Human Health Effects of Cyanobacterial Toxins in the Great Lakes Region: A Science and Monitoring Assessment

A report submitted to the
International Joint Commission by the
Health Professionals Advisory Board

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On the front cover: satellite image of algal blooms impacting Pelee Island in western Lake Erie for summer 2015, NASA.

Executive Summary

The International Joint Commission's Health Professionals Advisory Board has undertaken a science and monitoring assessment for the cyanobacteria and associated toxins common to the North American (Laurentian) Great Lakes basin, and the human health impacts arising from those toxins. This report, developed with the assistance of a contractor that surveyed available literature, describes challenges for the region in maintaining safe aquatic environments for recreation and the production of potable drinking water due cyanobacterial harmful algal blooms. This report builds upon the board's previous work on cyanotoxins and human health, and updates previously presented topics of interest to health professionals on the occurrence of cyanobacterial harmful algal blooms (cyanoHABs) in the Great Lakes, the prevalence and emergence of different cyanobacteria and associated cyanotoxins, and the human health effects of cyanotoxins (HPAB, 2013). This work expands the review to also consider topics of interest to water treatment plant managers: current methods for cyanotoxin detection, challenges to the removal of cyanotoxins during drinking water treatment, and current research on the status and application of numerical limits on several cyanotoxins in finished drinking water to protect human health. The full technical review is found in the report appendix.

Cyanobacteria are bacteria that commonly grow in environments around the world, including in the fresh waters of the Great Lakes. Where waters have abundant or excess nutrients, like nitrogen or phosphorus, cyanobacteria will grow rapidly and densely, or bloom. Such blooms in the Great Lakes often float on surface water as a mat of blue-green scum, though the blooms can extend deep below the surface. Due to the diversity of cyanobacteria, blooms can also be stimulated by factors other than nutrients, such as water temperature, wind or current patterns. Blooms often occur during sustained periods of warm surface water temperatures, such as the summer and early fall.

Some blooms of cyanobacteria may produce toxic compounds (cyanotoxins), which can harm animal and human health. The incidence of cyanoHABs and their toxins are increasing across the Great Lakes region as a result of increased nutrient pollution of waterways and climate change. The number of cyanobacterial species in the region is also increasing, with species from temperate and tropical environments recently identified in the Great Lakes (Liu et al., 2006;

Hong et al., 2006; Conroy et al., 2007; Boyer, 2008; Bridgeman and Penamon, 2010; Davis et al., 2015).

There are a multitude of cyanotoxins associated with cyanoHABs in the Great Lakes. Their effect upon human and animal health depends upon on the species and strain of bacteria present, and can damage the liver (liver toxins), the nervous system (neurotoxins), and the skin (dermatoxins). The most commonly detected cyanotoxins produced by cyanobacteria in the Great Lakes include microcystins (liver toxin), cylindrospermopsin (liver and dermatoxin), and anatoxin-a (neurotoxin) (Boyer et al., 2007), although the potential presence of other cyanobacterial toxins in freshwater lakes is an emerging health concern. For instance, the apparent spread of tropical or subtropical cyanobacterial species capable of producing saxitoxin into northern lakes, including the Great Lakes, suggests this toxin may become more prevalent in the Great Lakes (Boyer, 2008; Sinha et al., 2012; Davis et al., 2015). Research indicates the potential for other human health threats from cyanotoxin exposure, including cancers, and reproductive and developmental toxicity. Cyanotoxins may remain inside the cyanobacteria cells, may be expelled in large concentrations from the cell into their surrounding waters, or may be released when bacteria break down.

Ingestion of, or exposure to, water polluted by cyanotoxins can harm animals and humans. Consequently, cyanoHABs and cyanotoxins threaten recreational waters and drinking water supplies. It is very difficult to monitor beaches and drinking water to determine cyanotoxin presence for the many possible cyanobacterial species and toxins that might be present. A bloom may consist of multiple cyanobacterial strains and multiple forms of toxins, which complicates the search for simple testing strategies. A standard method to test for multiple bacteria and toxin forms is not presently available to water managers, though laboratory research pursuing standard methods is ongoing. There are considerable gaps in knowledge about optimal water treatment strategies for the multiple Great Lakes cyanobacteria and their varied toxic products. This is of considerable concern given the Great Lakes' role as the drinking water supply for 35 million people in both countries. These gaps highlight both the importance of preventing cyanoHABs and research and investment in water treatment technologies.

Health agencies are challenged to set general exposure limits for cyanotoxins due to the wide range of cyanobacteria and cyanotoxin strains and their broadly differing health impacts. World Health Organization set guidelines specifically for microcystin at 1 microgram per liter in

drinking water based on studies of adult liver toxicity. This standard is backed by sufficient data to broadly apply to adult populations, though there are questions on how well this guideline protects children. Limited experimental data and monitoring capacity makes it difficult to set exposure limits for individual cyanotoxins.

While health care providers understand many of the human health hazards associated with cyanotoxins, the ability to associate environmental cyanotoxin exposure with individual cases and illness diagnoses remains a public health challenge. Recent attempts to address this challenge by state and national health agencies include leveraging existing hospital admittance records as well as new national monitoring and surveillance systems.

The public health risk of cyanotoxin exposure can be reduced with improvements in drinking water monitoring and laboratory testing. While a thorough examination of management strategies is beyond the scope of this report, **the HPAB recommends the following strategic improvements to support public health protection**:

1) Improvements in drinking water treatment technologies or management of existing modern technologies to ensure cyanotoxins are efficiently removed without the production of toxic byproducts. There are multiple cyanotoxins and different treatment strategies affect them differently.
2) Drinking water treatment plant (DWTP) operations should include robust monitoring of prevalent cyanotoxins and optimization for different source conditions and treatment systems. Monitoring data suggests that current treatment strategies are not always effective at removing or destroying all types of cyanotoxins and occasionally fail to reduce concentrations to below the World Health Organization's safe drinking water level of 1 microgram per liter of microcystin per day. While the recent (August 2014) drinking water ban in Toledo, OH focused public and government attention, more activity is needed.
3) Further research on optimal drinking water treatment approaches for Great Lakes cyanotoxins should be developed. DWTPs currently practice a range of treatment regimes, but typical treatment processes may not be effective for cyanotoxins. Many uncertainties on effective removal of cyanotoxins from DWTPs remain.

4) Improvements are needed in cyanotoxins laboratory testing, and to establish uniform methods and practices across laboratories, including standards and quality assurance. Promising technologies include methods for multiple toxins and toxin congeners along with methods to measure cyanotoxins directly or indirectly.

5) An examination of drinking, source water and beach monitoring strategies should include provisions for regular and improved monitoring of cyanotoxins, reporting of such results to the public in a timely fashion, and continued development of predictive models for forecasting cyanoHABs and their toxins given predictions of increasingly warm waters for longer periods of the year.

6) Additional toxicity studies are needed to improve the development of strong numerical limits for a broader range of cyanotoxins beyond microcystins. These include using purified cyanotoxins in animal studies, additional work examining exposure from inhaled aerosols (e.g. from shower bathing), and skin irritation, and additional data on chronic effects from cyanotoxin exposure such as tumor promotion and cancer.

These recommendations seek to improve the monitoring and abatement of human risks from harmful cyanobacterial blooms. They should augment, but cannot replace, efforts to prevent the increasing severity and frequency of blooms by addressing nutrient pollution and climate-mediated temperature changes in the Great Lakes region.

Table of Contents

Abbreviations

HAB = Harmful Algal Bloom
cyanoHABs = cyanobacterial harmful algal blooms
N = Nitrogen
P = Phosphorus
MC = microcystin (all possible congeners)
OATP = organic anion transporting polypeptide
WHO = World Health Organization
DWTP = Drinking Water Treatment Plant
BMAA = Beta-Methylamino-L-alanine
LPS = Lipopolysaccharide
b.w. = body weight
DWI/BW = Drinking Water Intake to Body Weight Ratio
TTMCs = Total Toxic Microcystins

Tables

Figures

1. Introduction

The Health Professionals Advisory Board has undertaken a science and monitoring assessment for the cyanobacteria and associated toxins common to the North American (Laurentian) Great Lakes basin, and the human health impacts arising from those toxins. This report, developed with the assistance of a contractor that surveyed available literature, describes challenges for the region in maintaining safe recreational aquatic environments and the production of potable drinking water due to cyanobacterial blooms. This report builds upon the board's previous work on cyanotoxins and human health, and updates previously presented topics of interest to health professionals on the occurrence of cyanobacterial harmful algal blooms (cyanoHABs) in the Great Lakes, the prevalence and emergence of different cyanobacteria and associated cyanotoxins, and the human health effects of cyanotoxins (HPAB, 2013). This work also expands the review to also consider topics of interest to water treatment plant managers: current methods for cyanotoxin detection, challenges to the removal of cyanotoxins during drinking water treatment, and current research on the status and application of numerical limits on several cyanotoxins in finished drinking water to protect human health. The full technical review is found in the report Appendix.

