] or are selective based on specificity for its removal [3, 36, 37]. Delgado et al. identified activated carbon (AC) as a key component to removing cyanotoxins from finished drinking water, compared to its limited efficiency of removal of other endocrine disruptors and pharmaceutical compounds [38]. Chlorine is effective for removing MCs, CYN, and STX, and potassium permanganate can be used for MC and ATX removal ([39, 40]; reviewed by [3]).

The potential for chlorination to produce DBPs as breakdown products from FHAB toxins creates a separate issue for monitoring finished drinking water contaminants [41-43]. Differences in source water quality - such as variations in cyanobacterial species and densities [12], natural organic matter (NOM) [44] – or

characteristics of the treatment system – including amounts/types of flocculent(s) and activated carbon, pH, and the maintenance quality of the treatment system – can also impede potential cyanotoxin elimination [1, 45, 46].

Cyanobacteria also produce a variety of toxic or otherwise bioactive compounds that, though likely present, go undetected, including anabaenopeptins (Apts), cyanopepotolins (Cpts), and microginins (Mcgns), among others. These compounds receive little acknowledgement for bioactive or toxic effects that they could exert through finished drinking water exposure, resulting in little efforts to set guidelines for tolerable intake doses, despite known physiological effects. Such effects include neurotoxicity in crustacean or fish models [47, 48] and inhibition of serine/threonine proteases [49-52], exopeptidases [53, 54], and protein phosphatases [55].

Epidemiological studies have linked cyanobacterial contamination of drinking water with increased serum liver enzymes; gastrointestinal, skin, and ear symptoms with muscle pains; or liver and colorectal cancers in Serbia, China, Canada, and the United States [25, 56-58]. However, other studies have found no associations between such exposures and disease [59] or used weak variable measures to identify risk to cyanotoxin exposure in determining a lack of association [60-62].

Despite these potentially weak measures (i.e. distance of home zip code to that of a lake), one study found an interesting association between clusters of non-alcoholic liver disease in the United States and location to cyanobacterial blooms based on satellite imagery for the "blue-green" pigment, phycocyanin [62]. A regression analysis showed that risk of non-alcoholic liver disease increased by 0.3% with each 1% increase in bloom coverage in an affected county. Though MC concentrations were very

low in finished drinking water from this study, the long-term low-dose effects of MC

exposures are not as well studied. Nonetheless, low level PP1/2A inhibition is

associated with alterations in cell cycle regulation and development of carcinogenesis

[63]. Scarce information regarding cyanotoxin occurrence in finished drinking water, with

no required testing make identifying at-risk communities potentially more vulnerable to

harmful exposures. This disparity is potentially increased in less developed countries

lacking access to modern water treatment systems. Furthermore, until relevant,

established biomarkers are in place for measuring exposures to cyanotoxins, clinicians

and those trying to study long-term illness resulting from FHABs contamination of

surface waters must use what measures are available as surrogates for assessing

correlation.

We sought to investigate the types of cyanobacterial secondary metabolites,

toxic or otherwise bioactive peptides going into four drinking water plants drawing from

a eutrophic lake and their subsequent occurrence in finished drinking water. In contrast

to previous studies, we used HPLC with tandem mass spectrometry (MS/MS) to track

specific cyanopeptides throughout the DWTP processes and compare removal

efficiencies among modern technologies.


## METHODS

### Drinking Water Source & Sample Collection

Lake Winnebago is the largest inland lake in Wisconsin with an area of 557 km$^2$

and mean depth of 4.7 m. It is the end of a chain of lakes known as the Winnebago Pool

(Lake Butte des Morts, Lake Poygan, Lake Winneconne) and flows north through the

Fox River into Green Bay of Lake Michigan (Figure 4.1), which contributes to it approximately 20% of Lake Michigan's phosphorus [64]. Though classified as hypereutrophic, Lake Winnebago serves as a year-round recreational hub for boating, fishing, and swimming, and as a drinking water source for four municipalities in Oshkosh, Appleton, Neenah, and Menasha. The approximate locations of intakes for these DWTPs are in Figure 4.1. MC occurrence was previously investigated in source water, through treatment stages, and in finished water from Appleton DWTP in 1998 and from Menasha, Neenah, and Oshkosh DWTPs in 1998 and 1999 using ELISA detection [22]. Several upgrades and expansions have been conducted at each of these plants since that study. These DWTPs will be randomly identified as Plants 1, 2, 3, and 4 for the remainder of the text.

Plant treatment trains are illustrated in Figures 4.2 and 4.3; if known, typical chemical doses and contact times are included. DWTPs 1 and 2 use potassium permanganate to treat incoming water, as well as particulate activated carbon (PAC) and DWTP 3 uses sodium permanganate with PAC, while DWTP 4 treats raw water with citric acid and copper sulfate. These chemicals are added as raw water enters pretreatment contact basins. Different chemical additions then occur in each DWTP prior to water flowing into softening, recarbonation, aeration, or flocculation/settling basins, and/or another immediately following. Each plant then uses dual filtration systems after solids have been removed in softening/settling processes. DWTP 1 uses GAC and sand filters, whereas the other three DWTPs use sand/anthracite (dual media filtration; DMF). DWTP 1 is the only plant that uses ultrafiltration membranes following

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Figure 4.1 Lake Winnebago Pool
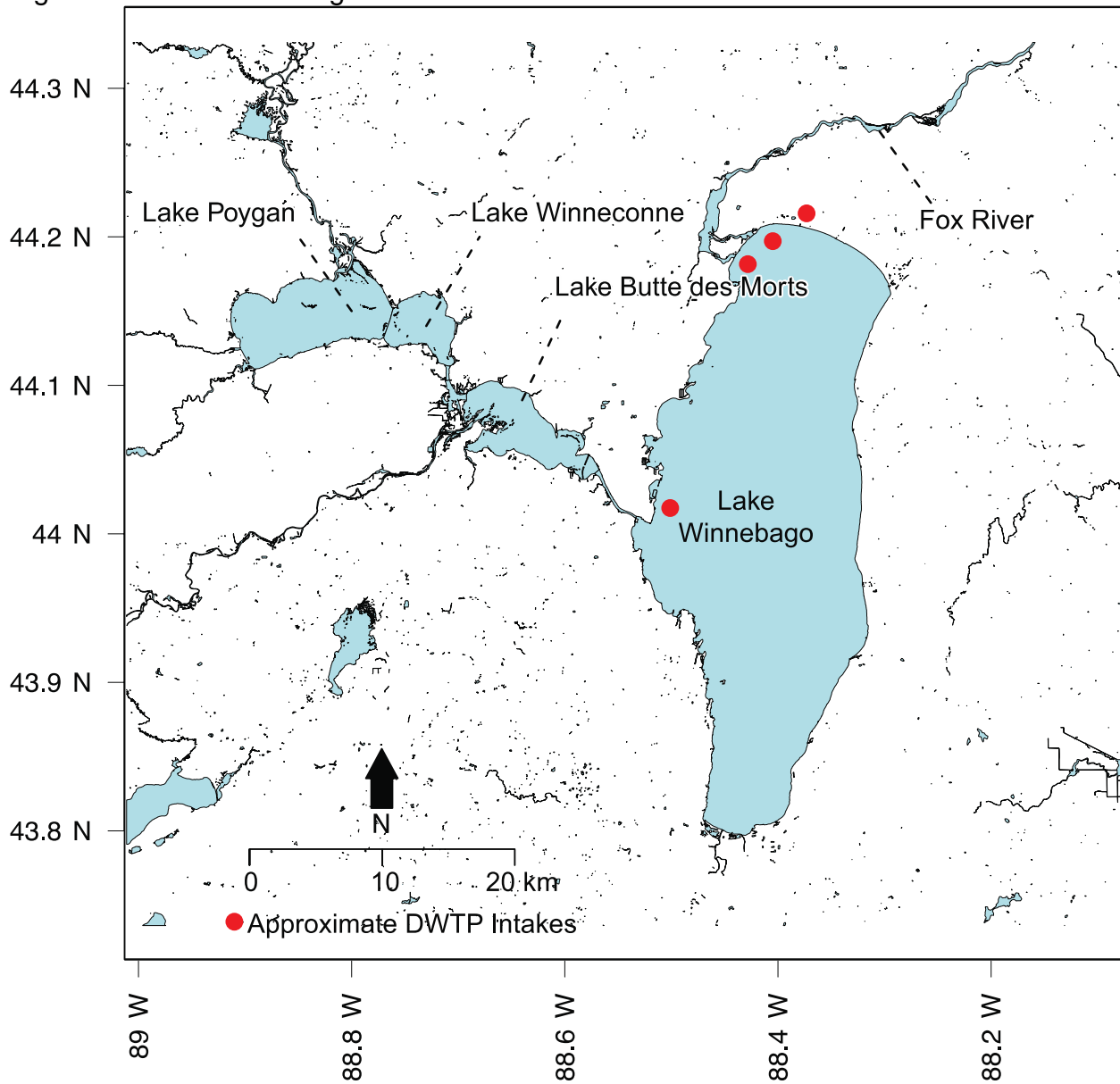


Figure 4.1 illustrates Lake Winnebago and other lakes in the Winnebago Pool. Red circles indicate approximate locations of the four DWTP intakes whose systems are studied in this text. This map was created using data from the United States Geological Survey National Hydrogeography Database (https://viewer.nationalmap.gov/basic/) using the Geospatial Data Abstraction Library (GDAL) in R (*gdal)* and PROJ.4 (*proj4*).

Figure 4.2 Treatment trains at Winnebago DWTPs 1 and 2

**DWTP 1**

Intake
Winnebago 2m
— KMnO4
— PAC (5 mg/L)

Pretreatment
Contact Basin
— Lime (200 mg/L)
— Fe2(SO4)3 (35 mg/L)
(coagulant)

Softening Basin
— CO2

Recarbonation Basin
— PO4
(sequestering agent)

GAC & Sand Filters  (5,040 gpd/sq. ft)

Ultrafiltration
Membranes
(29 million gpd/sq. ft
at 20 C)
— Corrosion inhibitor
— Caustic soda
(pH adjustment)
— NaClO
— Fluoride

Chlorine Contact
Basin
— NH3

Finished Water
Storage

Distribution
(73,000)

**DWTP 2**

Intake
Winnebago 0m
— KMnO4
— PAC

Pretreatment
Contact Basin
— KMnO4

Aeration Basin
— PAC
— Alum
— Polymer

Floc/Settling Basins
— Chlorine
— NH3

Dual Media Filtration

GAC Filtration

UV Treatment
— Chlorine

Chlorine Contact
Basin
— Sodium silicofluoride
— Sodium silicate
— Soda ash
— Chlorine
— NH3

Finished Water
Storage

Distribution
(17,000)

Figure 4.2 illustrates treatment processes at Plants 1 and 2. The water intake pipe depths are indicated at the top of Figure 4.2, followed by downstream treatments indicated along the arrow directions. Red circles indicate sampling points for cyanopeptide analysis. The distribution system feeds water to the approximate populations indicated in the bottom boxes. Abbreviations: $CO_2$=Carbon dioxide; DWTP=Drinking water treatment plant; $Fe_2(SO_4)_3$=Ferric sulfate; GAC=Granular activated carbon; gpd=Gallons per day; $KMnO_4$=Potassium permanganate; m=Meters; mg/L=Milligrams per liter; NaClO=Sodium hypochlorite; $NH_3$=Ammonia; PAC=Particulate activated carbon; $PO_4$=Phosphate; sq. ft=Square feet; UV=Ultraviolet.

Figure 4.3 Treatment trains at Winnebago DWTPs 3 and 4

**DWTP 3**

Intake
Winnebago 1m
— NaMnO4
— PAC

↓

Pretreatment
Contact Basin
— Alum
— Polymer
— Lime

↓

Softening Basin
pH=10.5-11
— CO2

↓

Recarbonation Basin
— Polymer

↓

Dual Media Filtration

↓

GAC Filtration
— Fluoride
— Corrosion inhibitor

↓

UV Treatment
— NaClO
— NH3
— Fluoride

↓

Chlorine Contact
Basin

↓

Finished Water
Storage

↓

Distribution
(25,000)

**DWTP 4**

Intake
Winnebago 4m
— Citric Acid
— Cu(SO2)4

↓

Pretreatment
Contact Basin
— KMnO4

↓

Rapid Mixing
Time=30 s
— PAC
— Alum
— Polymer
— NH3
— Chlorine

↓

Floc/Settling Basins
Time=176 min
— Polymer

↓

Dual Media Filtration (3.1 gpm/sq. ft)

↓

Ozonation
Time=25 min (5.5 mg/L)

↓

GAC Filtration
Time=19.4 min (3.1 gpm/sq. ft)
— Chlorine
— NH3
— Polyphosphate
— Fluoride

↓

Chlorine Contact
Basin

↓

Finished Water
Storage

↓

Distribution
(66,000)

Figure 4.3 illustrates treatment processes at Plants 3 and 4. The water intake pipe depths are indicated at the top of Figure 4.3, followed by downstream treatments indicated along the arrow directions. Red circles indicate sampling points for cyanopeptide analysis. The distribution system feeds water to the approximate populations indicated in the bottom boxes. Abbreviations: CO2=Carbon dioxide; Cu(SO2)4=Copper sulfate; DWTP=Drinking water treatment plant; GAC=Granular activated carbon; gpm=Gallons per minute; KMnO4=Potassium permanganate; m=Meters; mg/L=Milligrams per liter; min=Minute; NaClO=Sodium hypochlorite; NaMnO4=Sodium permanganate; NH3=Ammonia; PAC=Particulate activated carbon; sq. ft=Square feet; s=Seconds; UV=Ultraviolet.

GAC. DWTPs 2 and 3 use ultraviolet (UV) treatment, whereas DWTP 4 uses ozonation (OZO) as its oxidation process. DWTP 4 is unique in that it utilizes DMF, then OZO, and then GAC filtration, compared to the other plants. After filtration and oxidation processes, water is transported to a chlorine contact basin with the addition of chlorine, sodium hypochlorite, ammonia (to form chloramines), fluoride, corrosion inhibitors, and buffering chemicals. Samples were collected from all plants after the chlorine contact basin stage before water is transported to storage and distribution systems.

In 2013, quarter-liter samples were collected on a weekly basis from all four DWTPs between June 26th and September 25th, 2013, as well as daily samples at Plant 4 occurring between August 8th and October 25th, 2013. Samples were collected prior to or after substantial phases in the treatment process from each plant, including raw (incoming, minimally treated) water, dual media filtration (DMF), recarbonation (recombinant effluent = SRE), granular activated carbon (possibly combined with sand filtration; GAC), ozonation (OZO), after dual media filtration and before either GAC filtration or ozonation (FIN at DWTP 3 and DWTP 4, respectively; Figure 4.3). Samples were collected from taps in each DWTP laboratory in amber glass bottles, stored on ice during transportation, and frozen at -20°C until extraction. Sampling points are indicated by red circles in Figures 4.2 and 4.3.

Surface water was monitored on all weekly sampling dates at the approximate intake locations for Plants 1, 2, and 4 with 0 m grab samples collected for cyanopeptide analysis. Samples from these sites were collected in amber glass bottles and stored at -20°C until extraction.

**Cyanopeptide Extraction from Drinking Water Samples**

Cyanopeptide extraction methods varied depending on the stage of drinking water processing. 10 mL of each raw water sample was lyophilized and frozen-thawed in 0.1% formic acid for cell lysis. Cyanopeptides were extracted with 70% methanol (MeOH) and water bath sonication before LC-MS/MS analysis.

Finished drinking water samples were expected to contain much lower concentrations of cyanobacterial toxins and peptides, and were thus concentrated 100-fold. In some cases, 100 mL of sample was not available. The final volume of samples in these cases was measured to a finite value and the resulting concentration accounted for at the time of LC-MS/MS analysis. The majority of intermediate process and finished drinking water samples were lyophilized. The dried mass was washed from the freeze flasks with 10% acetic acid into two Falcon conical tubes per sample. Samples were then subjected to 60-minute freeze-thaws between a -80°C freezer and 50°C water bath. Approximately 15% of weekly samples were not lyophilized because instrumentation was unavailable. Instead, samples were subjected to freeze thaws, as described above, in the original sample bottles and prior to addition of 10 mL acetic acid to 100 mL of sample. Samples were incubated at room temperature with intermittent mixing for one hour, then placed in a heated (50°C) sonicating water bath and concentrated using solid phase extraction via reversed phase Bond Elut C18 cartridges (Agilent Technologies, Santa Clara, CA, USA). Cyanotoxins were eluted from the extraction cartridges with 100% MeOH, following the extraction procedure by Harada et al. [65]. Heat and $N_2$ gas were applied to extracts until a dry residue formed. Dried extracts were re-suspended in 70% MeOH with vortexing and waterbath sonication, and a post-centrifugation supernatant was used for LC-MS/MS analysis.

**Cyanopeptide Analysis via LC-MS/MS**

Cyanopeptide separation and detection was conducted using methods described previously in Chapter 3. Ion transitions for each cyanopeptide are included in Appendix B (Table B1). Cyanotoxin data was integrated using Analyst software.

**Statistical Analyses**

Mean, max, and standard deviation cyanopeptides concentrations were calculated to define basic characteristics of individual and groups of toxins (i.e. Total MCs, Total Cpts, Total Apts) after each treatment stage. In addition, frequency of occurrence and magnitude of cyanopeptide concentrations in terms of chronic exposure levels relevant to public health in finished drinking water are described.