Cyanobacteria are ubiquitous phototrophic bacteria that inhabit diverse environments across the planet. They dominate many eutrophic lakes impacted by excess nitrogen (N) and phosphorus (P) forming dense accumulations of biomass known as cyanobacterial harmful algal blooms or cyanoHABs. Their dominance in eutrophic lakes is attributed to a variety of unique adaptations including N and P concentrating mechanisms, nitrogen fixation, colony formation that inhibits predation, vertical movement via gas vesicles, and the production of toxic or otherwise bioactive molecules.

The ecology, toxicology, and physiology of cyanobacteria and cyanoHABs have been studied for more than a century providing rich information about their impacts on aquatic resources. Cyanobacteria (known colloquially as "blue-green algae") are a ubiquitous and diverse group of photosynthetic gram-negative bacteria that inhabit both terrestrial and aquatic habitats throughout the planet. These bacteria were responsible for the oxygenation of early earth

more than three billion years ago and are the precursors to chloroplast organelles in Eukaryotic algae and higher plants (McFadden, 2014).

An increasingly recognized characteristic of cyanobacteria is their ability to produce toxic or otherwise bioactive compounds that affect animal and human physiology. In most cases, these are secondary metabolites (not necessary for normal functioning of the bacterial cells) but presumably provide some largely unknown benefit to the organism). Multiple compounds are being discovered (including congeners, methylated and other variants) whose toxicity is incompletely characterized to date. Both the full diversity and toxicity of such compounds (in isolation and in mixtures) produced in nature is not known.

Many of these are known potent toxins or suspected as being harmful to a variety of organisms. The presence of these compounds in **a**quatic environments, particularly lakes, presents challenges for recreational water quality management and drinking water production. Until recently, measuring cyanotoxins in surface water was technically challenging. Present methods are able, using multiple methods in a step-wise fashion to measure low concentrations of a broad array of toxins with good accuracy, but a systematic cyanotoxin monitoring system has yet to be established. In the absence of such a monitoring system, little is known about spatial and temporal patterns of cyanotoxins in the Great Lakes. Triggers for toxin production and toxin release are poorly understood. These provide challenges for characterizing, monitoring, and predicting toxin formation in the environment. Cyanotoxins may remain in the bacterial cells (intracellular), be actively excreted, or be released by cell lysis. Human activities, including drinking water treatment, may affect these states. Another challenge is establishing human health standards (the state of current toxicological knowledge) for these toxins to allow for environmental assessment.

2. Health risks

Cyanotoxins associated with cyanoHABs in lakes are generally divided into three groups: liver toxins, neurotoxins and dermatotoxins. Less conclusive evidence associates toxins with carcinogenicity and reproductive and developmental toxicity. Recently discovered

cyanobacterial species in freshwater lakes, and the potential presence of their toxins, represent an emerging health concern.

2.1. Liver toxins

In the Great Lakes region, the most commonly observed or targeted cyanobacterial liver toxins are the microcystins (MCs) (Vanderploeg et al., 2001; Boyer, 2007; Rinta-Kanto et al., 2009). MCs and at least 85 different variants have been detected in lakes or cell cultures (Sivonen and Jones, 1999). MCs covalently bind to and inhibit protein phosphatases type 1 and 2A in eukaryotic cells, though other proteins and enzymes may also be inhibited (Honkanen et al., 1990). MCs are specifically transported into hepatocytes and across the blood-brain barrier by organic anion transporter polypeptides (OATPs) (Fischer et al., 2005).

While MCs appear to be the most prevalent cyanotoxins, cyanobacteria also produce other liver toxins. Nodularin is a hepatotoxic cyclic pentapeptide with similarity to MCs. It also inhibits protein phosphatases, but is primarily produced by Nodularia spumigena in brackish waters and may also occur in freshwaters (Beattie et al., 2000). Cylindrospermopsin and its analogs (e.g. deoxycylindrospermopsin) are produced by Cylindrospermopsis raciborskii and some other genera (e.g. Aphanizomenon) and they can cause liver and kidney toxicity by inhibiting the synthesis of protein and glutathione along with (see below and (Runnegar et al., 2002)). C. raciborskii is now considered an invasive species in temperate regions, including the Great Lakes (Hong et al., 2006; Conroy et al., 2007).

2.2. Neurotoxins

Anatoxin-a and homoanatoxin-a are nicotinic acetylcholine agonists and Anatoxin-a(s) is an organophosphate that irreversibly binds to acetylcholinesterase in peripheral nerve cells (Cook et al., 1988; Cook et al., 1989; Cook et al., 1991; Hyde and Carmichael, 1991; Thomas et al., 1993). The net effect of all anatoxins is uncontrolled activation of nicotinic and muscarinic acetylcholine receptors resulting in respiratory dysfunction and potentially paralysis. Anatoxin-a and homanatoxin-a are produced by species of *Anabaena*, *Oscillitoria* and *Aphanizomenon* among others (Devlin et al., 1977; Edwards et al., 1992). *Anabaena* species, particularly *Anabaena flos-aquae*, have been shown to produce anatoxin-a(s). (Carmichael et al., 1979).

Recently, the possible chronic neurotoxin beta-N-methylamino-L-alanine (BMAA) was found in diverse species of cyanobacteria (Cox et al., 2005). Cox et al. recently found that BMAA is also produced by every major order of cyanobacteria including common freshwater bloom forming species such as Microcystis, Anabaena and Planktothrix species (Cox et al., 2005). Saxitoxin and its more than 50 analogs are tricyclic alkaloid neurotoxins that permanently block voltage-gated sodium channels in nerve cells causing dysfunction and paralysis.

Similarly, historically, *Lyngbya wollei* was associated with lakes in the southwestern United States, but has now invaded some parts of the Great Lakes, especially the western basin of Lake Erie (Bridgeman and Penamon, 2010). This organism blooms in thick mats at the sediment surface in shallow zones, and it produces two common saxitoxin variants as well as six that are unique to this species (Carmichael et al., 1997; Onodera et al., 1997).

2.3. Dermatoxins

Rash and contact dermatitis are reported anecdotally in cases of human exposure to cyanoHABs (Stewart et al., 2006), and may be caused by one of hundreds of bioactive metabolites produced by cyanobacteria. It has been suggested that the lipopolysaccharides (LPS) of cyanobacteria may contribute to human illness, particularly epidermal allergic reactions. The origin of skin impacts from cyanoHABs exposure is an ongoing area of study.

Health agencies have issued guidelines for their jurisdictions to protect against exposure to individual cyanotoxins, which occur most commonly from water ingestion and recreational activities, as seen in Table 1. One of the longest standing guidelines for microcystins in drinking water, 1 µg/L from World Health Organization (WHO), has been in place for 16 years and is commonly applied when referencing dangerous or unsafe cyanotoxin levels, as the supporting data underlying the guideline is relatively substantial compared to those for other cyanotoxins. Although commonly used to indicate "safe" drinking water, this guideline is outdated and incomplete for this purpose, being silent on the presence of other toxins.

Table 1. Numerical limits for cyanotoxins in drinking water based on varying critical studies and toxic endpoints discussed in this report and appendix.

Toxin	Microcystins					Cylindrospermopsins			Saxitoxins
Source	WHO	EPA (U.S. and Canada)		This Report	This Report	EPA (U.S. and Canada)		This Report	This Report
Critical study	Fawell et al. 1999a	Heinze 1999		Li et al. 2015	Chen et al. 2011	Humpage and Falconer 2002,2003		Humpage and Falconer 2002,2003	CONTAM
LOAEL/NOAEL (µg/kg/day)	40	50		5	1	30		30	0.5
End point	Liver Toxicity	Liver Toxicity		Central Nervous System Toxicity	Male Reproductive Toxicity	Kidney Toxicity		Kidney Toxicity	Peripheral Nervous System Toxicity
Age of Exposed	Adult	<6 yrs	>6 yrs	<6 yrs	<6 yrs	<6 yrs	>6 yrs	<6 yrs	<6 yrs
DWI/BW/day (L/kg/d)	0.03	0.15	0.03	0.15	0.15	0.15	0.15	0.15	0.15
Uncertainty Factor	1000	1000	1000	1000	1000	300	300	1000	3
Guideline Value (µg/L)	1	0.3	0.7	0.03	0.01	0.7	3	0.2	0.3

Establishing numerical limits for individual cyanotoxins has many challenges. These include:

1. There have been few repeat oral dose animal studies using purified cyanotoxin. These studies have traditionally served as the basis for developing numerical limits since ingestion is likely the primary route of cyanotoxin exposure.
2. Toxicity studies are needed to assess toxicity, including inhaled aerosols (e.g. from shower bathing) (EPA, 2016) and skin irritation.
3. Chronic effects such as tumor promotion and cancer have not been considered in developing numerical limits for cyanotoxins, primarily due to a lack of data.
4. Guideline values should be matched closely with monitoring capabilities. At present it is not clear if this is the case. For example, there is currently no known method that targets total toxic microcystins (TTMC).
5. Not all current standards address vulnerable populations such as children consuming higher amounts of water.
6. Consideration is needed for persons with underlying conditions that may make them particularly sensitive to the effects of cyanotoxins.

7. Consideration should be given to the possible effects of mixtures of cyanotoxins commonly observed in nature.

While health care providers understand many of the human health hazards associated with cyanotoxins, the ability to associate environmental cyanotoxin exposure with individual cases and illness diagnoses remains a public health challenge. Recent attempts to address this challenge include leveraging existing hospital admittance records as well as new national monitoring and surveillance systems. In New York State, 228 hospital visit records were linked to environmental exposure to harmful algal blooms using the recorded World Health Organization's International Classification of Disease (ICD) (Figgat et al, 2016). The visits, recorded from 2008 - 2014, resulted in multiple diagnoses and occurred throughout all four seasons. Nationally in the United States, the US Centers for Disease Control and Prevention launched a monitoring framework in 2016, the One Health Harmful Algal Bloom System (OHHABS, https://www.cdc.gov/habs/ohhabs.html), to provide for long-term monitoring and reporting of potential public health events due to cyanotoxin exposure and support public advisories related to harmful algal blooms in the United States. Relying on data sharing from participating states and territories, the system enables reporting on human and animal illness from exposure to cyanoHAB events in marine, brackish and freshwater environments.

3. Drinking water management strategies

The Great Lakes are currently used as a source of drinking water for over 35 million people. Lake Erie is the most affected by cyanotoxins and an estimated 11 million people rely on Lake Erie for drinking water. As such there is great interest in drinking water treatment strategies to remove cyanotoxins in this region.

In a recent survey of finished drinking water supplies from 24 plants in the United States, 75% of samples tested positive for microcystins (MCs) and some samples contained concentrations unacceptable for human consumption (Carmichael, 2000). In 2013 MCs were detected in finished drinking water from the Carroll County Township drinking water facility in Ohio, which draws water from Lake Erie's western basin. Levels spiked to just over 3.5 μg/L in finished water coinciding with a large cyanoHAB event that produced over 15 μg/L in raw intake

water (Figure 1). Then in 2014 MCs were detected in finished drinking water at the Toledo drinking water treatment plant at nearly 2.5 µg/L, 1.5 µg/L and 1 µg/L on three separate occasions that did not necessarily coincide with spikes in raw water.

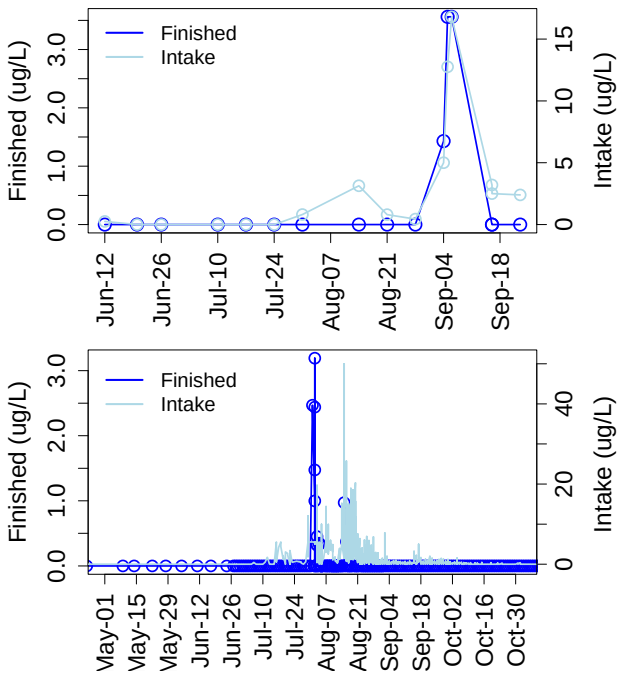

Figure 1. Concentrations of MCs in finished and intake drinking water at Carroll County (Top) and Toledo (bottom), OH plants in 2013 and 2014, respectively.

Optimal water treatment processes for one cyanotoxin may not work for another. Sedimentation alone does not remove dissolved cyanotoxins or those in buoyant cells. However, since Great Lakes microcystins are largely bound inside cyanobacterial cells, the bulk of MCs can be removed by this method. Rapid sand filtration, a common treatment technique, may actually lyse cells, releasing cyanotoxins. Chlorination has variable (species dependent) effects on destroying cyanotoxins, and the effectiveness of both chlorination and ozonation appear to be dependent on various factors including the species of chlorine, pH, temperature, and organic matter in the water being treated. Activated charcoal filtration, UV treatments (with additional catalysts), nanofiltration and reverse osmosis appear to hold promise, but ideal treatment strategies for many toxins are still unknown, and these are not in widespread use in the Great Lakes. Given that a particular treatment strategy may vary greatly in effectiveness with different cyanobacteria and raw water conditions, strategies may need to be tested and optimization of treatment may well vary from one location, plant or HAB event to another.

4. Toxin monitoring approaches

Cyanotoxin detection methods have been developed largely in the form of biological or immunological assays and analytical chemistry for laboratory measurements. The major caveats of these methods include inconsistency and unavailability among toxin standards (or suitable substitutes/surrogates), lack of standardization among laboratory measurements, costs of materials or instrument usage, and the capacity of each method to measure all fractions of the total toxin pool within a sample (i.e. intracellular, covalently-bound and dissolved) as well as all toxin variants that may exist in nature.

Typical analytical methods screen for a few select congeners, though there may be many potentially present or some structures that have yet to be described. There are some methods to address this analytically by converting all congeners to a common end-product. A tiered approach to testing may provide one practical workflow for assessing most variants of the most likely cyanotoxins to be encountered in the Great Lakes. However, the capacity for such testing in the region and procedures for standardizing assays and to ensure uniform testing procedures across the region would be needed to produce reliable and comparable results.

5. Environmental occurrence and tracking of CyanoHABs

Perhaps the most noticeable characteristic of many cyanoHAB species is their ability to multiply to high densities forming macroscopic colonies or groups of cells covered in a mucous polysaccharide sheath. In some cases, these colonies may coalesce into free-floating mats or "scums" on the surface of the lake with a bright blue or blue-green appearance due to the presence of C-phycocyanin, their major light harvesting pigment (Figure 2).

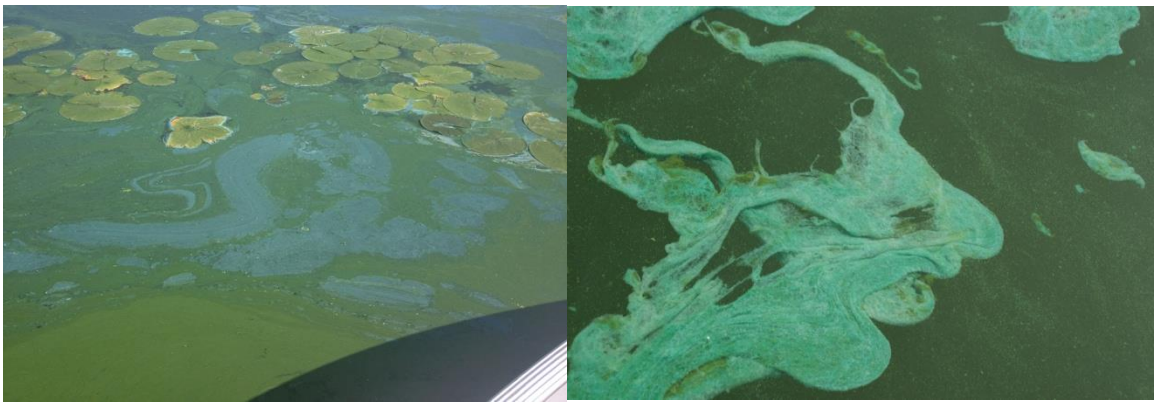

Figure 2 CyanoHAB floating scums in Lake Winnebago, WI in August 2013, left, and Lake Mendota, WI September 2008, right, showing the bright blue appearance due to C-phycocyanin.