Percent cyanopeptide removed (PCR) was calculated for groups of cyanopeptides: Total MCs (-LR, -RR, -YR, -LA, dm-MC-LR), Total Cpts (1007, 1020, 1041), Total Apts (B, F), Mcgn 690, and NOD. The sum for each Total group was calculated for each process on each sample date. Because some data was missing for some processes on some sample dates, the mean concentration of each cyanopeptide group was calculated across all dates for calculation of PCR. To account for non-detects, measurements equal to 0 ng/L were set to 1 ng/L when calculating PCR. The following equation for calculating PCR was multiplied by 100 as a percentage, in accordance with Mohamed et al. [21]:

$$\frac{(Cyanopeptide\ concentration\ sample\ point\ B - Cyanopeptide\ concentration\ sample\ point\ A)}{Cyanopeptide\ concentration\ sample\ point\ A}$$

Sample point A refers to a step prior in the DWTP train, and sample point B refers to a subsequent sampling point after the next immediate treatment process or one further downstream. Sample points A and B refer to sampling stages within the same DWTP.

**RESULTS**

**Cyanopeptide Detection Throughout Four DWTPs**

A total of 56 raw water samples and 56 finished drinking water samples were analyzed from among the four DWTPs. An overview of cyanopeptide removal in DWTPs is found in Table 4.1, examining differences among congeners and peptides in terms of frequency of detection and concentrations in raw water and finished drinking water.

MC-LR was the most frequently detected in raw water, averaging 85.72% detection in raw water samples. The mean concentration MC-LR detected in raw water was 1365.29 ng/L; in comparison, the maximum cyanopeptide concentration detected was 14,338 ng/L MC-LA. Cpt 1020 was only detected in raw water at DWTP 4. Cyanopeptides were only detected in finished drinking water from DWTPs 3 and 4, specifically MC-YR, Apt B, Cpt 1007, Cpt 1041, and Cpt 1020. The highest cyanopeptide concentration detected in finished drinking water was 7.3 ng/L.

Cyanopeptides have varying levels of hydrophobicity, which could potentially impact ease of removal from surface waters. Therefore, we described mean, standard error, and maximum concentrations of individual MCs, Apts, and Cpts detected at each sampling point in each DWTP train in Tables 4.2, 4.3, and 4.4, respectively. MCs were detected in raw water and the softening and recarbonation basin effluent (SRE) from

Table 4.1. Cyanopeptide Occurrence in Weekly Raw and Finished Water Samples from Four DWTPs

| Peptide | Raw (Untreated) Water (n=56) | | | | | | | Finished Drinking Water (n=54) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | % P1 (n=14) | % P2 (n=13) | % P3 (n=14) | % P4 (n=14) | Mean (ng/L) | Median (ng/L) | Max (ng/L) | % P3 (n=13) | % P4 (n=13) | Mean (ng/L) | Median (ng/L) | Max (ng/L) |
| MC-LR | 92.86 | 100 | 92.86 | 57.14 | 1365.29 | 345 | 12600 | 0 | 0 | 0 | 0 | 0 |
| MC-RR | 71.43 | 76.92 | 100 | 50 | 545.25 | 189 | 7165 | 0 | 0 | 0 | 0 | 0 |
| MC-YR | 35.71 | 53.85 | 35.71 | 9.09 | 68.46 | 0 | 954 | 7.69 | 0 | 0.11 | 0 | 5.79 |
| MC-LA | 7.14 | 15.38 | 35.71 | 21.43 | 441.18 | 0 | 14338 | 0 | 0 | 0 | 0 | 0 |
| dmMC-LR | 21.43 | 0 | 7.14 | 0 | 10.11 | 0 | 153 | 0 | 0 | 0 | 0 | 0 |
| Apt A | 42.86* | 92.3 | 28.57 | 21.43 | 1251.91 | 0 | 7002 | 0 | 0 | 0 | 0 | 0 |
| Apt B | 35.71 | 7.69 | 42.86 | 35.71 | 102.55 | 0 | 813 | 0 | 15.38 | 0.23 | 0 | 6.37 |
| Apt F | 28.57 | 38.46 | 21.43 | 7.14 | 49.26 | 0 | 711 | 0 | 0 | 0 | 0 | 0 |
| Cpt 1041 | 0 | 15.38 | 28.57 | 7.14 | 51.25 | 0 | 689 | 0 | 7.69 | 0 | 0 | 0 |
| Cpt 1007 | 57.14 | 61.54 | 64.29 | 27.57 | 493.91 | 79.05 | 4555 | 0 | 23.08 | 0.26 | 0 | 7.3 |
| Cpt 1020 | 0 | 0 | 0 | 14.29 | 2.64 | 0 | 78 | 0 | 7.69 | 0 | 0 | 0 |

The number of samples extracted from raw and finished water from each DWTP and the total from each treatment are indicated by (n=x) to note different numbers of samples processed in case of lost (broken/thawed) samples. P1 and P2 are excluded for finished drinking water because no cyanopeptides were detected in finished water from Plants 1 or 2; similarly, NOD and Mcgn 690 are excluded because they were not detected. Abbreviations: P1,2,3,4=Drinking water treatment plants 1, 2, 3, 4; %=Percent frequency of cyanopeptide detection

Table 4.2 Weekly microcystin occurrences after each plant treatment phase

| Plant Phase | Microcystin-LR | | Microcystin-RR | | Microcystin-YR | | Microcystin-LA | | dmMicrocystin-LR | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean ± SE | Max | Mean ± SE | Max | Mean ± SE | Max | Mean ± SE | Max | Mean ± SE | Max |
| Plant 1 RAW | 2406.14 ± 927.71 | 12600 | 1028.59 ± 493.36 | 7165 | 120.78 ± 67.89 | 953.5 | 111.79 ± 107.72 | 1565 | 28.75 ± 14.75 | 143.6 |
| Plant 1 SRE | 31.75 ± 23.15 | 342 | 0 | 0 | 0 | 0 | 47.21 ± 40.40 | 0 | 0 | 0 |
| Plant 1 GAC | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Plant 1 TAP | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Plant 2 RAW | 1969.27 ± 670.13 | 8760 | 684.81 ± 174.24 | 1785 | 108.8 ± 37.76 | 417 | 24.92 ± 16.89 | 205.5 | 0 | 0 |
| Plant 2 FIN | 1.06 ± 1.01 | 12.7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Plant 2 GAC | 1.12 ± 0.73 | 8.1 | 0 | 0 | 0.43 ± 0.41 | 5.59 | 0 | 0 | 0 | 0 |
| Plant 2 TAP | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Plant 3 RAW | 1025.79 ± 213.56 | 2438.8 | 438.58 ± 110.29 | 1648.2 | 40.46 ± 17.17 | 207.6 | 1576.49 ± 1069.55 | 14338 | 10.96 ± 10.56 | 153.5 |
| Plant 3 FIN | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Plant 3 GAC | 2.31 ± 1.53 | 13.8 | 2.31 ± 1.53 | 18.5 | 0.55 ± 0.53 | 7.74 | 0 | 0 | 2.31 ± 1.53 | 18.5 |
| Plant 3 TAP | 0 | 0 | 0 | 0 | 0.45 ± 0.43 | 5.79 | 0 | 0 | 0 | 0 |
| Plant 4 RAW | 103.10 ± 31.69 | 441.5 | 38.98 ± 12.43 | 163 | 6.68 ± 6.44 | 93.5 | 21.79 ± 13.91 | 192.5 | 0 | 0 |
| Plant 4 DMF | 3.13 ± 10.36 | 5.09 | 0.42 ±1.41 | 5.09 | 0 | 0 | 0 | 0 | 0 | 0 |
| Plant 4 OZO | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Plant 4 GAC | 0 | 0 | 0 | 0 | 7.55 ± 7.23 | 90.6 | 0 | 0 | 0 | 0 |
| Plant 4 TAP | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Table 4.2 concentrations are expressed in ng/L. Plant phases are according to the treatment process schemes in Figures 4.2 and 4.3. Abbreviations: A=Alanine; dm=Desmethyl (Dha[7]); DMF=Dual media filtration; FIN=Filter inlet; GAC=Granular activated carbon; L=Lysine; OZO=Ozonation; R=Arginine; RAW=Incoming (raw) water to drinking water treatment plants; SE=Standard error; SRE=(South) recarbonation effluent; TAP=Finished drinking water; Y=Tyrosine

Table 4.3 Weekly cyanopeptolin occurrences after each plant treatment phase

| Plant Phase | Cyanopeptolin 1007 | | Cyanopeptolin 1020 | | Cyanopeptolin 1041 | |
|---|---|---|---|---|---|---|
| | Mean ± SE | Max | Mean ± SE | Max | Mean ± SE | Max |
| Plant 1 RAW | 743.84 ± 361.92 | 4555 | 0 | 0 | 0 | 0 |
| Plant 1 SRE | 0.42 ± 0.41 | 5.92 | 0 | 0 | 39 ± 37.58 | 546 |
| Plant 1 GAC | 0 | 0 | 0 | 0 | 0 | 0 |
| Plant 1 TAP | 0 | 0 | 0 | 0 | 0 | 0 |
| Plant 2 RAW | 883.94 ± 298.75 | 3095 | 0 | 0 | 94.88 ± 61.86 | 655.5 |
| Plant 2 FIN | 0 | 0 | 0 | 0 | 0 | 0 |
| Plant 2 GAC | 0 | 0 | 0 | 0 | 0 | 0 |
| Plant 2 TAP | 0 | 0 | 0 | 0 | 0 | 0 |
| Plant 3 RAW | 312.10 ± 112.99 | 1608 | 0 | 0 | 100.5 ± 53.65 | 688.5 |
| Plant 3 FIN | 0 | 0 | 0 | 0 | 0 | 0 |
| Plant 3 GAC | 10.22 ± 9.35 | 136.2 | 2.31 ± 1.53 | 18.5 | 0 | 0 |
| Plant 3 TAP | 0 | 0 | 0 | 0 | 0 | 0 |
| Plant 4 RAW | 24.35 ± 10.79 | 120.6 | 10.36 ± 6.80 | 77.6 | 12.75 ± 12.29 | 178.5 |
| Plant 4 DMF | 0.97 ± 0.93 | 11.6 | 0 | 0 | 0 | 0 |
| Plant 4 OZO | 0 | 0 | 0 | 0 | 0 | 0 |
| Plant 4 GAC | 0 | 0 | 0 | 0 | 15.68 ± 15.01 | 188.1 |
| Plant 4 TAP | 1.06 ± 0.69 | 7.3 | 0 | 0 | 0 | 0 |

Table 4.3 concentrations are expressed in ng/L. Abbreviations: DMF=Dual media filtration; FIN=Filter inlet; GAC=Granular activated carbon; OZO=Ozonation; RAW=Incoming (raw) water to drinking water treatment plants; SE=Standard error; SRE=(South) recarbonation effluent; TAP=Finished drinking water

Table 4.4 Weekly anabaenopeptin occurrences after each plant treatment phase

| Plant Phase | Anabaenopeptin A | | Anabaenopeptin B | | Anabaenopeptin F | |
|---|---|---|---|---|---|---|
| | Mean ± SE | Max | Mean ± SE | Max | Mean ± SE | Max |
| Plant 1 RAW | 6173.57 ± 4266.52 | 33300 | 194.21 ± 73.94 | 758.5 | 90.37 ± 51.18 | 711 |
| Plant 1 SRE | 0 | 0 | 0 | 0 | 0 | 0 |
| Plant 1 GAC | 0 | 0 | 0 | 0 | 0 | 0 |
| Plant 1 TAP | 0 | 0 | 0 | 0 | 0 | 0 |
| Plant 2 RAW | 503.83 ± 95.10 | 1123.5 | 62.54 ± 216.64 | 813 | 74.36 ± 35.99 | 475.8 |
| Plant 2 FIN | 0 | 0 | 0 | 0 | 0 | 0 |
| Plant 2 GAC | 12.74 ±1 2.24 | 165.6 | 0 | 0 | 0 | 0 |
| Plant 2 TAP | 0 | 0 | 0 | 0 | 0 | 0 |
| Plant 3 RAW | 703.18 ± 489.99 | 7001.5 | 91.05 ± 33.45 | 419 | 29.72 ± 15.29 | 155.5 |
| Plant 3 FIN | 0 | 0 | 0 | 0 | 0 | 0 |
| Plant 3 GAC | 2.31 ± 1.53 | 18.5 | 2.31 ± 1.53 | 18.5 | 2.31 ± 1.53 | 18.5 |
| Plant 3 TAP | 0 | 0 | 0 | 0 | 0 | 0 |
| Plant 4 RAW | 34.46 ± 20.06 | 233.5 | 59.56 ± 22.74 | 220.5 | 4.37 ± 4.21 | 61.2 |
| Plant 4 DMF | 0 | 0 | 0 | 0 | 0 | 0 |
| Plant 4 OZO | 0 | 0 | 0 | 0 | 0 | 0 |
| Plant 4 GAC | 0 | 0 | 0 | 0 | 0 | 0 |
| Plant 4 TAP | 0 | 0 | 0.97 ± 2.28 | 6.37 | 0 | 0 |

Table 4.4 concentrations are expressed in ng/L. Abbreviations: DMF=Dual media filtration; FIN=Filter inlet; GAC=Granular

activated carbon; OZO=Ozonation; RAW=Incoming (raw) water to drinking water treatment plants; SE=Standard error;

SRE=(South) recarbonation effluent; TAP=Finished drinking water

DWTP 1, but not in GAC-treated or finished tap water at this plant (Table 4.2); Apts and Cpts also followed this trend (Tables 4.3, 4.4). dmMC-LR was only detected in raw water at DWTPs 1 and 3 and after GAC filtration in DWTP 3. In DWTP 2, MCs were detected in the DMF effluent/GAC filter inlet (FIN) (Table 4.2); however, Apts and Cpts were only detected in raw water and not after any treatment phases at DWTP 2 (Tables 4.3, 4.4). MCs were not detected in the GAC filter inlet (FIN) samples, but MC-YR was detected in the further downstream GAC and TAP water samples. This increase after GAC treatment also occurred for Cpt 1007 and Apt B in Plant 3, but neither of these compounds were detected in Plant 3's finished drinking water (Tables 4.3, 4.4). Apt B was detected in raw and finished water in DWTP 4, but not after any intermediate treatment processes. In DWTPs 3 and 4, cyanopeptides were not detected after OZO processes; However, all MCs and Cpt 1041 were detected after GAC treatment, and Apt B, Cpt 1007, and MC-YR were detected in finished water at either of these plants.

**Daily Sampling at DWTP 4**

Because samples were collected on more sample dates from DWTP 4, there were more opportunities to measure cyanopeptide occurrence throughout the drinking water treatment process. Thus, statistics for detection of cyanopeptides throughout DWTP among all dates are included in Table 4.5. NOD was not detected in any of the processes at DWTP 4 on any of the dates sampled. The highest Apt concentrations measured in raw water occurred on 9/5/13. Cpt maximums in raw water did not all occur on the same date, but all occurred in September. The highest raw water MC-LR and MC-RR concentrations occurred on 9/24/13 and 10/3/13, respectively, dates that were

Table 4.5 Cyanopeptides from All (Weekly and Daily) Samples at DWTP 4

| Toxin | Stat (ng/L) | RAW (n=70) | DMF (n=53) | OZO (n=53) | GAC (n=52) | TAP (n=71) |
|---|---|---|---|---|---|---|
| Apt A | Mean ± SE | 17.47 ± 6.2 | 0 | 0 | 0 | 0 |
| | Max | 241 | 0 | 0 | 0 | 0 |
| Apt B | Mean ± SE | 116.22 ± 15.16 | 0 | 0 | 0 | 0.66 ± 0.22 |
| | Max | 550 | 0 | 0 | 0 | 6.71 |
| Apt F | Mean ± SE | 17.11 ± 6.58 | 0 | 0 | 0 | 0 |
| | Max | 284 | 0 | 0 | 0 | 0 |
| Cpt 1007 | Mean ± SE | 130.97 ± 22.46 | 0.74 ± 0.36 | 0 | 0.51 ± 0.51 | 1.44 ± 0.45 |
| | Max | 723 | 11.6 | 0 | 27.2 | 21.5 |
| Cpt 1020 | Mean ± SE | 1.03 ± 1.03 | 0 | 0.29 ± 0.20 | 0 | 0.28 ± 0.28 |
| | Max | 73 | 0 | 8.65 | 0 | 20.2 |
| Cpt 1041 | Mean ± SE | 7.15 ± 3.15 | 0 | 0.37 ± 0.26 | 0.28 ± 0.28 | 0.44 ± 0.44 |
| | Max | 174 | 0 | 0 | 14.9 | 31.6 |
| dmMC-LR | Mean ± SE | 6.39 ± 4.63 | 0 | 0 | 0 | 0 |
| | Max | 283 | 0 | 0 | 0 | 0 |
| Mcgn 690 | Mean ± SE | 10.76 ± 7.68 | 0 | 0 | 0 | 0.56 ± 0.49 |
| | Max | 452 | 0 | 0 | 0 | 34.6 |
| MC-LA | Mean ± SE | 31.37 ± 7.88 | 0 | 0 | 0 | 0 |
| | Max | 210 | 0 | 0 | 0 | 0 |
| MC-LR | Mean ± SE | 575.93 ± 89.43 | 2.71 ± 1.11 | 0 | 0.41 ± 3.37 | 0 |
| | Max | 2690 | 39.1 | 0 | 21.6 | 0 |
| MC-RR | Mean ± SE | 234.15 ± 42.89 | 0.21 ± 0.21 | 0 | 0 | 0.25 ± 0.17 |
| | Max | 1805 | 6.17 | 0 | 0 | 9.84 |
| MC-YR | Mean ± SE | 83.80 ± 23.28 | 0 | 0 | 0 | 0 |
| | Max | 870 | 0 | 0 | 0 | 0 |

Abbreviations: A=Alanine; Apt=Anabaenopeptin; Cpt=Cyanopeptolin; dm=Desmethyl

(Dha[7]); DMF=Dual media filtration; GAC=Granular activated carbon; L=Lysine;

MC=Microcystin; Mcgn=Microginin; OZO=Ozonation; R=Arginine; SE=Standard error;

TAP=Finished drinking water; Y=Tyrosine.

not captured by weekly monitoring. Only Apt B, Cpts 1007, 1020, and 1041, Mcgn 690, and MC-RR were detected in finished drinking water at concentrations ≤34.6 ng/L.