Colony size affects cyanobacterial vertical movement, and aids in diffusion of nutrients and signaling molecules between cells (Wu and Kong, 2009; Misson et al., 2011). Large colony size resists predation by zooplankton (unlike other non-colonial algae) and filter feeding organisms including *Dreissenids* (e.g. zebra mussels) (Jarvis et al., 1987). The colonization of some lakes with *Dreissenids* has resulted in shifts in phytoplankton community composition to cyanobacterial dominance, such as in Lake Erie (Vanderploeg et al., 2001; De Stasio et al., 2008). Nearly all cyanoHAB species possess protein gas vesicles that may provide a competitive advantage over other photosynthetic life forms by allowing vertical movement of cells through the water column to adjust light exposure.

6. Physiology and ecology for CyanoHAB blooms: temperature and nutrient drivers

CyanoHAB events generally begin when water temperature is highest and may persist as water temperatures slowly decline (i.e. in autumn). For example, in Lake Erie water temperature is not conducive for cyanoHABs until June with peak biomass between July and October (Stumpf et al., 2012). Similar observations have been made for smaller lakes in the Great Lakes

region including Lake Mendota, WI and Lake Winnebago, WI (Miller et al., 2013; Konopka and Brock, 1978).

Water temperature is only one factor driving the growth of cyanobacteria in lakes. Nutrient availability either from external sources or internal recycling promotes cyanobacterial growth. Since the majority of external nutrients occur in spring when water temperatures are not conducive for cyanobacterial growth, spring nutrient runoff is not likely to be immediately consumed by cyanobacteria to produce cyanobacterial biomass. To a greater extent, cyanobacteria thrive off of internal (vs. external environmental) recycled phosphorus (P) from other organisms or sediment as well as nitrogen (N) fixation.

Cyanobacteria have a variety of mechanisms to compete for nutrients. Some cyanobacteria can fix atmospheric N providing a source of N when all other forms are scarce, particularly ammonium (Ohmori and Hattori, 1974; Beversdorf et al., 2013). In most cases, N fixation has been shown to benefit both N fixing and non-N fixing cyanobacteria, likely due to secretion of fixed organic N from N fixing cells. N fixation may explain why cyanobacterial dominance occurs in lakes with moderately low total N to total P ratios (< 29:1). However, the timing of N fixation events may be more important in stimulating and sustaining blooms of toxic cyanobacteria (Beversdorf et al., 2013).

It is important to recognize that genus and/or strain specific differences among cyanobacteria present challenges in making generalizations about environmental drivers of cyanoHABs. Optimal growth temperature, toxin production, predator avoidance, colony size, shape, and density are just a few of the characteristics that have been shown to vary by species, strain, and genotype (Robarts and Zohary, 1987; Otsuka et al., 2000; Jang et al., 2003; Xing et al., 2007; Lei et al., 2015; Li et al., 2015). The rate of lake mixing and/or oscillations between mixing and thermal stratification are important factors determining the occurrence of cyanoHABs (Dokulil and Teubner, 2000; Huisman et al., 2004).

The large diversity of cyanobacteria in nature prevents generalizations about their physiology and ecology. Efforts to model their responses to environmental variables at time scales relevant to human health (e.g. weekly if not hourly) are often site specific, largely non-transferrable between lakes. This may be in part due to a lack of sufficient data on a large number of lakes, or having the correct measurements. More research is needed in this regard.

Besides growth of new biomass, cyanoHAB events can also occur due to vertical movement (floating/sinking) of cyanobacteria, wind and/or currents pushing floating cells, processes that do not necessarily require growth.

7. Distribution of CyanoHABs in the Great Lakes region

Cyanobacteria are ancient organisms that have developed a number of adaptations that allow them to dominate nutrient rich lakes globally. CyanoHABs are a natural occurrence exacerbated by human activities including increased nutrient runoff, changes in land use, and climate change. In the Great Lakes region cyanoHABs most often occur in water bodies that maintain water temperatures above 20 °C for an appreciable period of time and that receive a large amount of nutrient input. Cyanobacteria that occur in the Great Lakes region produce hundreds, or perhaps even thousands of toxic or otherwise bioactive substances. Among the most commonly reported are MCs, anatoxins, saxitoxins, and cylindrospermopsins as well as a variety of bioactive peptides. With the exception of cylindrospermopsin, the molecular mechanisms of toxicity and acute pathogenesis of these cyanotoxins are well known. For some cyanotoxins (i.e. MCs and cylindrospermopsins) the molecular mechanism of toxicity and/or pathological effects indicates they are possible carcinogens, and indeed, tumor promotion has been demonstrated in animal studies. However, for purposes of developing numerical limits on cyanotoxin exposure more repeat oral dosing studies are needed. In addition, the development of numerical limits may require the use of epidemiological data to account for the possible contribution of some cyanotoxins to chronic diseases and cancer.

MCs are clearly the most often detected, or targeted cyanotoxins in the Great Lakes region. However, historically there have been no regular monitoring programs for cyanotoxins in the Great Lakes. At present our understanding of the variability in cyanotoxin diversity across spatial and temporal scales in the Great Lakes region is relatively unknown, and the rate at which humans are exposed to these toxins has not been adequately addressed due to a lack of monitoring tools. Biomarkers of cyanotoxin exposure are needed in order to develop diagnostic

tests and establish rates of human exposure to cyanotoxins. Such information will be useful in determining whether there are associations between cyanotoxin exposure and the development of chronic diseases.

There is no systematic regular monitoring program for cyanotoxins in the Great Lakes. As such, the majority of data on cyanotoxin distribution comes from select peer reviewed studies, primarily in Lake Erie and non-peer reviewed data from governmental agencies. Appreciable growth of cyanoHABs is foremost dictated by water temperature. CyanoHABs rarely form in areas that do not have sustained water temperatures above at least 20 ^{o}C, though most cyanoHAB species likely form blooms at temperatures less than their optimal growth temperature. The average daily surface water temperatures for the past 21 years (1992 – 2013) in the Great Lakes show that Lakes Erie, Ontario, and Michigan exceed 20 ^{o}C for 49 – 90 days (Figure 3).

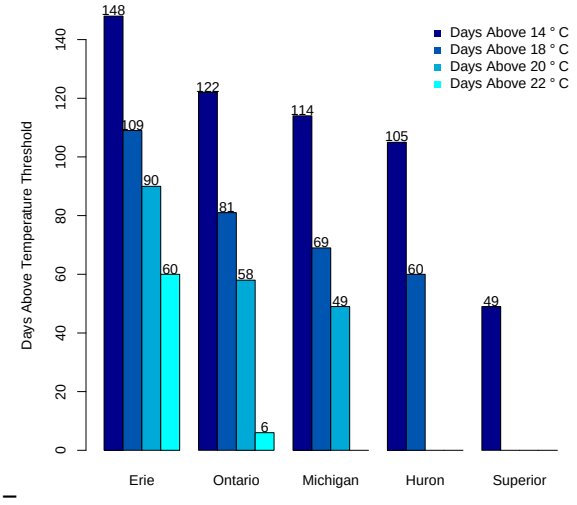

Figure 3. Number of days surface water temperature above thresholds in the Great Lakes, 1992 – 2013. Data from NOAA, Great Lakes Environmental Research Laboratory, Great Lakes Sea Surface Environmental Analysis (http://coastwatch.glerl.noaa.gov/statistic/statistic.html).

The timing of cyanoHAB events at weekly or even monthly scales is difficult to predict and despite decades of research efforts, the ability to predict cyanotoxin levels and the exact environmental conditions under which cyanotoxin production occurs remains elusive. Therefore, regular monitoring for cyanoHABs and their toxins is necessary.

8. Conclusion

CyanoHABs and their toxins are increasing across the Great Lakes region as a result of increased nutrient pollution of waterways and climate change. In some waterways it is likely that nutrient inputs and the availability of internal nutrients (e.g. in sediments) cannot be reduced low enough to completely halt the development of cyanoHABs and toxin production in the near term. For this reason, long-term strategies are needed for managing risk to human health from cyanotoxins.

While a thorough examination of these management strategies is beyond the scope of this report, **the HPAB recommends the following strategic improvements to support public health protection**:

1) Improvements in drinking water treatment technologies or management of existing modern technologies to ensure cyanotoxins are efficiently removed without the production of toxic byproducts. There are multiple cyanotoxins and thus different treatment strategies may affect them differently.
2) Drinking water treatment plant (DWTP) operations should include robust monitoring of prevalent cyanotoxins and optimization for different source conditions and treatment systems. The majority of DWTPs in the Great Lakes region that draw from source waters historically containing cyanoHABs are not effectively removing or destroying cyanotoxins, and occasionally fail to reduce concentrations to below the World Health Organization's safe drinking water level of 1 μg/L microcystin per day. While the recent (August 2014) drinking water ban in Toledo, OH have focused public and government attention, more activity is needed.
3) Further research on optimal drinking water treatment approaches for Great Lakes cyanotoxins should be developed. DWTPs currently practice a range of treatment regimes, but typical treatment processes may not be effective for cyanotoxins. Many uncertainties on effective removal of cyanotoxins from DWTPs remain.

4) Improvements are needed in cyanotoxins laboratory testing, and to establish uniform methods and practices across laboratories, including standards and quality assurance. Promising technologies include methods for multiple toxins and toxin congeners along with methods to measure cyanotoxins directly or indirectly.

5) An examination of source water monitoring strategies should include provisions for regular and improved monitoring of cyanotoxins, reporting of such results to the public in a timely fashion, and continued development of predictive models for forecasting cyanoHABs and their toxins given predictions of increasingly warm waters for longer periods of the year.

6) Additional toxicity studies are needed to improve the development of strong numerical limits for a broader range of cyanotoxins beyond microcystins. These include using purified cyanotoxins in animal studies, additional work examining exposure from inhaled aerosols (e.g. from shower bathing), and skin irritation, and additional data on chronic effects from cyanotoxin exposure such as tumor promotion and cancer.

These recommendations seek to improve the monitoring and abatement of human risks from harmful cyanobacterial blooms. They should augment, but cannot replace, efforts to prevent the increasing severity and frequency of blooms by addressing nutrient pollution and climate-mediated temperature changes in the Great Lakes region.

Ultimately the problem of reducing cyanoHABs and their toxins in the Great Lakes will be addressed through intensive nutrient abatement programs in the watershed. Climate change impacts were not a focus of this report but the increasing spread of warm water habitat with climate change serves to prioritize the impacts of cyanoHAB identified in this report and to underscore the need for long-term management strategies to control HABs.

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10. Appendix

Comprehensive Review of Cyanobacterial Toxins and Their Potential Human Health Effects in the Great Lakes Region

A report to the Health Professionals Advisory Board of the International Joint Commission

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Table of Contents

A. Abbreviations

HAB = Harmful Algal Bloom
cyanoHABs = cyanobacterial harmful algal blooms
N = Nitrogen
P = Phosphorus
MC = microcystin (all possible congeners)
MCLR = microcystin congener with leucine and arginine at variable positions
MCRR = microcystin congener with arginine and arginine at variable positions
LOAEL = Lowest Observed Adverse Effect Level
NOAEL = No Observed Adverse Effect Level

CONTAM = The Panel on Contaminants in the Food Chain
OATP = organic anion transporting polypeptide
WHO = World Health Organization
DWTP = Drinking Water Treatment Plant
BMAA = Beta-Methylamino-L-alanine
LPS = Lipopolysaccharide
PP1A = Protein Phosphatase 1A
PP2A = Protein Phosphatase 2A
PP1/2A = Protein Phosphatase 1A and/or 2A
i.p. = intraperitoneal injection
LD50 = Dosage Lethal to 50% of Animals
CIP2A = Cancerous Inhibitor Of PP2A
SNP = Single Nucleotide Polymorphism
b.w. = body weight
ROS = Reactive Oxygen Species
PSP = Paralytic Shellfish Poison
DWI/BW = Drinking Water Intake to Body Weight Ratio
EPA = Environmental Protection Agency
USDA = United States Department of Agriculture
TTMCs = Total Toxic Microcystins

B. Tables

C. Figures

D. Background

In freshwater environments, cyanobacteria (Cyanophyceae) dominate many nutrient rich lakes producing large accumulations of algal biomass. The accumulation of excess algal biomass, or "algal blooms" in lakes and other aquatic environments impacts ecological and human health as well as the socioeconomic value of our water resources. While a natural occurrence, the eutrophication of water bodies and global climate change promote the frequency, duration, and magnitude of these blooms. Excess bloom material is consumed via respiration by heterotrophic microorganisms consuming oxygen (i.e. increased biological oxygen demand) leading to anoxic/hypoxic conditions detrimental to fish and other wildlife. In addition, decaying algal biomass produces noxious or unpleasant odors inhibiting recreational activities. Potent toxins and other constituents of some algae are harmful to aquatic organisms, and other animals including humans. The presence of these toxins and odors associated with algal blooms presents challenges for the use of freshwaters for producing high quality, aesthetically pleasing drinking water (Zamyadi et al., 2012; Cheung et al., 2013). While many types of algae accumulate in aquatic environments, cyanobacteria are responsible for producing seasonal mass accumulations known as cyanobacterial harmful algal blooms or cyanoHABs. The focus of this review is on cyanoHAB occurrence and possible human health outcomes associated with cyanoHABs in the North American (Laurentian) Great Lakes (heretofore referred to as the Great Lakes), proposed limits for human exposure and issues related to monitoring and managing toxin levels in source and finished drinking water and lake water for protecting human health.

The ecology, toxicology, and physiology of cyanobacteria and cyanoHABs have been studied for more than a century providing rich information about their impacts on aquatic resources. Cyanobacteria (known colloquially as "blue- green algae") are a ubiquitous and diverse group of photosynthetic gram- negative bacteria that inhabit both terrestrial and aquatic habitats throughout the planet. These bacteria were responsible for the oxygenation of early earth more than 3 billion years ago and are the precursors to chloroplast organelles in Eukaryotic algae and higher plants (McFadden, 2014). They are among the most important, diverse, and abundant photosynthetic organisms on the planet with lifestyles that range from essential symbionts of lichens and plants to the most abundant phototrophs in the world's oceans (i.e. *Prochlorococcus*).

An increasingly recognized characteristic of cyanobacteria is their ability to produce toxic or otherwise bioactive compounds that affect animal and human physiology. In most cases, these are secondary metabolites (not necessary for normal functioning of the bacterial cells) but presumably provide some largely unknown benefit to the organism). Multiple compounds are being discovered (including congeners, methylated and other variants) whose toxicity is incompletely characterized to date. Both the full diversity and toxicity of such compounds (in isolation and in mixtures) produced in nature is not known. Genomic studies suggest some cyanobacteria are capable of producing hundreds of different bioactive molecules with varying degrees of toxicity (Mejean and Ploux, 2013; Calteau et al., 2014). While some such compounds are of interest to the pharmaceutical industry for their medical benefits, others are potent toxins harmful to a variety of organisms. The presence of these compounds in aquatic environments, particularly lakes, presents challenges for recreational water quality management and drinking water production.

Characteristics

Over their long history on this planet, cyanobacteria have evolved adaptations that favor their dominance in eutrophic lakes. Perhaps the most noticeable characteristic of many cyanoHAB species is their ability to multiply to high densities forming macroscopic colonies or groups of cells covered in a mucous polysaccharide sheath. In some cases, these colonies may coalesce into free-floating mats or "scums" on the surface of the lake with a bright blue or blue-green appearance due to the presence of C-phycocyanin, their major light harvesting pigment (Figure 1). Colony size affects cyanobacterial vertical movement, and aids in diffusion of nutrients and signaling molecules between cells (Wu and Kong, 2009; Misson et al., 2011). Large colony size resists predation by zooplankton (unlike other non-colonial algae) and filter feeding organisms including *Dreissenids* (e.g. zebra mussels) (Jarvis et al., 1987). The colonization of some lakes with *Dreissenids* has resulted in shifts in phytoplankton community composition to cyanobacterial dominance, such as in Lake Erie (Vanderploeg et al., 2001; De Stasio et al., 2008).

Figure 1 CyanoHAB floating scums in A) Lake Winnebago, WI in August 2013, and B) Lake Mendota, WI September 2008, showing the bright blue appearance due to C-phycocyanin.