**Calculating Removal Efficiency of Cyanopeptides**

Cyanopeptide removal efficiency was evaluated using a calculation for Percent Cyanopeptide Removed (PCR) after each treatment phase for each group of total peptides. PCRs for each peptide group and treatment phase at each DWTP are presented in Table 4.6. Negative values for PCR indicate removal to -100 or 100% removed; positive values indicate positive differences in cyanopeptide concentrations from the previous treatment phase.

All treatments in DWTP 1 had removal efficiencies greater than 94.70% among the three cyanopeptide groups. Because cyanopeptides were fully removed after GAC treatment, PCR calculated from the GAC and TAP samples was 100%. Apts were fully removed in DWTP 1 after sedimentation, filtration, and recarbonation (SRE). In DWTP 3, PCR in the FIN samples (after dual media filtration, before GAC treatment) was 100%. However, PCR increased after GAC treatment, indicating elevated cyanopeptide concentrations from the prior treatment phase among all three toxin groups (Table 4.6). PCR for Apts and MCs reverted back to 100% removal efficiency in remaining treatments before the finished drinking water stage (TAP), but PCR for Cpts was still reduced from full efficiency at -96.25%. DWTP 4 had four PCR values calculated for an additional sampling point along the DWTP process train. PCRs for MCs and Cpts were reduced from full efficiency after DMF treatment at 97.92 and 97.96%, respectively. OZO and GAC processes fully removed cyanopeptides compared to the prior

Table 4.6 Percent Cyanopeptide Removal (PCR) After Major Treatment Phases

| Plant | Phase | PCR MCs | PCR Apts | PCR Cpts |
|-------|-------|---------|----------|----------|
| DWTP 1 | SRE | -97.86 | -100 | -94.70 |
| | GAC | -100 | -100 | -100 |
| | TAP | -100 | -100 | -100 |
| DWTP 2 | FIN | -99.96 | -100 | -100 |
| | GAC | -99.96 | -100 | -100 |
| | TAP | -100 | -100 | -100 |
| DWTP 3 | FIN | -100 | -100 | -100 |
| | GAC | 1088.86 | 12427.86 | 6821.43 |
| | TAP | -100 | -100 | -96.25 |
| DWTP 4 | DMF | -97.92 | -100 | -97.96 |
| | OZO | -100 | -100 | -100 |
| | GAC | -100 | -100 | -100 |
| | TAP | -76.60 | 207.15 | -54.40 |

Abbreviations: Apts=Anabaenopeptins; Cpts=Cyanopeptolins; DWTP=Drinking water treatment plant; FIN=Filter inlet; GAC=Granular activated carbon; MCs=Microcystins; PCR=Percent Cyanopeptide Removal; SRE=(South) recombinant effluent; TAP=Finished drinking water. Negative values indicate removal, whereas positive values indicate increases in cyanopeptide concentrations after treatments, rather than removal. When a previous phase averaged 0 ng/L, 0.0001 was used as a replacement value to complete the division calculation.

treatments; however, PCR values in final TAP water were positive, indicating some final treatments caused detects of increased cyanopeptide concentrations in finished drinking water compared to the previous phase.

Changes in mean cyanopeptide groups (MCs, Apts, Cpts) throughout the DWTP processes are illustrated in Figure 4.4. These plots compare cyanopeptide concentrations in relation to new US EPA guidelines for MCs in finished drinking water of 300 ng/L for children <6 years old and 1600 ng/L for children >6 years old and adults [66]. Cyanopeptides were only detected at or near these concentrations in raw water from all plants. All plants showed a substantial decrease in peptide concentrations after the first treatment steps, but relative increases are visualized after GAC process in DWTPs 2 and 3 and in finished (TAP) drinking water in DWTP 4.

**DISCUSSION**

In this study, we sought to investigate types and concentrations of cyanopeptides detected in raw and finished drinking water from four modern DWTPs, as well as at intermediate stages of treatment. It is important to characterize this information in regard to the new guidelines from the US EPA in the form of a 10-day Health Advisory for MCs and CYN. CYN and ATX were not measured in this study because they were either not detected or not measured above 10000 ng/L, respectively, in Lake Winnebago water during the 2013 study period. We found that all four DWTPs removed MCs, Apts, and Cpts to levels far below the young child and adult health advisory guidelines for MCs.

Figure 4.4 Changes in mean cyanopeptide concentrations throughout drinking water
treatment processes
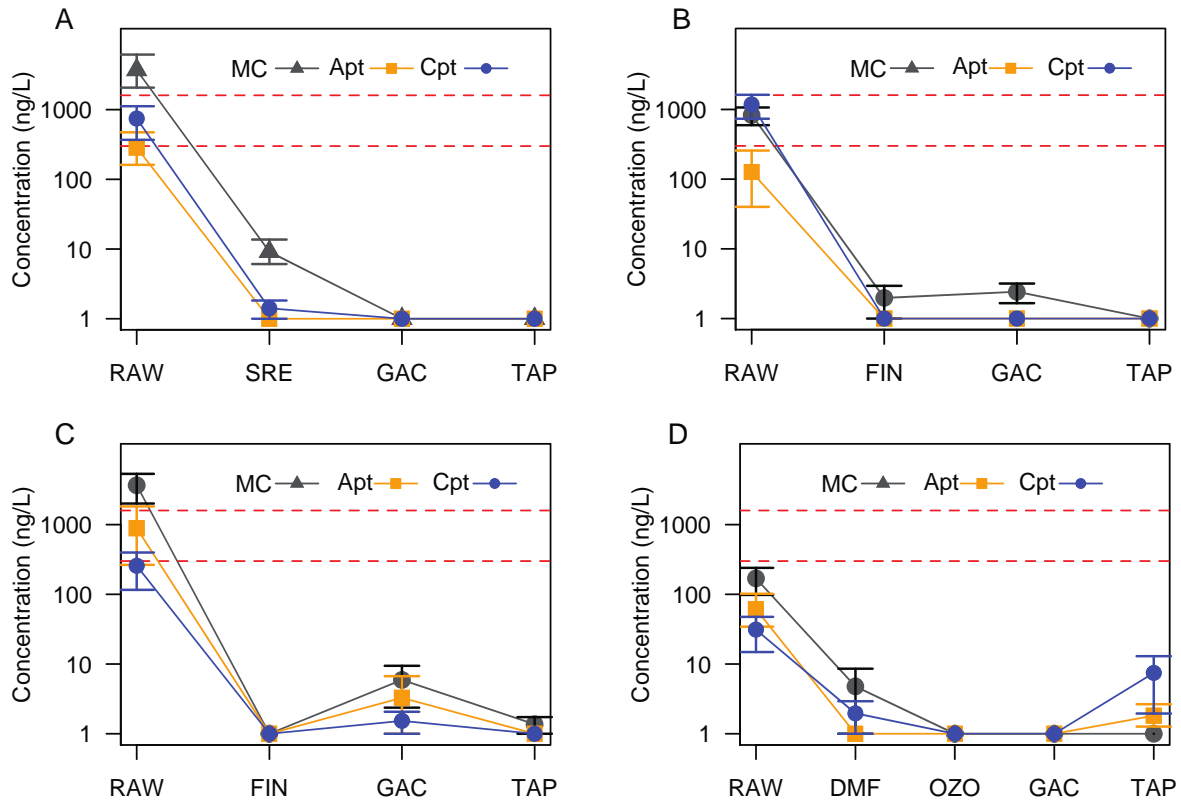


Figure 4.4 uses different shapes and colors to represent each cyanopeptide group.
Each point represents a mean concentration of the total cyanopeptide group with
resulting standard error bars. The red dashed lines in each plot represent the child- and
adult-recommended limits for MCs in finished drinking water based on a 10-day Health
Advisory by the US EPA [66]. A: DWTP 1; B: DWTP 2; C: DWTP 3; D: DWTP 4.

Though very few dates had detects of cyanopeptides in finished drinking water, we did detect increases in cyanopeptide concentrations at a few intermediate phases of the drinking water treatment process, particularly after GAC treatment.

Evidence has been presented for differences in adsorption to GAC among MC variants based on hydrophobicity. Newcombe et al. suggested MC-LA should adsorb better based on its lower molecular weight and hydrophobicity compared to MC-LR, MC-RR, and MC-YR, [67]. However, we observed increased MC-LR, MC-RR, and MC-YR concentrations, as well as increased Apts and Cpts, after GAC treatment in DWTP 3. We did not detect high concentrations of cyanopeptides prior to GAC treatment, suggesting that intracellular toxin was released upon GAC treatment or that dissolved toxin was released from GAC adsorption as a result of new water flowing through the filters. This resulted in positive removal efficiency calculation, rather than a negative removal efficiency calculation.

For the majority of finished drinking water samples, we did not detect any cyanopeptides in the finished extracts. One important thing to note is that we did not assess recovery between the ultrafiltration process at DWTP 1 and chlorination; therefore, we only know that the combined PCR was -100% for these processes. This is an example that should be considered when examining PCR of intermediate phases within these plants.

The recovery of toxins detected in each sample is also potentially dependent on final chlorine concentration and contact time prior to sample collection and freezing, as well as the final pH of the sample [68]. Variation in chlorine dose and time to freezing could have resulted in changes in contact time and resulting removal of cyanopeptides

without capturing actual peptide concentrations entering the storage/distribution systems, though these procedures should have been standardized. Standard operating procedures for sample procurement and measurement will be essential for the future if regulations are passed to monitor for cyanotoxins in finished drinking water.

No guidelines have been set or recommended by government agencies or researchers for other bioactive peptides in finished drinking water. It is likely that until there is more evidence for Cpts to exert toxic effects in animal models that they will not be considered for regulation or monitoring, until there is enough information that they may be placed on the EPA Candidate Contaminant List. More experiments are necessary for tracking the occurrence of these peptides throughout drinking water processes, as well as assessing the full spectrum of effects from mixtures of cyanotoxins and bioactive peptides.

Combined health effects from exposures to cyanotoxins, biologically active cyanopeptides, other unknown chemicals, and DBPs in finished drinking water are unknown. DWTP monitoring focuses on limits of known, regulated DBPs and pathogenic markers. The summation of an individual's lifetime exposures to these compounds as one's drinking water "exposome" [69] makes investigating health effects of these compounds at natural concentrations necessary, but difficult, to explore. Zebrafish (*Danio rerio*) have proven to be excellent models for assessing toxic effects from algal [70] and other compounds, particularly at sensitive time windows of early development. Though some studies using purified MC reference materials have not detected any effects on *D. rerio* embryos [71, 72], other studies have identified cellular effects of toxicity at lower dose exposures to MCs, intensified by inorganic pollutants

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and nutrients [73], as well as toxicity with exposures to bloom samples [70, 71, 74, 75]. Most of these experiments also exposed *D. rerio* embryos to much higher concentrations than those found in ambient lake water or finished drinking water (i.e. >100 μg/L). However, these animal models may provide a unique perspective for studying effects of long-term, low-dose toxin concentrations combined with other pollutants potentially present in finished drinking water, and how these contaminants affect cellular mechanisms of disease.

This study is the first to analyze concentrations of bioactive peptides through drinking water treatment processes at modern DWTPs. It also provides further information regarding another season of cyanotoxin occurrence in comparison to the previous study conducted at these plants 15 years prior [22], after several mechanical updates had taken place at each facility. Our data show that MCs, Apts, and Cpts were reduced to low (5-34.6 ng/L) or undetectable levels by conventional treatment processes including softening and recarbonation (SRE), dual media filtration (DMF), ozonation (OZO), granular activated carbon (GAC), and chlorination/chemical addition (TAP) prior to distribution. These results are comparable to the previous study of MCs by ELISA, yet higher levels of Cpts and Mcgn 690 were detected in finished drinking water compared to previously measured levels of MCs.

**CHAPTER 5:**

**Conclusions and Future Directions for the Study of CyanoHABs and**

**Cyanobacterial Secondary Metabolites**

This dissertation contributes information to the understanding of the succession of toxic cyanobacteria in lakes, global patterns of cyanotoxin occurrence in lakes, and the risk of algal toxin exposure from traditional drinking water systems in developed countries. Specifically, this work utilized a combination of long-term datasets to answer ecological questions regarding cyanobacterial perseverance, incorporated unique extraction and LC-MS/MS detection methods to measure cyanotoxins from a variety of sample matrices, and provided novel data regarding occurrence of cyanotoxins in drinking water from a hypereutrophic lake.

Despite these efforts and the work of other scientists, more research is necessary to fully understand the ecology of toxin production in cyanobacteria and accurately protect public health with recreational and drinking water exposures while improving management efforts. In this chapter, I describe recent changes in public health practices in response to evidence from scientific data and cyanotoxin drinking water exposures from a developed drinking water system, recent advances in knowledge regarding toxin production mechanisms and expanded ideas of toxic effects from cyanopeptides, as well as suggested future directions for the field.

A current focus regarding safety of freshwater harmful algal blooms (FHABs) involves providing early warning signs or indicators for the public of toxic blooms for recreational areas and drinking water. Previous guidelines suggested avoiding swimming in water if a person could not see their feet below the water surface,

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suggesting that unless the water is very turbid or opaque, little risk is present. To measure such risk, Hollister and Kreakle recently suggested that a 50% chance of exceeding MC advisory concentrations of 0.3, 1, 1.6, and 2 µg/L could be foreseen by measuring 23, 68, 84, and 104 µg/L chlorophyll-*a* (chl-*a*), respectively [1]. This association was drawn by assessing probability between single measurements of MCs and chl-*a* in lakes through the 2007 National Lakes Assessment, which is based on spatial seasonal differences rather than considering individual patterns in specific lakes.

In fact, weekly chl-*a* and MC data from six sites in northern Lake Winnebago in summer of 2013 show that suggested chl-a concentrations from this National Lakes Assessment dataset analysis far exceed these recent advisory levels (Figure 5.1). Furthermore, additional data collected from Lake Lillinonah's Water Quality Monitoring Volunteer program and analyzed for cyanotoxins through the GLEON Cyanotoxin Project (Chapter 3) suggests that elevated MC levels can be present even when visible algal mass is not observed (Table 5.1).

Thankfully, public health documentation is changing from the notion of still swimming in water that may appear clear to advising more caution. A recent guide distributed by the Wisconsin Department of Health Services regarding FHABs suggests rinsing off in case of personal or pet exposure to FHAB affected waters, checking beach and water quality postings, getting medical treatment in case of suspected poisoning, avoiding water recreation and drinking (persons and pets) when discolored water, foams, scums or algal mats are observed, and not letting pets lick off algae [3]. This updating and translation of science into factual, relatable knowledge is key to public health practice of protecting from FHABs. However, a key component to this practice is

Figure 5.1 Microcystins and Chlorophyll-*a* in Northern Lake Winnebago
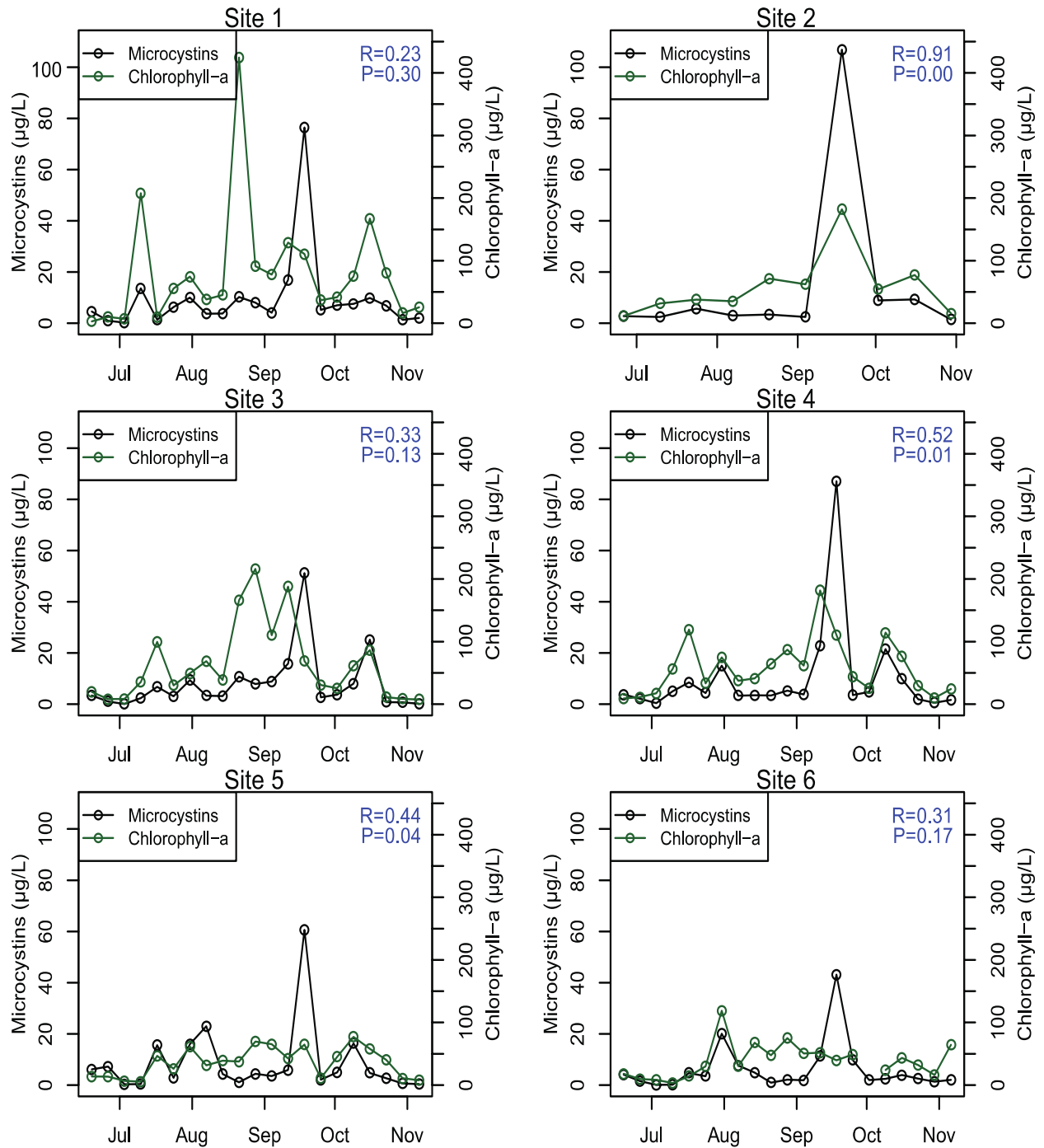
Figure 5.1 shows weekly (or biweekly in 5.1b) microcystin (MC) and chl-*a* concentrations. R- and p-values to measure correlation of these variables are results from *cor.test* in R. MCs were measured following toxin and liquid chromatography with tandem mass spectrometry methods in Chapter 3,and chl-*a* was measured spectrophotometrically after extraction with acetone [2] This study was conducted as assessment for a potential new drinking water plant intake.