Nearly all cyanoHAB species possess protein gas vesicles that provide buoyancy and thus drive vertical movement of cells through the water column. These vesicles are cylindrical shaped structures formed by two hydrophobic proteins that diffusively accept gas and repel water (Walsby, 1972a, b). With enough gas trapped in the vesicles, the cyanobacteria float upward toward the surface, where there is more photosynthetically active radiation. Photosynthesis produces carbohydrates, which may act as ballast, or increase cellular turgor

pressure (Grant and Walsby, 1977). Depending upon the width of the gas vesicle, this pressure may result in the irreversible collapse of the gas vesicle leading to a loss of buoyancy and sinking. As such, buoyancy or vertical movement in cyanobacteria is regulated by gene expression of vesicle proteins, width of the cylindrical gas vesicle, photosynthesis, metabolism of carbohydrate ballast, and sunlight.

It has been proposed that vertical movement of cyanobacteria is an adaptation that allows cyanobacteria to capture nutrients in dark deeper layers of the lake, then float to the sunlit surface waters where photosynthesis and carbon fixation takes place (Fogg and Walsby, 1971). This would give them a competitive advantage over other Eukaryotic phototrophs since long-term thermal stratification sequesters nitrogen (N) and phosphorus (P) in deeper waters at or below the thermocline while the nutrient-poor photic zone is generally limited to the upper few meters in most eutrophic lakes during the cyanobacterial growth season (Visser et al., 1996; Miller et al., 2013). Thus, cyanobacteria may have a competitive advantage by overcoming this separation in nutrients and sunlight through vertical movement driven by gas vesicles. However, it is not clear if cyanobacteria vertically migrate to such lower depths where nutrients are sequestered, especially in deep lakes where the thermocline depth is often far below the photic zone (Bormans et al., 1999). A more important advantage for vertical movement may be in obtaining optimal light conditions and avoiding ultraviolet radiation and oxidative stress. CyanoHAB species have low light requirements, but can adapt to high light by varying relative amounts of chlorophyll-a and their major light harvesting pigment C-phycocyanin. As such, some cyanobacteria are able to practice "self-shading," producing blooms at the surface that shade out other phototrophic competitors while also growing at a depth where light may be limited (Scheffer et al., 1997).

Some cyanobacteria can fix atmospheric N providing a source of N when all other forms are scarce, particularly ammonium (Ohmori and Hattori, 1974; Beversdorf et al., 2013). In most cases, N fixation has been shown to benefit both N fixing and non-N fixing cyanobacteria, likely due to secretion of fixed organic N from N fixing cells. N fixation may explain why cyanobacterial dominance occurs in lakes with moderately low total N to total P ratios (< 29:1). However, the timing of N fixation events may be more important in stimulating and sustaining blooms of toxic cyanobacteria (Beversdorf et al., 2013). Cyanobacteria also compete well for

low levels of P and/or have lower P requirements compared to other phototrophs (Kromkamp et al., 1989).

Most cyanobacteria also possess carbon, N, and P storage mechanisms. The carbon concentrating mechanism consists of protein transporters that concentrate bicarbonate within the cell to be used by RuBisCO (ribulose 1,5 –bisphosphate carboxylase oxygenase) for carbon fixation (Price et al., 2008). Fixed carbon and energy may then be stored in glycogen and polyhydroxyalkanoate. Similarly, in many bacteria including cyanobacteria, N and P are stored in cyanophycin and polyphosphate granules, respectively, thereby providing a source of N and P at a later time (Jacobson and Halmann, 1982; Mackerras et al., 1990).

The extent that these adaptations are expressed by cyanobacteria in nature favoring their dominance and resulting in cyanoHAB events depends on a variety of interacting physical, chemical, and biological factors that are only partially understood. Growth of cyanobacteria in nature is highly dependent on seasonal factors including water temperature, sunlight, and lake mixing. In north temperate environments, the optimal growth temperature for all cyanoHAB species is > 15 °C, and the most prevalent toxic cyanoHAB species (e.g. *Microcystis*) have optimal growth temperatures > 25 °C (Robarts and Zohary, 1987). As such, cyanoHAB events generally begin when water temperature is highest and may persist as water temperatures slowly decline (i.e. in autumn). For example, in Lake Erie water temperature is not conducive for cyanoHABs until June with peak biomass between July and October (Stumpf et al., 2012). Similar observations have been made for smaller lakes in the Great Lakes region including Lake Mendota, WI and Lake Winnebago, WI (Miller et al., 2013)(Konopka and Brock, 1978).

Water temperature is only one factor driving the growth of cyanobacteria in lakes. Nutrient availability either from external sources or internal recycling promotes cyanobacterial growth. Since the majority of external nutrients occur in spring when water temperatures are not conducive for cyanobacterial growth, spring nutrient runoff is not likely to be immediately consumed by cyanobacteria to produce cyanobacterial biomass. To a greater extent, cyanobacteria thrive off of internal recycled P from other organisms or sediment as well as N fixation. Nutrient speciation is also important (i.e. organic or inorganic, N versus P) for the metabolic needs of the cyanobacteria at the time nutrients become available. For example, lack of N promotes N fixation by diazotrophic cyanobacteria, but this is more associated with the lack of ammonia than other N sources (Flores and Herrero, 2004). As such, N fixation occurs in the

presence of nitrate (Spiller et al., 1986; Beversdorf et al., 2013; Paerl and Otten, 2016). Despite the association with eutrophic environments, on a temporal scale, cyanoHABs generally occur during periods when the standing stock of N and P are at their lowest (Gobler et al., 2007; Miller et al., 2013).

Besides growth of new biomass, cyanoHAB events can also occur due to vertical movement (floating/sinking) of cyanobacteria, wind and/or currents pushing floating cells, processes that do not necessarily require growth. Cells distributed throughout the water column can synchronize vertical movements thereby accumulating (i.e. bloom) simultaneously at the surface, often in the evening and on diurnal cycles (Ibelings et al., 1991). Similarly, currents and prevailing winds can cause biomass to accumulate, pool, or pile up along shorelines creating bloom conditions (Kanoshina et al., 2003). Predicting how these factors interact with elicitors of cyanobacterial growth (e.g. nutrients and temperature) at temporal and spatial scales is a rich area of study in cyanoHAB modeling efforts (Wallace and Hamilton, 2000).

It is important to recognize that genus and/or strain specific differences among cyanobacteria present challenges in making generalizations about environmental drivers of cyanoHABs. Optimal growth temperature, toxin production, predator avoidance, colony size, shape, and density are just a few of the characteristics that have been shown to vary by species, strain, and genotype (Robarts and Zohary, 1987; Otsuka et al., 2000; Jang et al., 2003; Xing et al., 2007; Lei et al., 2015; Li et al., 2015a). The rate of lake mixing and/or oscillations between mixing and thermal stratification are important factors determining the occurrence of cyanoHABs (Dokulil and Teubner, 2000; Huisman et al., 2004).

Bloom size and taxonomic identification of bloom species do not reliably predict the presence of toxins in a particular cyanoHABs bloom. The reasons for this are varied and in some cases not well understood. For example, toxigenic and non-toxigenic strains of cyanobacteria appear in the same species, depending on whether toxin genes are present (Pick, 2016). While several genera and species of cyanoHABs produce microcystins, production of this toxin occurs only when genes for its synthesis are fully functional (Pick, 2016). Also, the physiological role of cyanotoxins within cells is unclear, for instance whether toxins are consistently produced in response to stress due to lack of nitrogen (Gagnon and Pick, 2012).

Oscillatoriales and other filamentous cyanobacteria are often associated with shallow turbid lakes that are rarely if ever thermally stratified (Scheffer et al., 1997). These cyanobacteria

are shade tolerant allowing them to outcompete other phototrophs in low light, turbid conditions. Reynolds et al. (Reynolds et al., 1987) demonstrated that cell shape, size, and density are principal factors in determining buoyancy of cyanobacteria or resistance to sinking and dispersal during lake mixing events. *Oscillatoria* compared to *Microcystis* and *Anabaena* have the lowest ballast per unit cell volume making them more resistant to sinking and dispersal during frequent mixing events that occur in shallow polymictic (i.e. frequently mixed) lakes. In contrast, blooms of spherical colonial species including *Microcystis* (such as occur in Lake Erie) are associated with infrequent mixing, thermally stratified conditions, which is likely due in large part to their relatively high ballast to cell volume ratio in both high and low light conditions. For example, cyanoHABs of *Microcystis* in Lake Erie and other lakes (e.g. Lake Taihu) are associated with low wind stress and thermal stratification (Stumpf et al., 2012).

E. Distribution of cyanoHABs in the Great Lakes Region

There is no systematic regular monitoring program for cyanotoxins in the Great Lakes. As such, the majority of data on cyanotoxin distribution comes from select peer reviewed studies, primarily in Lake Erie and non- peer reviewed data from governmental agencies. Appreciable growth of cyanoHABs is foremost dictated by water temperature. CyanoHABs rarely form in areas that do not have sustained water temperatures above at least 20 °C, though most cyanoHAB species likely form blooms at temperatures less than their optimal growth temperature. The average daily surface water temperatures for the past 21 years (1992 – 2013) in the Great Lakes show that Lakes Erie, Ontario, and Michigan exceed 20 °C for 49 – 90 days (Figure 2).

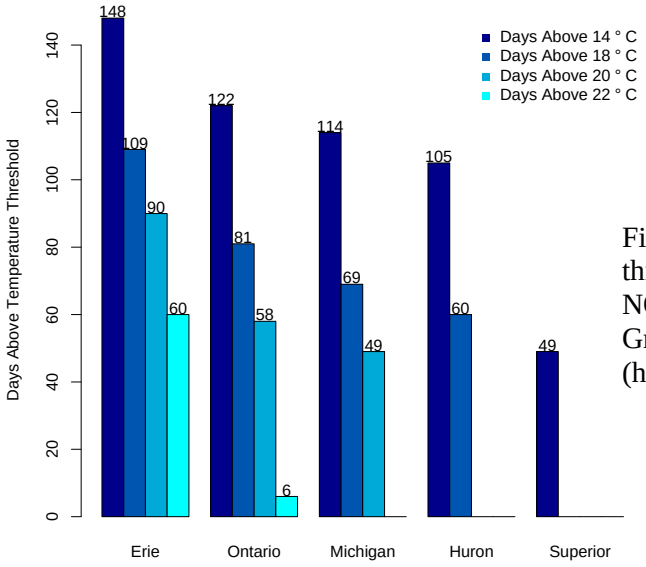

Figure 2. Number of days surface water temperature above thresholds in the Great Lakes, 1992 – 2013. Data from NOAA, Great Lakes Environmental Research Laboratory, Great Lakes Sea Surface Environmental Analysis (http://coastwatch.glerl.noaa.gov/statistic/statistic.html).

Only Lake Erie experiences surface water temperatures above 22 °C for any appreciable period of time. In other Great Lakes, certain embayments and near shore habitats reach temperatures well above 20 °C and sustain these temperatures into August. This includes Green Bay, Sandusky Bay, Grand Traverse Bay, Saginaw Bay, and other near-shore habitats particularly in Southern Lake Michigan. Connecting water bodies to Great Lakes may also contribute biomass and/or toxins including Lake Winnebago to Green Bay, Lake St. Clair to Lake Erie and Lake Macatawa to Lake Michigan among others.

Cyanobacterial toxins have been detected in Lakes Erie, Ontario, and in certain areas of Lake Michigan including Grand Traverse Bay and Little Traverse Bay (Makarewicz et al., 2009; Rediske et al., 2010). Green Bay is a shallow eutrophic embayment where cyanoHABs have been previously reported (Stasio et al., 2008). However there have been no published studies on

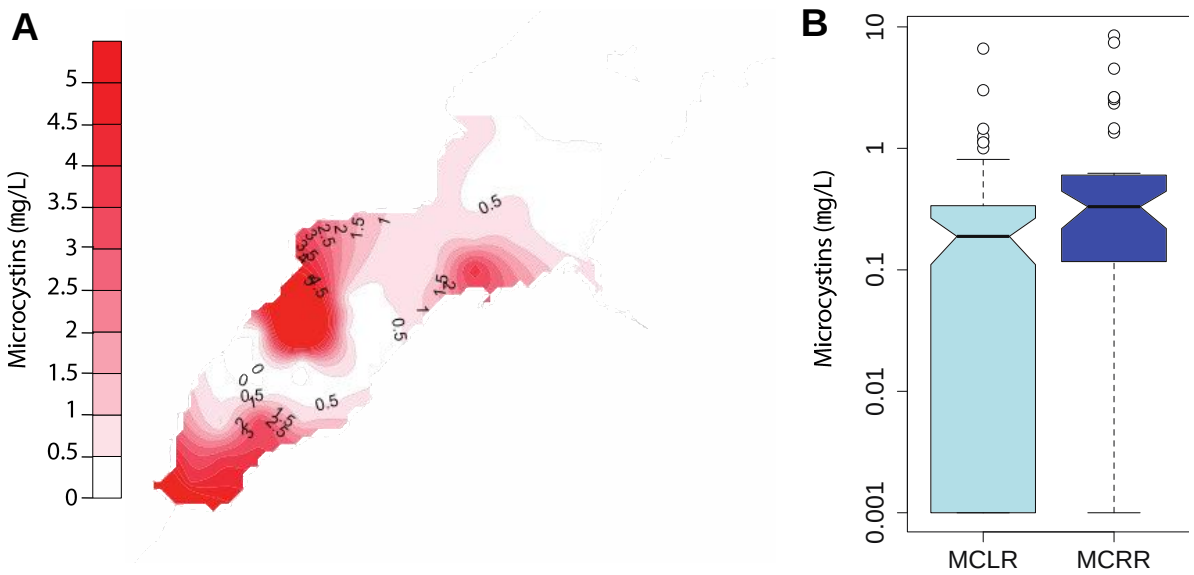

Figure 3. A) Concentrations (µg/L) of all microcystin congeners detected in a transect across Green Bay in August, 2014 and B) boxplot of microcystin congeners detected showing mean values in Green Bay plus outliers. MCLR = microcystin-LR, MCRR = microcystin- RR. In this transect the data indicate that cyanotoxins are heterogeneously distributed in Green Bay with highest levels in lower Green Bay. While 6 different congeners were targeted only MCLR and MCRR were detected.

cyanotoxin levels in Green Bay. Cyanotoxins have recently been measured in transects of Green Bay (Figure 3) (Miller 2016, unpublished). The mean and max levels of MCRR (0.97 and 8.5 µg/L) and MCLR (0.47 and 6.6 µg/L) were just under recreational risk levels proposed by the

WHO. However, this was only one time point and blooms and toxins can vary widely over temporal scales (Hotto et al., 2008; Pawlik-Skowrońska et al., 2008; Miller et al., 2013; Beversdorf et al., 2015).

Of all the Great Lakes, the Western Basin of Lake Erie and its connecting water bodies (i.e. Lake St. Clair, Maumee River) experience the most extensive cyanoHABs (Boyer, 2008). Historically, a variety of cyanobacterial species have been responsible for cyanoHABs in Lake Erie including filamentous N fixing cyanobacteria such as *Anabaena* and *Aphanizomenon*, as well as the non-N fixing *Microcystis* (Rinta-Kanto et al., 2005). Early studies suggested that filamentous forms were dominant at times in Lake Erie in the 1970's and 1980's (Munawar and Munawar, 1976). One study of preserved and recently collected sediment from Lake Erie suggests that *Microcystis* has always been the dominant MC producer since the 1970's (Rinta-Kanto et al., 2008), although it is unclear how well cyanobacterial cells survive in deep sediments over the course of decades. In any case, CyanoHABs of toxic *Microcystis* species have escalated such that it is now clearly the dominant taxa during the primary growth season in the Western Basin of Lake Erie.

The single largest source of nutrients to Lake Erie is the Maumee River, which drains a 16,835 square kilometer watershed, 80% of which is fertile agricultural land. This river enters the southwestern end of Lake Erie. Mapping Spring P levels across the lake, there is a clear gradient from low to high P in an East- West direction (Figure 4). Similarly, data from the Ohio Environmental Protection Agency (http://epa.ohio.gov/ddagw/HAB.aspx) shows that the highest MC levels detected in the lake are found in the Western Basin (Figure 4), though some of the highest detects in this dataset are on the eastern edge of the Western Basin.