Table 5.1 Lillinonah Samples with Elevated Microcystins Not Associated with Poor

Water Quality

| Sample (Date/Sample Location) | Microcystins (µg/L) |
|---|---|
| JK002 (6/30/2012 Lover's Leap) | 38.03 |
| JK047 (6/17/2010 Shepaug) | 977.52 (some colored material in bottle but secchi = 3.25) |
| JK046 (7/1/2010 Shepaug) | 113 |
| JK080 (8/16/2011 Shepaug) | 288.64 |

Table 5.1: Poor water quality measures were assessed by sampling volunteers,

including lowered secchi depth, notes of algal mass, or decreased recreation potential.

This table includes samples from two sites in Lake Lillinonah (Lover's Leap and

Shepaug); Shepaug sites usually have lower nutrient concentrations and higher water

clarity compared to three other sites sampled on Lake Lillinonah. (Descriptions of

sample sites and water quality measures from Jennifer Klug, PhD, Fairfield University).

effectively communicating this information. Placing permanent signage at affected lakes assumes the general public will be attentive to the message and heed the warnings, if necessary. Better active communication with the general public is necessary to convey the risks of swimming in water with blue-green algae or the potential for cyanobacterial growth, as well as in instances where cyanotoxins may exceed the US EPA Health Advisory limits in finished drinking water. The former scenario presents an opportunity for collaboration among public health professionals, scientists, natural resource departments, and lake stakeholder groups to pool resources and not only provide multiple means of communication, but also assist with monitoring strategies.

Key lessons regarding public health communication of FHABs resulted from a 2014 Drinking Water Advisory (DWA) issued for several counties in Ohio retrieving surface water from Lake Erie containing high amounts of cyanobacterial biomass and extracellular toxins [4]. The Great Lakes provide drinking water for at least 35 million people in the United States and Canada [5]. Communities outside of the Great Lakes watershed rely on inland lakes that are smaller, likely shallower, and of higher trophic status. However, results from Chapter 3's semi-global survey of cyanopeptides in lakes show that eutrophication is unnecessary for cyanotoxin occurrence in lakes. The following examples present ideas regarding planning and assessing communication strategies following drinking water advisories for affected lake communities large and small.

A Community Needs Assessment for Public Health Emergency Response (CASPER) survey was conducted in Lucas County, Ohio, affected by the 2014 DWA [4]. This assessment was conducted to evaluate communication strategies, water

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exposures, and household needs in response to the do-not-drink advisory. Survey results found that both "traditional" and "nontraditional" media outlets were important in communicating details to the public regarding such announcements; 41.8% and 54.5% of acknowledgement of the advisory resulted from social media and word-of-mouth, respectively. Multiple respondents reported drinking and brushing teeth with municipal water during the advisory period and/or becoming ill, while others refrained from using municipal water even after the advisory was lifted. This suggests that more focus on community education is also important not only during, but after such advisories are lifted.

Another recent article described how the Water Safety Planning (WSP) approach by the World Health Organization could help prevent such risk to consumers in Lake Erie-based drinking water treatment plants (DWTPs) [6]. The authors provided information on how regular assessments of surface waters, equipment, and relevant risks, identifying water quality standards, establishing a stakeholder and resource team, and incorporating key plans for control measures, effectiveness evaluation, documentation and management, supporting programs, and periodic reviews and revisions of such plans could prevent future cyanotoxin exposures from this lake and others. This article also describes inherent challenges to creating these types of plans, which may not be feasible in smaller communities (or those that lack a federal spotlight). Included within this description is also the idea of reframing lake eutrophication as a *public health problem*, rather than an ecological/limnological problem. Moving forward from such an idea can involve extension of best management practices (BMP) and

evaluation of DWTP processes drawing water from FHAB-affected lakes to consumer toxin exposures in finished drinking water based on community education and WSP.

Regarding BMP, results from Chapter 2's long-term study of bloom-forming cyanobacteria in Lake Mendota did not indicate any decreases in total cyanobacterial biovolume in the last 20 years, despite statewide nutrient loading reductions to achieve phosphorus (P) water quality standards (NR102 and NR217). A meta-analysis of 35 long-term (5-35 years) lake studies demonstrated equilibration to re-oligotrophication P values after 10-15 years of reduced nutrient loading [7]. P loading from point and nonpoint source runoff has been adding to Lake Mendota since the 1800's, which has internally contributed to its issues with algae and macrophyte growth [8]. Nutrient deposits in sediment and croplands will likely continue to delay P decreases in Lake Mendota [9] and in similar agricultural areas.

Some researchers have called for nitrogen (N) management in addition to attempting to control P inputs into freshwater bodies to slow or potentially reverse eutrophication and decrease FHAB impacts (see review by [10]). Controlling N inputs would be challenging because cyanobacteria have demonstrated use of multiple forms of bioavailable N when all present in Lake Erie [11]. Additionally, the use of urea in agriculture may impact dependence of cyanobacteria and other phytoplankton on dissolved organic N (DON) illustrated in Chapter 2 (correlations with Kjeldahl N), even when these organisms are considered N-limited by inorganic N sources [12]. Furthermore, changes in DON (and transformation processes to other forms of N) may impact not only changes in cyanobacterial community composition (CCC), but also toxicity of resulting FHABs in Lake Mendota and others [13].

Ideally, to fully capture changes in CCC, cyanotoxin production, influences of the environment on CCC and toxin production, and how these processes change over time, long-term datasets would capture or incorporate nutrient concentrations and loading rates, weather-specific environmental parameters in relation to changes in cyanobacterial biovolume. Changes in toxin production could be incorporated by quantifying of transcription factors for toxin production (i.e. signaling molecules involved in N and iron stress: ntcA [14, 15] and fur (iron-ferric uptake regulator) for MCs [16, 17]), identifying species-specific changes in biomass and toxin production (to identify changes in toxic and non-toxic CCC), and monitoring a large range of cyanobacterial secondary metabolites using liquid chromatography coupled with tandem mass spectrometry. These data would *ideally* also be available from multiple lakes of varying trophic status from around the globe with or without one or more nutrient control strategies and other BMPs in place. Finally, such measurements would include strict quality control measures, including highly tested extraction procedures with reproducible recoveries and internal standards, such as $^{15}N$-labeled MC-LR and $^{13}C$-phenylalanine, ultimately adapted into internationally accepted standard operating procedures for extracting and measuring cyanotoxins and other secondary metabolites.

Several limitations in resources and collegial, governmental, and citizen stakeholder networking decrease the ability to meet this ideal scenario of connecting key ecological measures of toxin production and CCC on a global scale to identify possible patterns. A lack of research funding, reference materials for all possible congeners of cyanopeptides and other toxins, lack of training and availability of LC-

MS/MS technology at low cost, and complicated shipping/transportation procedures as described in Chapter 3 are examples of these limitations. Additional complexity involves extending from these ecological concepts to understanding how these patterns of toxic FHABs impact human disease.

Enormous strides have been made in the past two decades, gathering novel information regarding the toxicological mechanisms of cyanotoxins, diversity of cyanobacterial secondary metabolites, and increasing awareness of monitoring blue-green algal blooms for protecting public health. However, several questions remain to be answered in regards to long-term human health exposures to cyanotoxins and the resulting health effects. A need also exists for development of practical, effective, and affordable methods for analyzing the full suite of cyanobacterial secondary metabolites affecting animal and human health. Finally, we need a better understanding of how to manage FHABs appropriately with the perspective of eutrophication of freshwater lakes and FHABs as a public health concern, and appropriately communicate that concern and risk to the general public. Ultimately, these efforts need to be addressed by engaging interdisciplinary work from local and national government officials, professionals from public health, natural resources, and water treatment departments, chemists, toxicologists, microbiologists, doctors and veterinarians, lake scientists and ecologists, farmers, and lake stakeholders.

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# APPENDIX A: CHAPTER 2 SUPPLEMENTAL DATA

**Figure A1. Monthly mean depths of the epilimnion, hypolimnion, and photic zone receiving photosynthetically active radiation.**
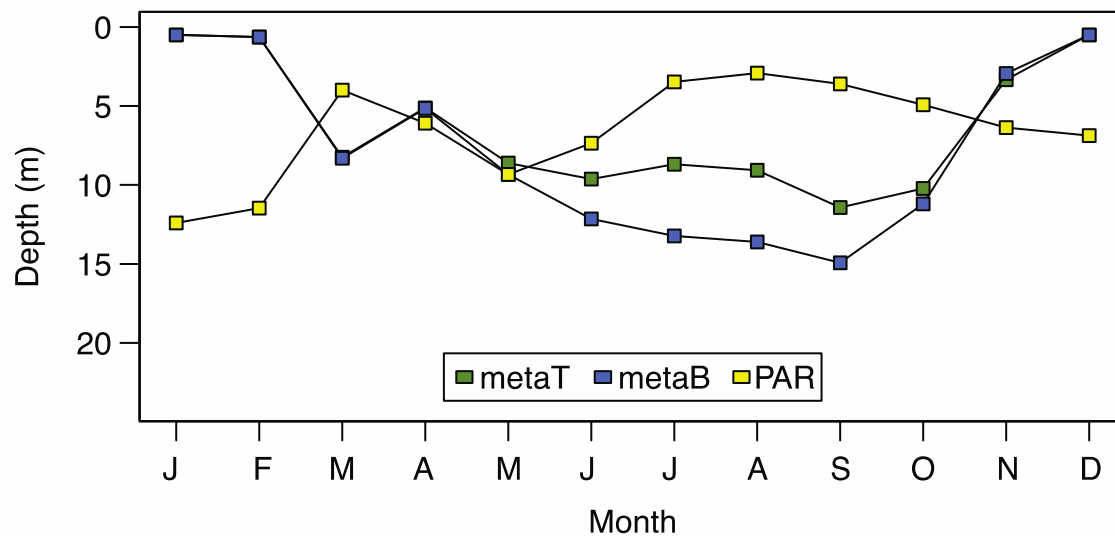


Abbreviations on the x-axis represent months of the year, starting with January and ending with December. PAR was estimated by multiplying secchi depth by 1.6. All depths are in meters. Legend abbreviations: metaT=Top depth of metalimnion, bottom depth of epilimnion; metaB=Bottom depth of metalimnion, top depth of hypolimnion; PAR=Depth of photic zone receiving 1% photosynthetically active radiation

**Table A1. Datasets used to analyze interannual correlations between cyanobacteria and environmental variables**

| Dataset/Source | Variables Used | Description | Variable(s) Created |
|---|---|---|---|
| NTL LTER: Phytoplankton – Madison Lakes Area 1995 – current (www.lter.limnology.wisc.edu/datacatalog) | Division; Biovolume Conc; Genus | Biovolume of each cyanobacterial genus ($\mu m^3$/mL) from an integrated sample (0-8 m) taken each sample date; Phytoplankton data also categorized by Phylum (Division) | *Cyanophyta*=Sum of all BV with Division "Cyanophyta"; *Microcystis*=Sum of all BV with Genus "Microcystis"; *Aphanizomenon*=Sum of all BV with Genus "Aphanizomenon"; Relative BV=Genus BV/Sum *Cyanophyta* BV |
| NTL LTER: Chemical Limnology of Primary Study Lakes; Nutrients, pH and Carbon 1981 – current (www.lter.limnology.wisc.edu/datacatalog) | NO3NO2_SLOH; NH4_SLOH; KJDL_N_SLOH; TOTPUF-SLOH; DRP_SLOH | Nutrient concentrations (mg/L) measured from samples collected at the water surface, measured from each sample date | TN=KJDL N + $NO_3^-NO_2^-$ |
| NTL LTER: Physical Limnology of Primary Study Lakes 1981 – current (www.lter.limnology.wisc.edu/datacatalog) | Wtemp | Water temperature (°C) collected at discrete depths from 0-24 m, measured each sample date | $Wtemp_8$; St; metaT; metaB; Lake Number |
| NTL LTER: Secchi Disk Depth; Other Auxiliary | Secview; Secnview | Secchi depths (m) | PAR=Secchi |

| | | | |
|---|---|---|---|
| Base Crew Sample Data 1981 – current (www.lter.limnology.wisc.edu/datacatalog) | | measured each sample date | depth*1.6=Photic zone depth with 1% photosynthetically active radiation; PAR:Epi=PAR/metaT |
| Wisconsin Climatology Office: History of Freezing and Thawing of Lake Mendota, 1852-53 to 2015-16 (www.aos.wisc.edu/lakes/Mendota-ice.html) | Ice-on date; Ice-off date; Number of days of ice cover | For each winter (year-year), dates recorded for ice formation and ice break-up on Lake Mendota, visualized from Picnic Point | Days ice cover from January 1st each year to date of Ice-off; Days since October 31st each year to ice-on; Open water days (inverse of days ice cover) |
| Dane County Regional-Truax Airport Weather Station, Madison, Wisconsin (KMSN): Weather History for KMSN (Daily History or Custom History) (www.wunderground.com/history/airport/KMSN) | Rainfall; Maximum wind speed; Maximum air temperature | Daily (matched with LTER sample dates) rainfall (converted to mm), and maximum wind speed (converted to m/s) and air temperature (converted to °C) | Lake Number (wind speed) |
| Lake Mendota Bathymetry | | Depth of the lake by multiple perimeters of lake shape | St; metaT; metaB; Lake Number |

Table A1 describes each dataset accessed to create monthly and cyanobacterial-growing season (June – October) means for specific variables, which variables were used from the dataset, units in which the variables are defined, and any manipulation of these to create new variables. Abbreviations: BV=Biovolume; Conc=Concentration; DRP_SLOH=Dissolved reactive phosphorus from Wisconsin State Lab of Hygiene; KJDL_N_SLOH=Kjeldahl nitrogen (unfiltered) from Wisconsin State Lab of Hygiene; metaB=Bottom of metalimnion (hypolimnion depth); metaT=Top of metalimnion (epilimnion depth); NH4_SLOH=Ammonium from Wisconsin State Lab of Hygiene; NO3NO2_SLOH=Nitrate plus nitrite from Wisconsin State Lab of Hygiene; NTL LTER=North Temperate Lakes Long Term Ecological Research (Program); Secview=Secchi depth measured with viewer; Secnview=Secchi depth measured without viewer; St=Schmidt Stability; TOTPUF_SLOH=Total phosphorus, unfiltered, from Wisconsin State Lab of Hygiene; $Wtemp_{(8)}$=Water temperature (8=mean water temperature from 0-8 m)

**Table A2. Annual Data Used to Identify Environmental Correlates of Cyanobacterial Biovolume**

| Year | Cyanophyta | Microcystis | Aphanizomenon | Spring TP | Spring TN | Spring DRP | Spring KJDL N | Spring $NH^+_4$ | Spring $NO^-_3NO^-_2$ |
|---|---|---|---|---|---|---|---|---|---|
| 1995 | 2820617.97 | 979016.71 | 1536445.80 | NA | NA | NA | NA | NA | NA |
| 1996 | 1506076.30 | 544874.36 | 719124.68 | 0.123 | 1.557 | 0.065 | 0.9 | 0.191 | 0.657 |
| 1997 | 1371762.30 | 580845.64 | 562282.25 | 0.148 | 1.7 | 0.099 | 1.1 | 0.201 | 0.6 |
| 1998 | 3087453.82 | 1675430.49 | 1045757.12 | 0.144 | 1.468 | 0.111 | 1 | 0.246 | 0.468 |
| 1999 | 3699219.39 | 414675.39 | 2714254.79 | 0.115 | 1.458 | 0.096 | 0.73 | 0.13 | 0.738 |
| 2000 | 3850794.33 | 246859.92 | 1804991.01 | 0.094 | 1.367 | 0.055 | 0.9 | 0.242 | 0.467 |
| 2001 | 2634220.14 | 322772.21 | 1089981.09 | 0.098 | 1.582 | 0.053 | 0.78 | 0.05 | 0.802 |
| 2002 | 1029535.54 | 164659.48 | 100234.01 | 0.096 | 1.722 | 0.056 | 0.74 | 0.048 | 0.982 |
| 2003 | 1318311.93 | 422998.25 | 631421.43 | 0.083 | 1.284 | 0.068 | 0.84 | 0.33 | 0.444 |
| 2004 | 1819033.27 | 1052895.92 | 596776.00 | 0.085 | 1.4 | 0.046 | 0.85 | 0.117 | 0.562 |
| 2005 | 2783053.12 | 407593.67 | 2160018.87 | 0.135 | 1.732 | 0.098 | 0.81 | 0.108 | 0.922 |
| 2006 | 1321074.55 | 242389.26 | 660425.70 | 0.144 | 1.258 | 0.124 | 0.91 | 0.474 | 0.348 |
| 2007 | 1917829.69 | 729221.86 | 954909.31 | 0.129 | 1.428 | 0.105 | 0.98 | 0.184 | 0.499 |
| 2008 | 5898166.64 | 1199914.70 | 4191722.03 | 0.141 | 1.953 | 0.024 | 1 | 0.044 | 0.953 |
| 2009 | 3237951.46 | 633324.72 | 1730586.98 | 0.165 | 2.51 | 0.133 | 1.05 | 0.112 | 1.74 |
| 2010 | 1937271.79 | 465939.68 | 996783.35 | 0.103 | 1.769 | 0.018 | 0.95 | 0.076 | 0.819 |
| 2011 | 3014718.23 | 269818.75 | 2554954.00 | 0.066 | 1.635 | 0.007 | 0.72 | 0.029 | 0.915 |
| 2012 | 552307.09 | 69810.04 | 76826.60 | 0.051 | 1.42 | 0.032 | 0.8 | 0.093 | 0.62 |
| 2013 | 1545725.66 | 346177.21 | 924343.36 | 0.073 | 1.158 | 0.014 | 0.59 | 0.104 | 0.568 |
| 2014 | 4497867.09 | 457405.89 | 2316994.78 | 0.084 | 1.613 | 0.035 | 0.81 | 0.098 | 0.803 |

| Year | Spring TN:TP | CGS TP | CGS TN | CGS DRP | CGS KJDL N | CGS $NH^+_4$ | CGS $NO^-_3NO^-_2$ | CGS TN:TP | Rainfall | Apr-Jun Rainfall |
|---|---|---|---|---|---|---|---|---|---|---|
| 1995 | NA | 0.06 | 0.96 | 0.027 | 0.863 | 0.134 | 0.11 | 21 | 3265.93 | 723.9 |
| 1996 | 13 | 0.065 | 1.03 | 0.034 | 0.79 | 0.103 | 0.243 | 23 | 1704.85 | 467.87 |
| 1997 | 11 | 0.078 | 1.024 | 0.046 | 0.85 | 0.098 | 0.174 | 15 | 631.19 | 223.78 |
| 1998 | 28 | 0.072 | 1.143 | 0.035 | 1.048 | 0.075 | 0.095 | 20 | 866.14 | 328.93 |

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| 1999 | 14 | 0.078 | 1.023 | 0.04 | 0.874 | 0.101 | 0.149 | 15 | 783.84 | 397 |
| 2000 | 18 | 0.084 | 1.12 | 0.05 | 0.974 | 0.172 | 0.146 | 15 | 692.66 | 326.9 |
| 2001 | 18 | 0.082 | 1.088 | 0.05 | 0.935 | 0.186 | 0.153 | 17 | 952.5 | 312.42 |
| 2002 | 19 | 0.054 | 0.96 | 0.031 | 0.751 | 0.105 | 0.209 | 25 | 654.3 | 257.3 |
| 2003 | 19 | 0.045 | 0.778 | 0.025 | 0.696 | 0.117 | 0.082 | 26 | 784.1 | 222.76 |
| 2004 | 19 | 0.044 | 0.876 | 0.015 | 0.739 | 0.047 | 0.137 | 23 | 995.43 | 419.86 |
| 2005 | 14 | 0.047 | 0.873 | 0.011 | 0.708 | 0.022 | 0.165 | 20 | 627.63 | 185.17 |
| 2006 | 9.3 | 0.055 | 0.859 | 0.025 | 0.803 | 0.061 | 0.056 | 22 | 928.37 | 305.31 |
| 2007 | 15 | 0.05 | 0.831 | 0.021 | 0.753 | 0.062 | 0.078 | 23 | 1128.01 | 276.86 |
| 2008 | 45 | 0.086 | 1.299 | 0.039 | 1.037 | 0.143 | 0.262 | 18 | 1119.12 | 505.71 |
| 2009 | 17 | 0.107 | 1.527 | 0.051 | 1.126 | 0.166 | 0.401 | 15 | 984 | 311.91 |
| 2010 | 25 | 0.045 | 0.941 | 0.007 | 0.831 | 0.065 | 0.11 | 23 | 961.64 | 401.83 |
| 2011 | 25 | 0.046 | 1.032 | 0.019 | 0.789 | 0.126 | 0.243 | 31 | 775.72 | 242.82 |
| 2012 | 28 | 0.027 | 0.672 | 0.004 | 0.603 | 0.045 | 0.069 | 28 | 669.54 | 161.29 |
| 2013 | 16 | 0.039 | 0.932 | 0.012 | 0.816 | 0.112 | 0.116 | 30 | 1152.65 | 590.8 |
| 2014 | 19 | 0.048 | 1.024 | 0.014 | 0.946 | 0.091 | 0.078 | 26 | 896.87 | 461.01 |

| Year | Wind speed | $Wtemp_8$ | St | Lake Number | Photic Zone Depth | Photic: MetaT | MetaT | Days Ice Cover Winter Prior | Ice-free Days | Days ice-on since 10/31 | Days since 1/1 to ice-off |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1995 | 7.18 | 19.8 | 357 | 5 | 6.1 | 0.4 | 9.6 | 46 | 298 | 67 | 52 |
| 1996 | 6.04 | 20.3 | 375 | 14 | 5.7 | 0.5 | 11.6 | 119 | 256 | 40 | 98 |
| 1997 | 6.44 | 19.1 | 349 | 53 | 5.2 | 0.7 | 8.3 | 102 | 274 | 50 | 91 |
| 1998 | 5.48 | 20.6 | 408 | 42 | 4.7 | 0.5 | 11.9 | 47 | 317 | 72 | 58 |
| 1999 | 5.86 | 19.5 | 313 | 8 | 4.4 | 0.4 | 10.6 | 82 | 285 | 60 | 80 |
| 2000 | 5.4 | 18.4 | 260 | 33 | 4.8 | 2.2 | 9.7 | 53 | 294 | 75 | 67 |
| 2001 | 7.23 | 18.8 | 280 | 6 | 4.8 | 0.3 | 9.9 | 115 | 268 | 43 | 97 |
| 2002 | 7.38 | 20.8 | 357 | 3 | 4.3 | 0.5 | 6.9 | 21 | 341 | 63 | 12 |
| 2003 | 5.53 | 18.6 | 367 | 21 | 5.8 | 0.5 | 7.6 | 89 | 276 | 65 | 93 |
| 2004 | 6.12 | 20.6 | 315 | 14 | 4.3 | 0.4 | 13.3 | 80 | 279 | 68 | 87 |
| 2005 | 7.26 | 21.9 | 509 | 46 | 4.2 | 0.6 | 8 | 101 | 252 | 55 | 95 |

| 2006 | 6.17 | 20.8 | 441 | 29 | 4.7 | 0.5 | 7.9 | 95 | 282 | 49 | 83 |
|------|------|------|-----|----|-----|-----|------|-----|-----|----|-----|
| 2007 | 6.17 | 21.9 | 451 | 17 | 4.2 | 0.4 | 10.8 | 66 | 294 | 81 | 86 |
| 2008 | 7.02 | 20.3 | 339 | 13 | 3.8 | 0.3 | 8.6 | 106 | 249 | 56 | 101 |
| 2009 | 6.12 | 18.8 | 279 | 46 | 5.3 | 0.4 | 8.2 | 97 | 281 | 46 | 82 |
| 2010 | 6.35 | 21.8 | 441 | 42 | 2.7 | 0.3 | 9.5 | 87 | 264 | 59 | 85 |
| 2011 | 6.35 | 21.2 | 453 | 26 | 4.8 | 0.5 | 9.3 | 109 | 272 | 45 | 93 |
| 2012 | 8.45 | 22.3 | 467 | 32 | 3.4 | 0.3 | 9.5 | 57 | 308 | 75 | 71 |
| 2013 | 7.51 | 20 | 443 | 1 | 4.2 | 2.6 | 6.7 | 87 | 278 | 75 | 101 |
| 2014 | 7.02 | 19.8 | 430 | 2 | 3.4 | 2.8 | 6.9 | 117 | 319 | 46 | 102 |

This table represents all annual data used in correlation tests with strength measured by Kendall's tau-b between cyanobacterial biovolume (BV) and environmental variables. Abbreviations: CGS=Cyanobacterial-growing season (June – October); TP=Total phosphorus (mg/L); TN=Total nitrogen (mg/L); DRP=Dissolved reactive phosphorus (mg/L); KJDLN=Kjeldahl nitrogen (mg/L); $NO_3^-NO_2^-$=Nitrate, plus nitrite (mg/L); $NH_4^+$=Ammonium (mg/L); $Wtemp_8$=Water temperature (°C), average from 0-8 m; St=Schmidt stability; MetaT=Top depth of the metalimnion (depth of epilimnion, m). All BV measurements are in $\mu m^3/mL$

**Figure A2. Monthly mean biovolume of total *Cyanophyta* and dominant genera in**

**Lake Mendota, 1995-2014**
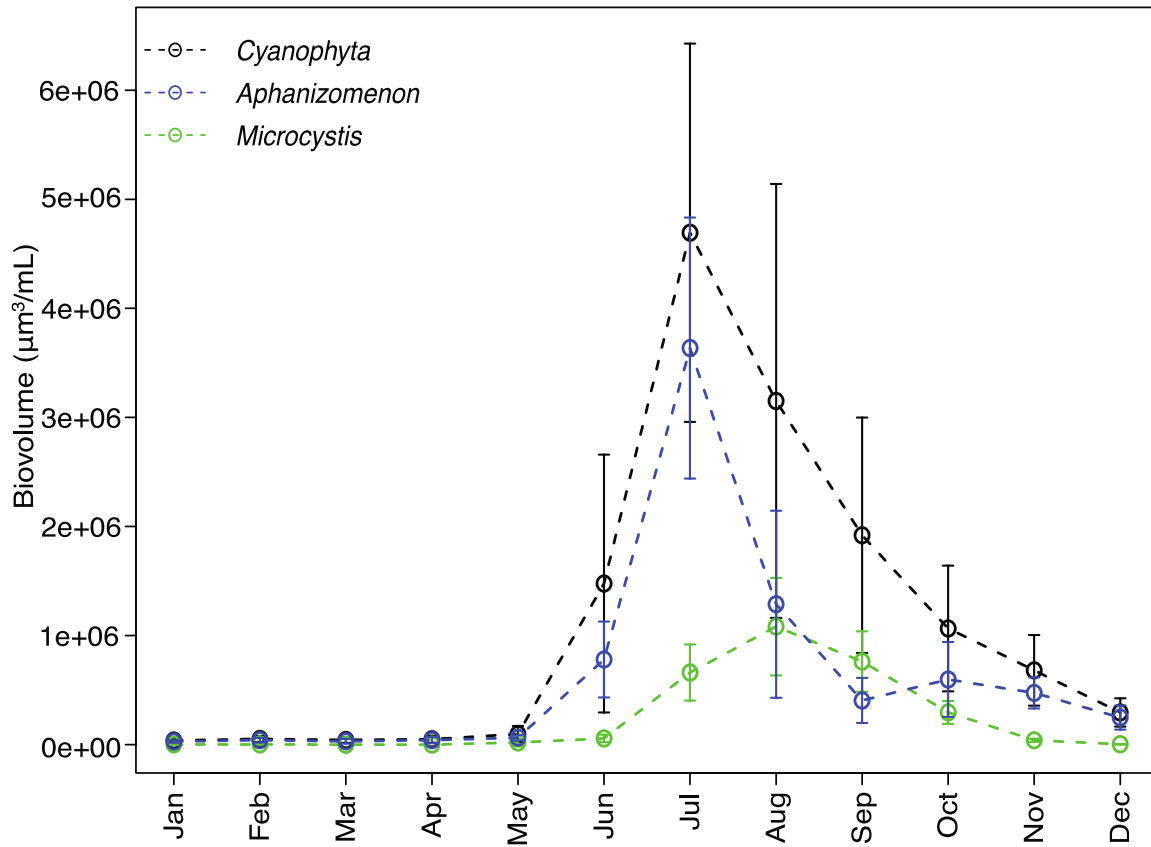


Average monthly BVs of total cyanobacteria, *Aphanizomenon spp.*, or *Microcystis spp.* are expressed in μm³/mL.

**Figure A3. Relative annual biovolume for outlier genera (*Planktothrix,
Synechococcus*) and environmental correlates**
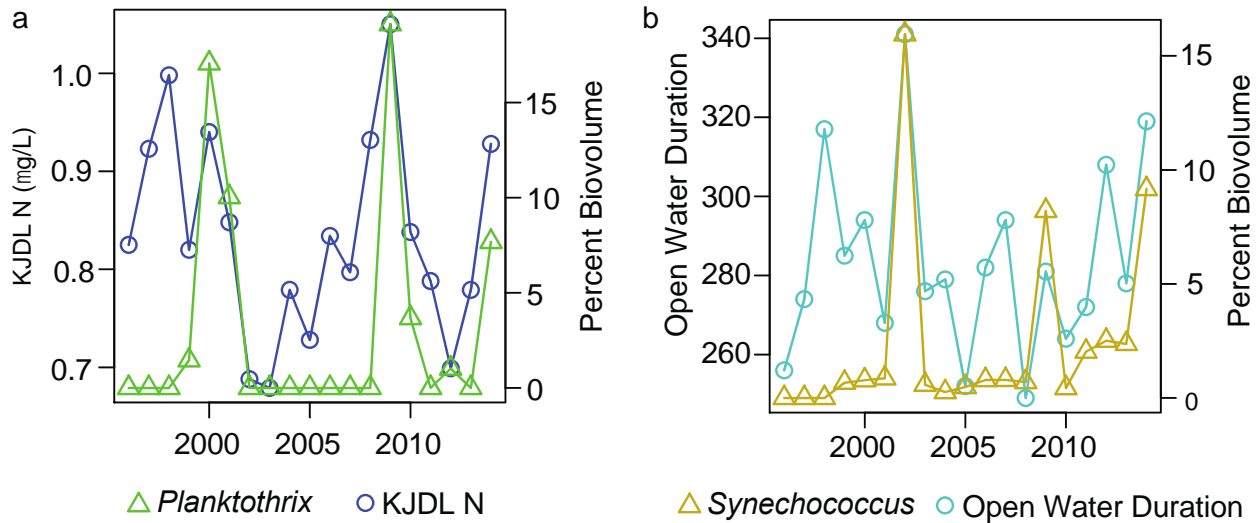


Figure A3 illustrates key genera impacting Lake Mendota's cyanobacterial community
composition in years of their exceptionally high biovolume (BV; $\mu m^3$/mL) and their
strongest, significant environmental correlates. Relative BV was calculated by dividing
the BV of each genus by the BV of all cyanobacterial species counted, per sample date,
then converted to a percentage. This percentage was then averaged by month, and
then those numbers used to calculate an annual mean relative BV for the
cyanobacterial growing season in Mendota, June through October. Left y-axes
represent environmental correlates (circles), and right y-axes represent relative genus
BV (triangles). **A3a**: Average relative *Planktothrix* BV with mean Kjeldahl nitrogen
(mg/L). **A3b**: Average relative *Synechococcus* BV with open water duration (days).

# APPENDIX B: CHAPTER 3 SUPPLEMENTAL DATA

## Table B1. Cyanotoxin-specific tandem mass spectrometry parameters

| Parent Mass/Charge ($m/z$) | Daughter Mass/Charge ($m/z$) | Analyte | Declustering Potential (volts) | Collision Energy (volts) |
|---|---|---|---|---|
| 837.544 | 70 | AptB-1 | 106 | 129 |
| 837.544 | 201.4 | AptB-2 | 106 | 57 |
| 851.757 | 175.1 | AptF-1 | 121 | 53 |
| 851.757 | 201 | AptF-2 | 121 | 53 |
| 1007.54 | 776.3 | Cpt1007-1 | 131 | 59 |
| 1007.54 | 989.6 | Cpt1007-2 | 131 | 51 |
| 1021.6 | 776.4 | Cpt1020-1 | 131 | 63 |
| 1021.6 | 989.6 | Cpt1020-2 | 131 | 57 |
| 1042.528 | 828.3 | Cpt1041-1 | 131 | 51 |
| 1042.528 | 1024.5 | Cpt1041-2 | 131 | 51 |
| 981.531 | 103.2 | dmMC-LR-1 | 126 | 129 |
| 981.531 | 135.3 | dmMC-LR-2 | 126 | 101 |
| 910.617 | 135.2 | MC-LA-1 | 106 | 87 |
| 910.617 | 776.4 | MC-LA-2 | 106 | 27 |
| 995.619 | 127.1 | MC-LR-1 | 126 | 115 |
| 995.619 | 135.3 | MC-LR-2 | 126 | 115 |
| 520 | 70.1 | MC-RR-1 | 81 | 129 |
| 520 | 135.1 | MC-RR-2 | 81 | 43 |
| 1045.633 | 127.1 | MC-YR-1 | 141 | 123 |
| 1045.633 | 135.3 | MC-YR-2 | 141 | 107 |
| 691.368 | 343.1 | Mcgn690-1 | 96 | 37 |
| 691.368 | 510.2 | Mcgn690-2 | 96 | 31 |
| 825.522 | 135.3 | NOD-1 | 116 | 129 |
| 825.522 | 103.2 | NOD-2 | 116 | 83 |

"Analyte" refers to each individual cyanobacterial peptide; the -1 and -2 after each of

these abbreviations represent notation two unique ion transitions used per toxin.

Abbreviations: Apt=Anabaenopeptin; Cpt=Cyanopeptolin; dm=Desmethyl ([Dha[7]]);

MC=Microcystin; L=Leucine; R=Arginine; A=Alanine; Y=Tyrosine; Mcgn=Microginin;

NOD=Nodularin

**Figure B1. Cyanopeptides produced by University of Texas Culture Collection**

***Microcystis aeruginosa* 2385 during spike-recovery experiments**
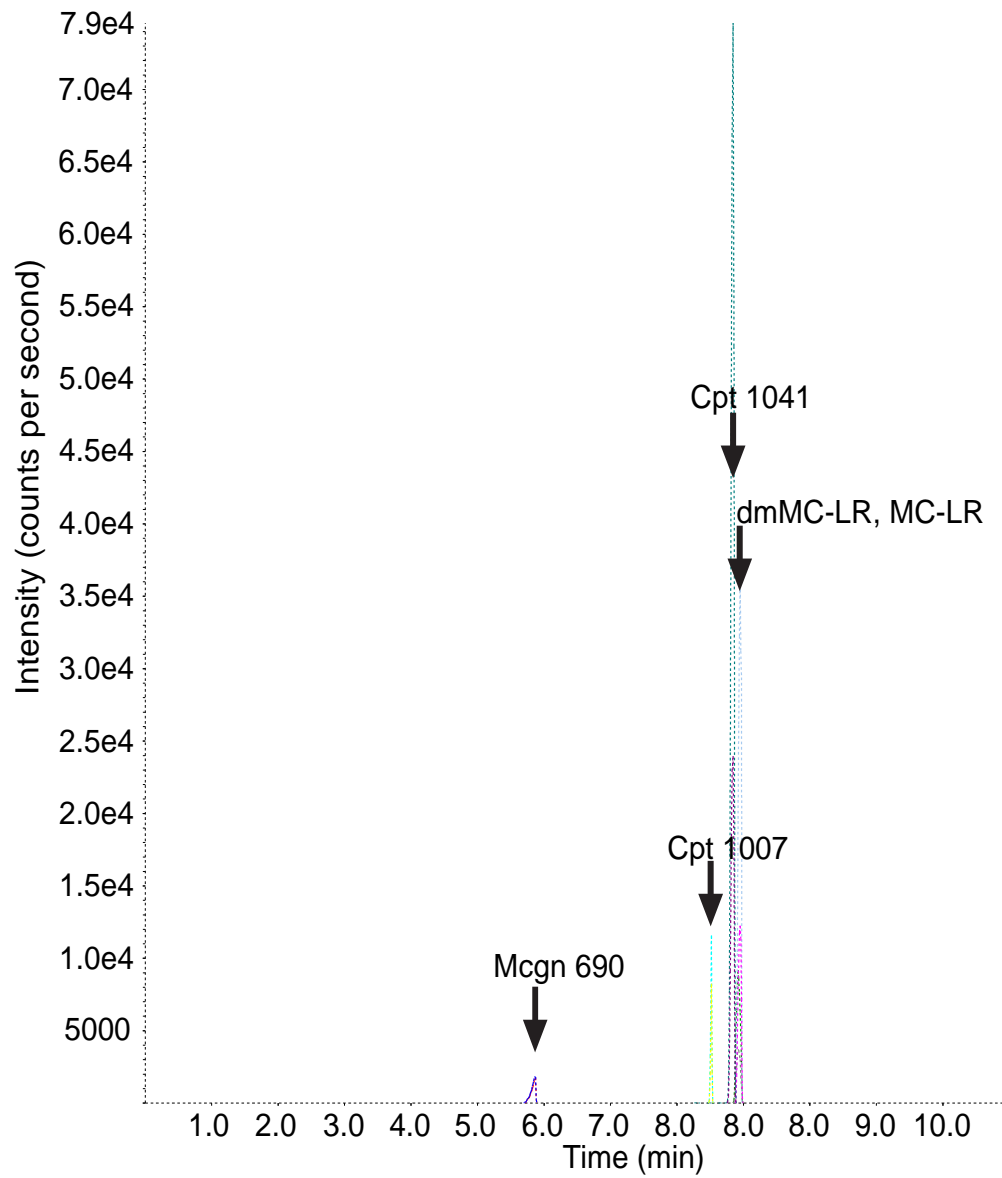


Abbreviations: Cpt=Cyanopeptolin; dm=Desmethyl ([Dha$^7$]); L=Leucine;

MC=Microcystin; Mcgn=Microginin; R=Arginine

**Table B2. Cyanopeptides measured from integrated depth sample collection in Lake Stechlin, Germany**

Samples were collected biweekly from Lake Stechlin as integrated samples from the full lake depth, and on some dates, also from the metalimnion. Approximately 70 mL of sample was lyophilized or dried to a residue for shipment. Samples were resuspended using water bath sonication in 0.1% formic acid in water and extracted as described with freeze-thaws and ~67% MeOH. Apt A and dmMC-LR were not measured in these samples. MC-RR, MC-LA, NOD, Mcgn 690, and Cpts 1007, 1020, and 1041 were not detected in Lake Stechlin. Apts were most frequently detected (Apt B, 76.5%; Apt F, 47.1%) from Lake Stechlin, also in higher concentrations than MCs (Table 2). Apt B was detected in higher concentrations than Apt F, with mean concentrations of 1.49 and 2.5 µg/L detected in full-depth and metalimnion integrated samples, respectively.

| Date | Depths (m) | MC-LR (µg/L) | MC-YR (µg/L) | Apt B (µg/L) | Apt F (µg/L) |
|---|---|---|---|---|---|
| 4/14/13 | 0-25 | 0 | 0 | 0 | 0 |
| 5/7/13 | 0-25 | 0 | 0 | 2.286 | 0.12204 |
| 5/22/13 | 0-25 | 0.001359375 | 0 | 0.23625 | 0 |
| 6/4/13 | 0-25 | 0.02175 | 0.016125 | 2.9375 | 0.0042125 |
| 6/10/13 | 0-25 | 0 | 0 | 1.6375 | 0 |
| | 5-10 | 0 | 0.0038875 | 0 | 0 |
| 7/9/13 | 0-25 | 0 | 0 | 2.85 | 0.11675 |
| | 5-10 | 0.001975 | 0 | 0 | 0 |
| 7/30/13 | 0-25 | 0 | 0 | 3.5875 | 0.029625 |
| | 5-15 | 0 | 0 | 4.175 | 0 |
| 8/8/13 | 0-25 | 0 | 0 | 0.80875 | 0.043875 |
| | 5-10 | 0 | 0 | 0.13 | 0.05425 |
| 8/22/13 | 0-25 | 0 | 0 | 2.35 | 0.10475 |
| | 5-15 | 0 | 0 | 8.2625 | 0.38125 |
| 9/3/13 | 0-25 | 0.00265 | 0 | 0 | 0 |
| 9/18/13 | 0-25 | 0 | 0 | 0.077625 | 0 |
| 10/8/13 | 0-25 | 0 | 0 | 1.07875 | 0 |

Abbreviations: m=meters; Apt=anabaenopeptin; MC=microcystin; L=leucine; R=arginine; Y=tyrosine

**Tables B3 and B4. Cyanopeptides analyzed from lakes in Estonia**

Samples were received from seven lakes in Estonia: Lake Kaiavere, Lake Uljaste, Lake Mäeküla, Lake Ähijärv, Lake Pühajärv, Lake Tündre, Lake Vanamõisa; most samples were collected at two depths. Lake Kaiavere had the highest number of biweekly surface samples (0.3 m) collected; all other lakes had between two and four monthly surface samples submitted for analysis, sometimes with other depths also included. Only MC-RR and Apts were detected in these lakes. No cyanopeptides were detected in lakes Uljaste, Pühajärv or Vanamõisa. Lake Ähijärv had Apt A detected in one (4 m) sample at 0.912 µg/L on July 8, 2013; two other samples were collected at 0.3 and 4 m on August 7 and September 9, 2013 with no cyanopeptides detected.

**Table B3. Cyanopeptides detected in Lake Tündre**

| Date | Depth (m) | Apt A (µg/L) | Apt B (µg/L) |
|------|-----------|--------------|--------------|
| 7/3/13 | 0.3 | 0 | 1.914 |
| | 3 | 3.63 | 6.6 |
| | 7 | 0 | 0 |
| 8/5/13 | 0.3 | 0 | 0 |
| | 3 | 1.578 | 4.71 |
| | 7 | 0 | 0 |
| 9/4/13 | 0.3 | 0.4392 | 0 |
| | 4 | 0 | 0 |

Abbreviations: m=meters; Apt=Anabaenopeptin

**Table B4. Cyanopeptides detected in Lake Mäeküla (depth=3 m)**

| Date | MC-RR (µg/L) | Apt A (µg/L) | Apt B (µg/L) |
|------|--------------|--------------|--------------|
| 7/3/13 | 0 | 0 | 0 |
| 8/5/13 | 0 | 0 | 1.938 |
| 9/4/13 | 1.086 | 3.492 | 6.84 |

Abbreviations: Apt=Anabaenopeptin; MC=Microcystin; R=Arginine

**Other Data Summarized from GLEON Cyanotoxins Project (Chapter 3)**

**Parentis Lake cyanopeptides at multiple depths**

Parentis Lake was sampled monthly from January through December 2013 at three depths: the surface, secchi depth, and integrated depth of the photic zone (Supplemental Fig. 2). Apt concentrations were always higher than MCs; NOD was only detect on one sample date in an integrated euphotic zone sample (bottom panel, Supplemental Fig. 2). Highest MC concentrations were detected in the beginning of November, likely during a FHAB die-off, season turn-over, and subsequent intracellular toxin release. Highest Apt concentrations were detected, however, in the January prior. Concentrations of Apts and NOD indicate potential Type I error of using a protein phosphatase inhibition assay for MC detection.

**Anabaenopeptins detected at two sites in Lake Wallenpaupack**

Two sites were sampled monthly at Lake Wallenpaupack in 2013; sample Site 3 was the middle of the reservoir, whereas sample Site 5 was near the most northwest point of the reservoir, near one of many recreational sites surrounding Wallenpaupack. None of the individual MCs for which reference materials were available were detected in this analysis; only Apt B and Apts A and B were detected at Sites 3 and 5, respectively. Highest Apt concentrations were detected at both sites in October 2013; Apt B concentrations were consistently higher than Apt A concentrations (Supplemental Figure 3).

**Argentinian Lakes**

In addition to Laguna Grande and Laguna Chascomús, Argentinian lakes Laguna Carpincho and Laguna Gómez were also sampled for cyanotoxin analysis in January

and February 2013. Two monthly samples were collected from each lake; neither

sample from January 2013 contained any detected cyanopeptides. However, in both

February lake samples, MC-RR was detected at 1.02 and 0.896 μg/L in Laguna

Carpincho and Laguna Gómez, respectively.

# APPENDIX C: REFERENCE MATERIALS AND SOLUTIONS

**Table C1. Mixed working stock (1000 µg/L) of cyanotoxins reference materials**

| Cyanopeptide | Supplier | Concentration (µg/L) | Volume (µL) |
|---|---|---|---|
| Microcystin-RR | Sigma Aldrich | 25,000 | 40 |
| Microcystin-YR | Sigma Aldrich | 100,000 | 10 |
| Microcystin-LA | Sigma Aldrich | 100,000 | 10 |
| Anabaenopeptin F | MARBIONC | 100,000 | 10 |
| Microginin 690 | MARBIONC | 100,000 | 10 |
| Cyanopeptolin 1020 | MARBIONC | 100,000 | 10 |
| Cyanopeptolin 1041 | MARBIONC | 100,000 | 10 |
| Cyanopeptolin 1007 | MARBIONC | 100,000 | 10 |
| Anabaenopeptin A | MARBIONC | 100,000 | 10 |
| Anabaenopeptin B | MARBIONC | 100,000 | 10 |
| Microcystin-LR | National Research Council Canada | 10150.7 | 98.6 |
| Nodularin | National Research Council Canada | 10229.75 | 97.8 |
| [Dha$^7$]-Microcystin-LR | National Research Council Canada | 9399.47 | 106.4 |
| Total Volume Cyanopeptide Standards | | | 432.8 |
| Volume 70% Methanol | | | 567.2 |

Abbreviations: MARBIONC=Marine Biotechnology in North Carolina Development

Group, LLC; Dha=Dehydroalanine

**Tables C2-C9. Modified BG11 Medium**

1.  Make up the following solutions and **<u>autoclave</u>**. Store at 4˚C.

**Table C2. 100X Base BG11**

| Chemical | Amount |
|---|---|
| $NaNO_3$ | 1.5 g |
| $MgSO_4\ 7H_2O$ | 0.75 g |
| $CaCl_2\ 2H_2O$ | 0.36 g |
| Citric acid | 0.06 g |
| 0.25 M NaEDTA, pH 8.0 | 110 µL |
| $ddH_2O$ | Bring up to100 mL |

**Table C3. 1000X Ferric Ammonium Citrate ($Fe(NH_4)_3(C_6H_5O_7)$)**

| Chemical | Amount |
|---|---|
| $Fe(NH_4)_3(C_6H_5O_7)$ | 0.6 g |
| $ddH_2O$ | Bring up to100 mL |

**Table C4. 1000X Sodium Carbonate ($Na_2CO_3$)**

| Chemical | Amount |
|---|---|
| $Na_2CO_3$ | 2.0 g |
| $ddH_2O$ | Bring up to100 mL |

**Table C5. 1000X Dipotassium Hydrogen Phosphate ($K_2HPO_4$)**

| Chemical | Amount |
|---|---|
| $K_2HPO_4$ | 3.1 g |
| $ddH_2O$ | Bring up to100 mL |

**Table C6. 1M Sodium Bicarbonate ($NaHCO_3$)**

| Chemical | Amount |
|---|---|
| $NaHCO_3$ | 8.4 g |
| $ddH_2O$ | Bring up to100 mL |

2.  Make up the following solutions and **<u>filter sterilize.</u>** Store several 1 mL aliquots at -

    20˚C, freeze the rest in bulk.

**Table C7. Trace Metals**

| Chemical | Amount |
|---|---|

| | |
|---|---|
| $H_3BO_3$ | 2.86 g |
| $MnCl_2 \cdot 4H_2O$ | 1.81 g |
| $ZnSO_4 \cdot 7H_2O$ | 0.222 g |
| $Na_2MoO_4 \cdot 2H_2O$ | 0.39 g |
| $CuSO_4 \cdot 5H_2O$ | 0.079 g |
| $Co(NO_3)_2\ 6H_2O$ | 0.049 g |
| $ddH_2O$ | Bring up to 1 L |

**Table C8. Vitamins Stock**

| Chemical | Amount |
|---|---|
| Thiamin $B_1$ | 50 mg |
| d-Biotin/ Cyanocobalamin solution (0.25mg each/mL) | 1 mL |
| $ddH_2O$ | Bring up to 500 mL |

3.  Autoclave an appropriate amount of $ddH_2O$ and add ingredients:

**Table C9. Working BG11 Medium**

| Stock Solution | Amount |
|---|---|
| 100X base BG11 | 10 mL |
| 1000X Ferric Ammonium Citrate | 1 mL |
| 1000X Sodium Carbonate | 1 mL |
| 1000X Dipotassium Hydrogen Phosphate | 1 mL |
| 1M Sodium Bicarbonate | 10 mL |
| Trace Metals | 0.5 mL |
| Vitamins | 1 mL |
| $ddH_2O$ (autoclaved) | Add to 975 mL |

**Table C10. Cyanotoxin Reference Materials in E2 Zebrafish Medium**

| Toxin | Exposure Details | Low Dose (µg/L) | Lake Dose (µg/L) | High Dose (µg/L) |
|---|---|---|---|---|
| Apt A | Media Concentration | 0.3 | 3 | 30 |
| | Intermediate Dilution | 1000 | 10000 | 100000 |
| | Reference Volume (µL) | 15 | 15 | 15 |
| | MeOH (µL) | 85 | 85 | 85 |
| Apt B | Media Concentration | 0.3 | 3 | 30 |
| | Intermediate Dilution | 1000 | 10000 | 100000 |
| | Reference Volume (µL) | 15 | 15 | 15 |
| | MeOH (µL) | 85 | 85 | 85 |
| ATX (Tocris Bioscience) | Media Concentration | 0.2 | 2 | 20 |
| | Intermediate Dilution | 1000 | 1000 | 10000 |
| | Reference Volume (µL) | 10 | 100 | 100 |
| | MeOH (µL) | 90 | 0 | 0 |
| Cpt 1007 | Media Concentration | 1 | 10 | 100 |
| | Intermediate Dilution | 10000 | 10000 | 100000 |
| | Reference Volume (µL) | 5 | 50 | 50 |
| | MeOH (µL) | 95 | 50 | 50 |
| Cpt1020 | Media Concentration | 0.01 | 0.1 | 1 |
| | Intermediate Dilution | 100 | 1000 | 1000 |
| | Reference Volume (µL) | 5 | 5 | 50 |
| | MeOH (µL) | 95 | 95 | 50 |
| Cpt 1041 | Media Concentration | 0.3 | 3 | 30 |
| | Intermediate Dilution | 1000 | 10000 | 100000 |
| | Reference Volume (µL) | 15 | 15 | 15 |
| | MeOH (µL) | 85 | 85 | 85 |
| [Dha$^7$]-MC-LR | Media Concentration | 0.04 | 0.4 | 4 |
| | Intermediate Dilution | 1000 | 1000 | 9400 |
| | Reference Volume (µL) | 2 | 20 | 21.3 |
| | MeOH (µL) | 98 | 80 | 78.7 |
| MC-LA | Media Concentration | 0.3 | 3 | 30 |
| | Intermediate Dilution | 1000 | 10000 | 100000 |
| | Reference Volume (µL) | 15 | 15 | 15 |
| | MeOH (µL) | 85 | 85 | 85 |
| MC-RR | Media Concentration | 1 | 10 | 100 |
| | Intermediate Dilution | 10000 | 10000 | 100000 |
| | Reference Volume (µL) | 5 | 50 | 50 |
| | MeOH (µL) | 95 | 50 | 50 |
| MC-YR | Media Concentration | 0.1 | 1 | 10 |
| | Intermediate Dilution | 1000 | 10000 | 10000 |
| | Reference Volume (µL) | 5 | 5 | 50 |
| | MeOH (µL) | 95 | 95 | 50 |

| Mcgn 690 | Media Concentration | 0.01 | 0.1 | 1 |
|---|---|---|---|---|
| | Intermediate Dilution | 100 | 1000 | 1000 |
| | Reference Volume (µL) | 5 | 5 | 50 |
| | MeOH (µL) | 95 | 95 | 50 |

## APPENDIX D: DETAILED PROTOCOLS

## Steps for Optimizing Compounds Using Miller LC-MS

## Compound Optimization by Infusion

1. Put compound into solvent potentially to be used for LC-MS at a concentration of 10-20 ug/L. Draw this up in the 1.0 mL glass syringe and set this up to pump at 20-40 uL/minute on the infusion pump.

2. Open Analyst on the computer. Go to the Hardware Configuration section in the lefthand panel and activate the "4000Qtrap Only" option.

3. Go to Manual Tuning and choose Q1 MS (Q1) for the Scan Type. Enter in appropriate start and stop values for the scan (bottom hundred and top hundred for compound, i.e. if looking for compound with m/z=459, start=400 and stop=500). Enter in 1 sec for Time. Hit start and look for the parent ion m/z value. Change to positive or negative mode depending on compound.

4. Do another type of scan called a Product Ion (MS2) scan. Enter in the parent mass in the Product of: box. Choose appropriate Start and Stop m/z values for expected daughter ions and 1 sec for Time. Turning on MCA might be helpful here to determine the most prevalent ion transitions.

5. Staying in Manual Tuning mode (note purple "T" button indented in top icon menu) and click on Compound Optimization under "Tune and Calibrate" on the lefthand panel. Select infusion and click "Next" twice. For the final window, input the compound name, the molecular weight in Daltons (note: most literature identifies the value searched for with the H+ added, so subtract 1 from the m/z value), and the number of charges. Click Finish. This will give the most prevalent

ion transitions and parameters such as Collision Energy, Declustering Potential, etc. (things that affect breaking up the ions).

**FIA Optimization - Gases**

6. Go back to Hardware Configuration and activate the option called MS + LC. Create an MRM Acquisition Method by going to "Build Acquisition Method" on the left under Acquire. Enter in the top three highest transition ions as (Compound)q, (Compound)c1 and (compound)c2, example: MCLRq, MCLRc1 and MCLRc2. Also enter in DP, CE and CXP per ion transition. Make the method run for two minutes at 50% B (organic). Save the Acquisition Method in the format: date_nameofcompound.

7. Check the column oven to see if there is a column inserted. If there is, remove the solvent lines from the buffers and put both A and B into the appropriate storage solvent (85% acetonitrile for HILIC and 100% acetonitrile for C18). Run the pumps for five minutes. Stop the pumps and remove the column from the oven, making sure to put the caps on top and bottom and putting it in the correct storage box.

8. Replace the column with a small metal tubular insert.

9. Click again on Compound Optimization in the lefthand panel. This time select FIA. You must select the appropriate method (the one that you just created). Continue clicking next and finish. All the gases that will be optimized for should already be selected. FIA infusion will run for at least an hour and produce a final method optimized for all transition ions.

**Sequence of steps for optimizing compounds on AB Sciex 400 QTrap**

1. Q1 scan in manual tuning

2. Product ion scan in manual tuning

3. Compound optimization (syringe infusion)

4. Further compound optimization (optional; multiple injections without column using shortened optimized method)

5. Full acquisition method with column and adjusting retention time separation

**Column Care**

**Changing Columns:**

1. Make sure the LC is turned on and neither of the pumps are flowing. Take both pump lines and put them in an appropriate storage solvent for the column currently in the column oven:

   a. HILIC: 85% acetonitrile

   b. C18: 100% ACN or methanol (MeOH)

   c. C8: 100% ACN or MeOH

   Pump this solvent through the lines for at least five minutes.

2. Remove the column from the oven, replace the appropriate caps (red for C18 and C8 columns, beige for HILIC), and put back into the appropriate box.

3. Change the mobile phase buffers to the appropriate ones for the method, and purge both the pump valves and storage solution in the autosampler. To purge the lines, turn both knobs on the pumps at least one full rotation to the left.

4. During the purging of the lines, insert the new column into the column oven. Be careful not to lose the small caps on either the LC lines (they tend to fall off) or

for storing the column – it's a good idea to place these in the small beaker next to the LC.

**Cleaning columns:**

- Turn the column upside down and run the appropriate solvent through into a waste beaker:

    o HILIC: 45% ACN

    o C18/C8: 100% ACN or MeOH

- If there is a guard column, remove the regular column and put it right-side up. Run solvents through the attached guard column/column. Let the waste drip into a beaker.

**LC General Care:**

- Purge waste should be emptied into the waste carboy into the hood. Solvents to include on the chemical list are methanol, acetonitrile, formic acid, water, ammonium formate (no abbreviations).

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

Note: Concentration units are in ng/mL (=µg/L)

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; date formate should be mmddyy).

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.

Step #2: Creating a Results File

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

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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

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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 ☹

Step #4: Calculating Percent Peak Area for Noting Ion Suppression

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.

Step #5: Calculating Concentrations of Analytes in Your Samples

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. <u>Buttons:</u> In the tan area above the chromatogram, click on the button with the counter-clockwise 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 3$^{rd}$ 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. (4$^{th}$ 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 & Cyanobacterial Peptides with 70% Methanol**

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 (two 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 vacuum pressure 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 55° 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 ~67%).

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 surrogate standard: 5 µL of 1000 µg/L Nodularin or $^{13}C_6$-phenylalanine to each sample in LC vial. Vortex.

## Extraction of Neurotoxins and Cylindrospermopsin from Lake Water Samples

1. Transfer 1 mL of sample to a 1.5mL tube.

   a. Add 10 µL of 0.5 mg/L $^{13}C_6$-phenylalanine in 0.1% Formic Acid.

   b. Add 1 µL of formic acid.

   c. Vortex to mix.

2. Freeze samples in -80° C freezer for 30 minutes; thaw in 55° C water bath for 5 min.

   a. Perform three cycles of freeze/thaws.

   b. Vortex after each thaw.

3. Place samples in sonicating water bath (45° C) for 10 minutes.

4. Vortex.

5. Centrifuge for 15 minutes at max speed.

6. Transfer the top 500 µL of supernatant to an LC vial for analysis.

7. Freeze the remaining sample at -80° C.

## Dual Extraction of Cyanobacterial Neurotoxins and Hepatotoxins from Filtered Biomass

1. If filter is wrapped in foil or in a container other than a 15 mL conical, transfer to a clean, re-labeled 15 mL conical tube using forceps sterilized with 70% EtOH

2. Add 4 mL 0.1% formic acid in water to the tube and vortex well.

3. Freeze sample(s) in -80° C freezer for 30 minutes; thaw in 55° C water bath for 5 minutes.

   a. Perform three cycles of freeze/thaws.

   b. Vortex after each thaw.

4. During freeze-thaw cycles:

   a. Label LC vials for HILIC analysis and add 300 µL vial inserts to the vials.

   b. Preheat sonicating water bath to 45° C.

5. After the final sample thaw, place sample(s) 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).

6. Centrifuge samples for 15 minutes at maximum speed.

7. Remove 100 µL of supernatant and add to the LC vials. Freeze until LC-MS/MS analysis using the ZIC-HILIC column. (Assume removal of cyanobacterial peptides in small sample volume is negligible.)

8. Add 8 mL MeOH to each 15 mL tube and vortex well – make sure to cap tubes tightly.

9. Sonicate in a 45° C water bath for 10 minutes and vortex well.

10. Centrifuge samples for 15 minutes at maximum speed.

11. Remove 500-1000 µL of supernatant and add to a labeled LC vial for LC-MS/MS analysis with C18 column for cyanobacterial peptides.

12. If 1000 µL volume added, spike with 5 µL of 1000 µL stock $^{13}C_6$-phenylalanine as a surrogate standard; spike accordingly for smaller sample volumes.

## Dual Extraction of Cyanobacterial Neurotoxins and Hepatotoxins from Samples Preserved with EtOH to 50%

1. Transfer 10 mL 50% EtOH:50% sample to 20 mL glass scintillation vial.

2. Dry in evaporator with $N_2$ gas and low heat to a dry residue.

3. Add 1 mL 0.1% formic acid in water to the tube and vortex well.

4. Sonicate in a 45° C water bath for 10 minutes and vortex well.

5. Freeze sample(s) in -80° C freezer for 30 minutes; thaw in 55° C water bath for 5 minutes.

   a. Perform three cycles of freeze/thaws.

   b. Vortex after each thaw.

6. During freeze-thaw cycles, label LC vials for HILIC analysis and add 300 µL vial inserts to the vials.

7. After the final sample thaw, place sample(s) back 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).

8. Transfer 100 µL to 1.5 mL centrifuge tubes and centrifuge samples for 15 minutes at maximum speed.

9. Transfer supernatant to labeled LC vial inserts. Freeze until LC-MS/MS analysis using the ZIC-HILIC column. (Assume removal of cyanobacterial peptides in small sample volume is negligible.)

10. Add 2 mL MeOH to each scintillation vial and vortex well – make sure to cap tubes tightly.

11. Sonicate in a 45° C water bath for 10 minutes and vortex well.

12. Transfer 500 µL of sample to 1.5 mL centrifuge tubes and centrifuge samples for 15 minutes at maximum speed.

13. Transfer supernatant to a labeled LC vial for LC-MS/MS analysis with C18 column for cyanobacterial peptides.

14. Add 5 µL $^{13}C_6$-phenylalanine to each sample; make sure to calibrate with blank appropriately for analysis of ion suppression (500 µL blank volume + $^{13}C_6$-phenylalanine).

# Extracting Cyanopeptides from Intermediate Process and Finished Drinking Water Samples

*If starting extraction procedure with lyophilization:*

1. Add 100 mL sample to 300 mL freeze flask for lyophilization. If lyophilizing multiple samples (in separate flasks), either use multiple graduate cylinders or rinse graduated cylinder with 100% methanol and let dry briefly in between pouring samples.

2. Freeze sample(s) at -80° C on an angle for at least 1.5 hours, without rubber caps.

3. When sample is completely frozen, place rubber top in -80° freezer for ~5-10 minutes before placing onto freeze flask, and then add flask directly to lyophilizer.

4. Lyophilize for 24-48 hours.

5. Wash cap of freeze flask with 10% acetic acid in water in squeeze bottle and wash down sides. Fill up the freeze flask to the 50 mL mark with 10% acetic acid/water.

6. Transfer the ~50 mL acetic acid volume to two 50 mL Falcon tubes, labeled with the drinking water sample ID and A/B to help identify the two parts of the sample.

7. Cap the tubes and place in the -80° C freezer. Meanwhile, turn on the incubating water bath and set to 50° C.

8. Thaw the sample(s) in the water bath after one hour, vortex, and return back to the -80° C freezer. Repeat freeze thaw cycle two more times.

*If not lyophilizing sample(s) (i.e. the lyophilizer is waiting for maintenance and cannot be used) prior to solid phase extraction (SPE), conduct freeze-thaw cycles on whole samples prior to acidification:*

1. Transfer samples from -20° C freezer into -80° C freezer for 1-2 hours so they fully freeze.

2. Remove samples from -80° C freezer and allow to thaw slightly on benchtop for 30-60 minutes.

3. Put samples in warmed water bath until fully thawed.

4. Remove samples from water bath and allow to cool before putting back into -80° C freezer for an overnight freeze.

5. Complete steps 1-4 until samples have undergone three full freeze-thaw cycles.

6. Once a sample has undergone its third thaw, measure out 100 mL of sample in a graduated cylinder. Add 10 mL glacial acetic acid. Mix and separate into three conical tubes, and incubate on the benchtop for one hour.

7. Before putting amber bottle samples back into the -20° freezer for storage:

    a. Wrap a piece of label tape around the bottle and write "freeze-thaws completed mm/dd/yy"

    b. Make sure to freeze the bottle on its side – you can move other bottles around in the freezer, but try to keep on the same shelf. If you freeze the sample upright, the water will expand differently and the amber bottle is more likely to break.

*After completing freeze-thaw cycles/acidification:*

8. Sonicate the samples in the sonicating water bath at 45° C for 10 minutes.

9. Centrifuge the samples for 15 minutes at maximum speed.

    a. Before centrifuging, weigh each sample using the balance and write the weight on the cap. To appropriately balance the centrifuge, volume may need to be added to some samples to make the sample weights equal – this can be done using the 10% acetic acid from the squeeze bottle.

*While the centrifuge is going…*

10. Grab the following: one 50 mL glass test tube, one Bond-Elut C18 SPE cartridge and one glass scintillation vial per sample.

11. Set up the vacuum manifold for SPE:

    a. Add tips to bottoms of the openings for the SPE tubes.

    b. Attach the C18 SPE tubes into as many openings as there are samples.

    c. Label SPE tubes to help keep track of samples.

    d. Apply two 6-mL volumes distilled water to the SPE tubes. **The flow rate of any liquid through the tubes should be about 1 drop per second at all times.**

    e. Apply two 6-mL volumes 20% methanol (MeOH) to the SPE tubes.

*Solid Phase Extraction:*

12. After centrifuging, pour off supernatant from one half of the sample into a clean glass test tube.

13. Apply the samples to the appropriately labeled SPE tubes.

    a. This will have to be done in several volumes – transferring the supernatant to the clean tubes will allow you to pour into the SPE tube, set down, and pour again without disturbing the pellet from centrifugation.

b. When sample volume in the glass test tube runs out, either close the valve to not let the SPE tube dry out or add more volume from the second 50-mL conical to the glass test tube and keep applying to the C18 cartridge.

14. Once each of the ~50-mL sample extracts are poured through the SPE tubes, apply another 2 6-mL volumes of 20% MeOH to the SPE tubes to wash the C18 material.

15. Label six (20-mL) glass scintillation vials. After the 20% MeOH has gone through all the SPE tubes, release the vacuum and put the scintillation vials under the appropriate SPE tubes.

16. Apply two 6-mL volumes of 100% MeOH to the SPE tubes and let them run drip-dry.

17. Remove the scintillation vials from the vacuum manifold and put them into the evaporator. Dry them down with heat and nitrogen gas (will take several hours).

18. When the extracts are just barely dry, remove them from the evaporator. Resuspend the extracts in 1 mL 70% ethanol.

19. Capping the scintillation vials tightly, sonicate the samples for 10 minutes.

20. Centrifuge the extracts in 1.5 mL tubes for 12 minutes to remove suspended particulates.

21. Transfer the supernatant to labeled amber LC vials.

**Early Life Stage Toxicity Evaluation with Cyanobacterial Toxin Exposures**

**Prep day (Wednesday):**

a. Set up four EK zebrafish spawning tanks (tanks 1-4) in afternoon or evening before experimental start.

b. Bring fresh E2 medium up from fish room 133 for rinsing during chorion removal.

**0 hpf (Thursday):**

Prep

a. Transfer fish to clean water at 8:00 am.

b. Bring fresh E2 medium up from fish room 133 for making exposure media.

c. Make three doses of toxin standard and control media (E2) with the same concentration of methanol (MeOH; 0.2%, unless microcystin(MC)-LR at 0.4%)[1]. Leave media at room temperature of 2084 experimental room for incubation, prior to experiment.

d. Thaw and dilute pronase to 2 mg/mL, making a total of 5 mL (240 uL of 41 mg/mL pronase into 4.76 mL E2). Incubate pronase in behavioral experiment room in lab 2084.

e. Move shaker into behavioral experiment room.

f. Label 12 100 mm glass petri dishes (glass required for pronase treatments) with treatments: Control 1-3, each toxin 1-3.

g. Fish should start spawning ~9am. Monitor fish at 9:10 - if spawning well, collect between 9:20-9:30. A minimum of 1200 embryos are required for each set of experiments.

h.  Once eggs are collected, remove unfertilized and dead embryos, scales, algae and fish waste.

Chorion Removal

i.  Count 150 embryos into each of 12 glass petri dishes[2].

j.  Add 1 mL of Pronase (50 mg/mL) to one petri dish for 2-3 minutes. Hold petri dish at an angle, swirling pronase back and forth. Remove pronase and start rinsing with E2 when two or more chorions come off embryos.

k.  Rinse 3 times with E2 into a waste container.

l.  With final rinse in petri dish, place dish on shaker for 15-20 minutes, observing frequently for number of embryos without chorions.

m.  Rinse 2 more times with E2 into a waste container.

Exposure Start

n.  After final rinse with E2, dose embryos with control media (0.2% MeOH in E2) or cyanotoxin standard treatment (0.2% MeOH in E2 with one of three dose concentrations per toxin).

o.  Immediately place petri dish on a red tray labeled "CAUTION: CYANOTOXINS" in 28 deg. C incubator in 2084 behavioral room[3].

**24 hpf (Friday – start of organogenesis; brains visible):**

a.  Count number of surviving embryos with chorions removed in petri dishes, out of original 150[4] (for all toxins except MC-LR).

b.  Each plate will have the following exposure layout: A-C1 – control, A-C2 – lowest toxin concentration, A-C3 – lake toxin concentration, A-C3 – 10X lake toxin

concentration. Transfer 12 (surviving, dechorionated) embryos for the appropriate concentration into each well.

c. Add fresh media to the appropriate wells – a 2 mL dropper-full into each well should be sufficient.

d. Score embryos using stereomicroscope in room 261 for the following morphologies: delayed development; abnormal pigment, body axis, somites, tail, brain, eyes, ears; edemia: cardiac, yolk sac, abdominal, cranial.

**72 hpf (Sunday – adult feature formation, hatching, transition to movement)**

a. Change media in each of 12 wells.

b. Count, discard and record dead larvae.

c. Record number of larvae per treatment with following morphologies: # normal larvae; # dead; # hatched; delayed development; abnormal circulation, pigment, body axis, somites, tail, brain, eyes, ears, heart, face, fins; edemia: cardiac, yolk sac, abdominal, cranial.

**120 hpf (Tuesday)** – change media and observe for dead larvae

**144 hpf (Wednesday – starting to look for food)**

Scoring

a. Anesthetize one row of larvae using cold (4 C) tricaine[4].

b. Transfer individual larvae onto a slide for microscropic examination.

c. Record number of larvae per treatment with following morphologies: # normal embryos; # dead; air bladder inflation; delayed development; abnormal circulation, pigment, body axis, somites, tail, brain, eyes, ears, heart, face, liver, fins; edemia: cardiac, abdominal, cranial.

d. Freeze 12-well plates before discarding fish waste on Monday.

e. Prep EK zebrafish for next spawning round.

Notes

1. The total volume of medium per toxin will be 60 mL media that will be used throughout the entire six-day experiment for consistency (40 mL for initial exposure in 100 mm glass petri dish; 18 mL for three media transfers in each of three wells).

2. All petri dishes need to have chorions removed and exposures started by 1 pm.

3. Normal survival rate is 65-75% based on experiments with E2.

4. Tricaine will have been made in bulk and stored in the fridge. Typically, "old" tricaine exposures can have adverse effects on the fish, but it is okay to use stored tricaine since this is the last experimental endpoint.

## **Preparation of E2 Medium Spiked with Cyanopeptide Extracts**

- o Samples in LC vials should ideally have a minimum volume of 950 µL for zebrafish exposures and enzymatic bioassays.

1. Pipet the appropriate amounts of sample extract (in 70% MeOH; see table below) into four scintillation vials per sample.

2. Dry the sample extracts in scintillation vials using the evaporator with heating block and $N_2$ gas.

3. Turn on sonicating water bath.

4. As samples dry, remove them from the evaporator.

5. Add E2 medium to 2X volumes, according to the appropriate scintillation vial.

6. Resuspend in sonicating water bath for 10 minutes. After removing from bath, vortex again.

7. Transport samples immediately to Carvan laboratory and/or freeze at -20 C until use.

| Scintillation Vial – Point of Exposure | Total Volume (mL) Needed | Volume to Resuspend/Rinse w/ (mL) | Fraction of Total Volume (47.5 mL) | Volume Extract to Dry (µL) |
|---|---|---|---|---|
| 0 hpf | 25 | 12.5 | 0.526 | 394.7 |
| 24 hpf media change | 7.5 | 3.75 | 0.158 | 118.4 |
| 72 hpf media change | 7.5 | 3.75 | 0.158 | 118.4 |
| 120 hpf media change | 7.5 | 3.75 | 0.158 | 118.4 |

**CURRICULUM VITAE**

# Chelsea A. Weirich

## Education

**Doctor of Philosophy, Environmental Health Sciences**                    **May 2017**

University of Wisconsin (UW)-Milwaukee, Milwaukee, WI

Dissertation: "Cyanobacteria and Cyanotoxin Ecology in Lakes and Drinking Water"

**Bachelor of Science, Biological Sciences**                    **May 2011**

St. Norbert College, De Pere, WI

**Study Abroad**                    **Fall 2010**

Lancaster University, Lancaster, United Kingdom

## Research Experience

**Sr. Research Associate**          **S. C. Johnson & Son, Inc. (February 2017-Current)**

**Research Assistant**          **University of Wisconsin-Milwaukee (May 2011 – February 2017)**

## Professional Development and Training

❖ Basic Training: Good Laboratory Practice (Society of Quality Assurance, March 2017)

- ❖ Essentials of LC-MS (Pittsburgh Conference & Exposition on Analytical Chemistry and Applied Spectroscopy, March 2017)

- ❖ Basic HPLC Method Development (Pittsburgh Conference & Exposition on Analytical Chemistry and Applied Spectroscopy, March 2017)

- ❖ Basic HPLC – Fundamentals, Applications, and Troubleshooting (Pittsburgh Conference & Exposition on Analytical Chemistry and Applied Spectroscopy, March 2017)

- ❖ Course: Teaching and Learning in College (UW-Milwaukee, Spring 2016)

- ❖ LC/MS/MS Basic Triple Quadrupole Training Program (AB Sciex, January 2012)

- ❖ Responsible Conduct of Research: "Science and Research Integrity Professional Development Series" (UW-Milwaukee, Fall 2011)

## **Publications**

1. Miller, T. R., L. J. Beversdorf, **C. A. Weirich**, and S. L. Bartlett. 2017. Cyanobacterial toxins of the Laurentian Great Lakes, their toxicological effects, and numerical limits in drinking water. In Press at *Marine Drugs*.

2. Beversdorf, L. J., **C. A. Weirich**, S. L. Bartlett, and T. R. Miller. Occurrence of 15 cyanobacterial toxins and bioactive compounds in six eutrophic Wisconsin lakes. *Toxins* 9(2):1-21.

3. Ross, J. J., D. H. Zitomer, T. R. Miller, **C. A. Weirich** and P. J. McNamara. 2016. Pyrolysis removes common microconstituents triclocarban, triclosan, and

nonylphenol from biosolids. *Environmental Science: Water Research & Technology* 2:282-289.

4. **Weirich, C. A.** and T. R. Miller. 2014. Freshwater harmful algal blooms: toxins and children's health. *Current Problems in Pediatric and Adolescent Health Care* 44(1):2-24.

## Manuscripts In Preparation and Review

1. **Weirich, C.A.** and T. R. Miller. Long-term (20-year) interannual variation in bloom-forming cyanobacteria and nutrients, climate, and limnology in a eutrophic lake. In preparation for *Hydrobiologia*.

2. Brentano, D. M., E. Petry, **C. A. Weirich**, T. R. Miller, and M. M. Petrucio. What is the density effect of the dominant freshwater *Cylindrospermopsis raciborskii* on the concentration of Saxitoxin? In preparation for *Water Research.*

3. Beversdorf, L. J., K. Rude, **C. A. Weirich**, S. L. Bartlett, M. Seaman, P. Biese, T. Gosz, M. Suha, C. Stempa, C. Shaw, and T. R. Miller. Indicators of cyanobacterial peptide levels in raw drinking water. In preparation for *Water Research*.

4. Bartlett, S. L., **C. A. Weirich**, L. J. Beversdorf, and T. R. Miller. High resolution monitoring of microcystins shows significant sub-daily variation. In preparation for *Environmental Science and Technology*.

## Published Reports

Miller, T. R., L. J. Beversdorf, **C. A. Weirich**, and S. L. Bartlett. Cyanobacterial harmful algal blooms and human health in the Great Lakes region. Published as an appendix to: Health Professionals Advisory Board. (2017). "Human Health Effects of Cyanobacterial Toxins in the Great Lakes Region: A Science and Monitoring Assessment".

## Teaching Experience

❖ **Guest Lecturer:**

- "Legionella: Mechanisms of Infectious Disease", Biomedical Science 775 – Mechanisms of Infectious Disease at University of Wisconsin-Milwaukee (Spring 2016)

- "Detecting Cyanotoxins in Lakes and Drinking Water", Public Health 101 at University of Wisconsin-Milwaukee (Fall 2015)

- "Water Quality and Waterborne Disease", Public Health 101 at University of Wisconsin-Milwaukee (each semester, Fall 2013-Spring 2015)

- "Water Quality and Waterborne Disease", Public Health 302 at Carroll University (Fall 2013)

❖ **Discussion Leader, Environmental Health Seminars:** Cyanobacteria and Cyanotoxins (Fall 2015); Ebola (Spring 2015); Public Health Microbiology (Fall 2014)

❖ **Private Tutor, Basic Statistics (Summer 2013):** 25 hours working with Masters in Public Health Student to prepare for statistics course

❖ **Laboratory Teaching Assistant, St. Norbert College Department of Biological Sciences (Spring 2011):**

- Assisted with BIOL 365 Immunology – equipment/laboratory prep, mouse handling, peer student mentorship

- Skills learned: Northern blotting and enzyme-linked immunosorbent assays

## Student Mentoring

Summer 2016      **Eugene Kim**, Elmbrook High School: Growth Curves for Cyanobacterial Cultures, University of Texas Culture Collection

Summer 2016      **Allison Tomczyk,** Pio Scholar, Carroll University: Lake Winnebago and Green Bay Nutrients, Algal Pigments, and Cyanotoxins Analysis 2016

Summer 2016      **Rachel Kutzner,** Pio Scholar, Carroll University: Lake Winnebago and Green Bay Nutrients, Algal Pigments, and Cyanotoxins Analysis 2016

Fall 2015- Summer 2016      **Chelsea Moore**, Undergraduate, UW-Milwaukee: Total Organic Carbon Analysis of Raw and Finished Drinking Water; Lake Victoria Cyanotoxin Profile Using LC-MS/MS; Temporal *Escherichia coli* Monitoring for Three Milwaukee Beaches

Spring – Summer 2016      **Bridget Griffin,** Undergraduate, UW-Milwaukee: Nitrite by Depth in Lake Winnebago in 2012 and 2014

Summer 2015 - Spring 2016      **Joseph Weirich**, UW-Eau Claire (2013): Cyanotoxins in Wisconsin Sportfish Tissues

Summer 2015/      **Ben Weirich**, Undergraduate, UW-Stevens Point: Chemical

Winter 2016          Analysis of *Microcystis* Culture; Ammonia Profile by Depth in

Lake Winnebago

Summer 2015/      **Cara Ahrenhoerster**, Undergraduate, Hamline University:

  Winter 2016        Cyanotoxins and Total Organic Carbon in Raw Drinking

Water; Total Dissolved Phosphorus Depth Profile in Lake

Winnebago

Summer 2015       **Allison Driskill**, Undergraduate, Marquette University: Cyanotoxins

in Finished Drinking Water

Spring 2015          **Keaunis Grant, Brian Gayfield, Ame Xiong, Neziah Bowers**,

UW-Milwaukee McNair Scholars: Pharmaceutical and

Personal Care Products in the Milwaukee River

Summer 2014       **Kayla Rude**, Pio Scholar, Carroll University: Algal Toxin Dynamics

in a Eutrophic Lake and Indicators of Toxins in Raw Drinking

Water

Summer 2014       **Sheila Marton**, Pio Scholar, Carroll University: Environmental

Monitoring of Lake Michigan Beach Water for *Escherichia*

*coli*

Summer 2013       **Nicholas Myers, Kimberly Siegler, Jason Zellmer**, UW-

Milwaukee BioMath: Numerical Simulation of Nutrient

Dependent Competitive Algal Growth in a Eutrophic

Environment

Summer 2013       **Angelica Sanchez**, UW-Milwaukee McNair Scholar:

Spatiotemporal Cyanotoxin Dynamics in Wisconsin
Eutrophic Lakes

Summer 2013  **Clayton Wollner,** UW-Milwaukee McNair Scholar: Temporal
Variation of *Escherichia coli* Levels at a Local Beach in
Response to Hourly Environmental Changes

Summer 2012  **Oluwadamilola R. Oluwatosin**, UW-Milwaukee Undergraduate
Research Program: *Escherichia coli* and Fecal Coliform
Bacteria at Popular Milwaukee Beaches

## **Invited Talks**

1. "A Local-to-Global Characterization of Cyanotoxins in Freshwater Lakes". Society
   for Freshwater Science, Milwaukee, WI (2015)

2. "Linking Limnology to Cyanotoxins in Drinking Water". On Public Health Seminar
   Series, UW-Milwaukee Joseph J. Zilber School of Public Health, Milwaukee, WI
   (2015)

3. "Cyanobacterial Toxins in Lake Winnebago Surface Waters and Drinking Water".
   Riveredge Nature Center Student Research Symposium, Saukville, WI (2014)

4. "Covariates of Cyanobacterial Biomass Over 1.5 Decades In A Eutrophic Lake".
   Ecological Society of America, Minneapolis, MN (2013)

## **Posters**

1. "A Survey of Blue-Green Algal Toxin Monitoring and Detection in Lake Winnebago". Wisconsin Public Health Association 2016 Annual Conference

2. "Interannual Correlates of Cyanobacterial Biomass". Global Lakes Ecological Observatory Network 17th All-Hands Meeting, Chuncheon, South Korea (2015)

3. "Toward a Global Characterization of Toxins in Lakes". Gordon Research Conference: Mycotoxins and Phycotoxins, Easton, MA (2015)

4. "Cyanotoxins in the Lake Michigan Watershed and Drinking Water". Great Lakes Beach Association, Toronto, Montreal, Canada (2014)

5. "Challenging the Chlorophyll Management Practice: A Call for Different Water Quality Monitoring Strategies for Blue-Green Algae". Midwest Aquatic Plant Management Society, Lombard, IL (2014)

6. "Towards A Global Characterization of Cyanotoxins in Freshwater Lakes – GLEON Project Update". Global Lakes Ecological Observatory Network 15th All-Hands Meeting, Bahía Blanca, Argentina (2013)

7. "Home Grown Research: Current Environmental Health Projects from PhD Students at Zilber School of Public Health". Wisconsin Public Health Association (WPHA)-Wisconsin Association of Local Health Departments and Boards Annual Meeting, Wisconsin Dells, WI (2013)

8. "Scales of Variability in Microcystin Concentrations Related to Limnological Variables in Lake Winnebago". Global Lakes Ecological Observatory Network (GLEON) 14th All-Hands Meeting, Mulranny, Ireland (2012)

**<u>Awards</u>**

- ❖ **Public Health Doctoral Student Award** (December 2015; $1669)

- ❖ **Science Across Virtual Institutes (SAVI) Travel Award:** GLEON 17 All-Hands Meeting (October 2015)

- ❖ **South Milwaukee Lion of the Year Award** (May 2015)

- ❖ **Leo Club Advisor Outstanding Service Award** (May 2015): Lions Clubs International Award provided to advisors of teen volunteer clubs for exceptional service

- ❖ **UW-Milwaukee Graduate Student Travel Award:** Gordon Research Conference (Spring 2015)

- ❖ **"Crowd Pleaser" Award:** Presentation at Zilber School of Public Health First Annual Graduate Student Research Symposium (January 2014)

- ❖ **UW-Milwaukee Graduate Student Travel Award:** GLEON 15 All-Hands Meeting (Fall 2013)

- ❖ **Lion Paw Award:** From Lions Club President (Lions Clubs International), "Young Lion with loyalty, eagerness to learn and enthusiasm for South Milwaukee Lions Club and Lions International, Inc. Organization" (May 2013)

- ❖ **Winner, WPHA Student Graphic Contest for National Public Health Week** (Spring 2013)

- ❖ **UW-Milwaukee Graduate Student Travel Award:** GLEON 14 All-Hands Meeting (Fall 2012)

- ❖ **Chancellor's Graduate Student Award,** University of Wisconsin-Milwaukee (2011-2012)

- ❖ **Dean's Honors List**, St. Norbert College (Spring 2011)

**Outreach**

❖ **Diversity Matters 1ˢᵗ Annual Public Health Internship and Career Fair** (April 2016)

❖ **Ambassador, Miller Laboratory: Wisconsin Lakes Partnership Network meeting** (January 2015)

❖ **Mentor, Wisconsin Allegiance for Minority Participation (WiscAMP):** Presentation at WiscAMP Boot Camp January 2015; Assistance during Fall 2014 fieldwork with students in Milwaukee River and Spring 2015 laboratory analysis

❖ **Guest speaker, Carroll University Chemistry Club (Fall 2014):** Presentation on "Graduate School Opportunities"


**Professional Societies**

❖ **SQA** – Society of Quality Assurance (March 2017-Present)

❖ **MAPMS** – Midwest Aquatic Plant Management Society (February 2014-Present)

❖ **APHA** – American Public Health Association (February 2014-Present)

❖ **WEHA** – Wisconsin Environmental Health Association (September 2013-Present)

❖ **WEHN** – Wisconsin Environmental Health Network (February 2013-Present)

❖ **GLEON** – Global Lakes Ecological Observatory Network (June 2012 – Present)

❖ **WPHA** – Wisconsin Public Health Association (April 2012 – Present)


**Service & Leadership**

1. **Lions Club International**, South Milwaukee Lions Club (2011-Present):

- Advised teens as part of youth volunteer initiative in South Milwaukee and Southeastern Wisconsin
- Volunteer Coordinator, South Milwaukee Lionsfest (2015-2017)

2. **American Public Health Association (APHA) Campus Liaison** (2014-2016)
   - Coordinated activities and promoted student membership for APHA for graduate students in Public Health at UW-Milwaukee

3. **Zilber Accreditation Self-Study Research Chapter 3.1 Workgroup,** Joseph J. Zilber School of Public Health (2015-2016): Providing input on measurable objectives for research within School of Public Health

4. **Graduate Program Committee Doctoral Student Representative**, Joseph J. Zilber School of Public Health (2014-2015)

5. **Public Health Graduate Student Association (PHGSA)** at UW-Milwaukee (2013-Present): Treasurer (2014-2015), Social Media Chair (2013-2014)

6. **Graduate Student Advisory Council (GSAC)** at UW-Milwaukee (2012-2013)

7. **Colleges Against Cancer**, St. Norbert College Chapter (2007-2011). Secretary and Co-President