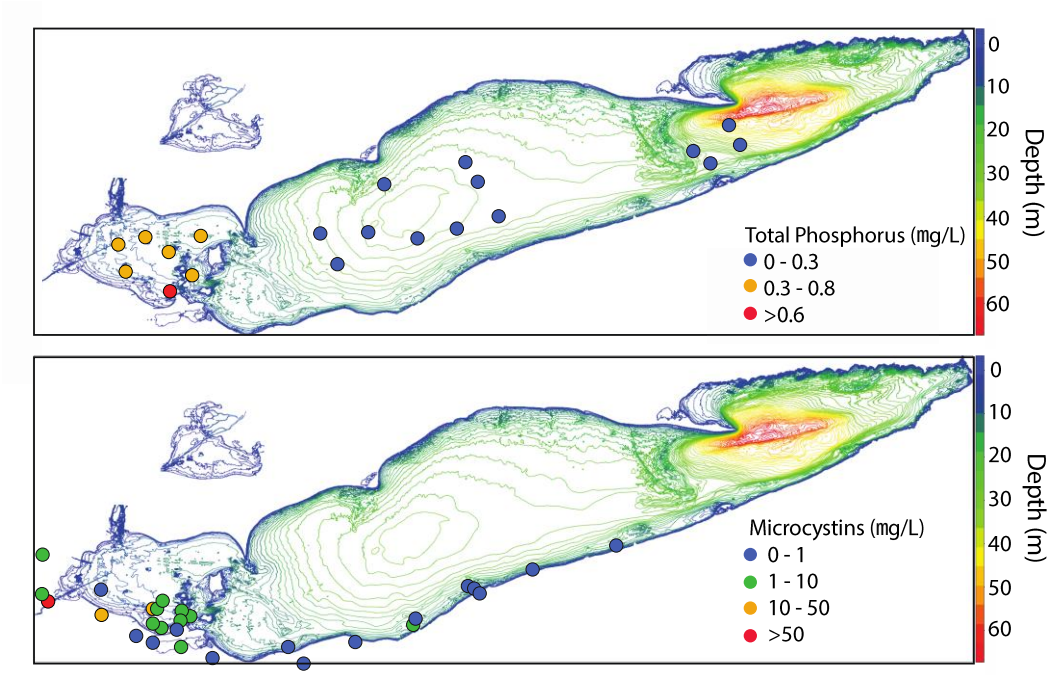

Figure 4. Distribution of total Phosphorus and microcystin across Lake Erie. Total P is the mean spring concentration measured in 2008 – 2012. Microcystin data spans 2010 – 2015 from the Ohio EPA. No data is provided for Lake St. Clair north of Lake Erie.

This is the shallowest end of the lake allowing water temperatures to rise the fastest. Periodic mixing events resuspend nutrients while calm periods allow for the accumulation of biomass at the surface in sunlit waters. While this is a relatively shallow part of the lake, it is still deep enough to thermally stratify during summer months, which favors *Microcystis* dominance. Warming in deeper parts of the lake in the central and eastern basins is inhibited by cold deep water. As such cyanoHABs occur less frequently in these areas.

Since *Microcystis* is the dominant cyanobacteria in Lake Erie, the most commonly measured cyanotoxins are MCs. Surprisingly few peer- reviewed studies have published MC concentrations from Lake Erie or other Great Lakes. Rinta-Kanto et al. (Rinta-Kanto et al., 2009) measured MCs by protein phosphatase inhibition assay at various stations in the Lake Erie Western Basin at eight time points over 3 years and found maximum concentrations (14 µg/L) occurred in August. Millie et al. (Millie et al., 2009) measured intracellular MCs at sampling stations across the Western Basin of Lake Erie during single time points in 2003, 2004, and 2005. Intracellular MCs peaked at 0.13, 1.64, and 0.14 µg/L in 2003, 2004, and 2005, respectively. Dyble et al. (Dyble et al., 2008) report a maximum of 4 and 0.38 µg/L intracellular

and extracellular MCs, respectively in Western Lake Erie on a single cruise in 2004. One scum sample contained 58 µg/L intracellular MCs. Hu et al. (Hu et al., 2016) reported a maximum of approximately 2 µg/L MCs at a beach near the Ottawa National Wildlife Refuge on the Southwestern shore of Lake Erie in samples collected over a season (May- November) in 2012 with weekly sampling. In a survey of the Western Basin of Lake Erie, Wang et al. (Wang et al., 2009) detected MCLR in 16 of 36 samples in 2007. Concentrations reported in µg/g dry weight were highly variable from 0.5 – 3,000 µg/g dry weight.

By comparison, recent data (non-peer reviewed data) reported by the Ohio EPA (http://epa.ohio.gov/ddagw/HAB.aspx) spanning 2010 – 2015 shows similar or slightly higher concentrations of MCs at surface water locations in Lake Erie. Table 1 shows mean and maximum MC levels reported at each site. Most locations are in the Western Basin except for sites 4, 13, 14, 18, 20, and 25, which are in the middle and eastern basins. The overall mean and maximum MC concentrations at all surface water locations, combined is 126.9 and 3,144 µg/L, respectively. This maximum of 3,144 µg/L and the next highest data point at 570 µg/L are clear outliers in the dataset, though important indicators for extreme toxin loads that typically occur during peak bloom conditions or in wind- blown accumulations of biomass. Removing these outliers produces an average of 1.81 µg/L. Highest monthly max and mean MC concentrations occurred in July (34.49 and 3,144 µg/L, respectively), followed by August (9.45 and 570 µg/L, respectively), and September (3.02 and 220 µg/L, respectively). With the outliers removed highest monthly averages occurred in August and September, each at ~3 µg/L.

Nine data points are reported for 2010 and 44 for 2011, otherwise, a similar number of data points are reported for 2012 – 2015 (62 – 77). The highest mean and maximum MC levels were over ten times greater in 2015 (52 µg/L) compared to 2014, 2013, and 2012 (4.00, 1.68, and 1.40 µg/L, respectively). Again, this result is driven by the two outliers. Removing these produces a mean MC level for 2015 (1.12 µg/L) similar to the previous three years. The Ohio EPA data suggest typical MC concentrations in the Western Basin of Lake Erie for the past several years of ~1 – 2 µg/L, with occasional extremes of one hundred to thousand fold higher concentrations.

Table 1. Mean and maximum MC concentrations (µg/L) at surface water locations in Lake Erie reported by Ohio EPA.

Site	Lake Erie Surface Water	Mean	Max	N
1	Lake Erie @ Gibralter Island Docks	3144.69	3144.69	1
2	Maumee Bay State Park Lake Erie Beach	21.38	570.00	52
3	Camp Perry Beach - Lake Erie	2.10	2.10	1
4	Lake Erie (Open Lake) East of Fairport Harbor	1.70	1.70	1
5	Lake Erie between Toledo/Oregon WTP Intakes	0.62	2.20	6
6	Lake Erie Ambient Site - Off Maumee Bay	0.58	3.20	30
7	Lake Erie Ambient Station - West Sister Island	0.31	2.80	34
8	Lake Erie Ambient Site - Port Clinton	0.28	2.00	33
9	Lake Erie North of Port Clinton	0.26	2.00	16
10	Lake Erie Off Detroit Near Canadian Border	0.19	2.00	22
11	Lake Erie @ Meinke Marina West	0.13	0.96	12
12	Lake Erie Ambient Station, Off Cedar Point	0.11	0.79	13
13	Lake Erie Ambient Station - Conneaut	0.10	0.49	5
14	Lake Erie Ambient Station - Fairport North	0.08	0.31	4
15	Lake Erie Ambient Site - Off Sandusky Bay	0.03	0.49	17
16	Lake Erie Ambient Station - Huron	0.02	0.58	30
17	Lake Erie Ambient Station - Rocky River	0.00	0.00	7
18	Lake Erie Ambient Station - Lorain West	0.00	0.00	7
19	Lake Erie Ambient Station - Wildwood	0.00	0.00	4
20	Lake Erie Fairport Transect Station 3	0.00	0.00	1
21	Lake Erie @ Channel Grove Marina	0.00	0.00	16
22	Lake Erie @ Wild Wings Marina	0.00	0.00	10
23	Lake Erie @ Lakefront Marina	0.00	0.00	3
24	Lake Erie @ Brands Marina	0.00	0.00	4
25	Lake Erie Ambient Station - Geneva North	0.00	0.00	4

A recent study by the U.S. Geological Survey (Francy et al., 2015) measured MCs in Maumee Bay, Port Clinton, and Sandusky Bay (all in Lake Erie) in 2013 and 2014. Median levels were highest in Maumee Bay (6.8 µg/L), followed by Sandusky Bay (3.6 µg/L) and Port Clinton (0.4 µg/L). MC concentrations in Maumee Bay reached a maximum of 240 µg/L in late August of 2014 and were above 20 µg/L in five samples over a three- week period. In 2013 MCs were below 30 µg/L on all dates.

F. Toxin Removal from Drinking Water

The Great Lakes are currently used as a source of drinking water for over 35 million people. Lake Erie is the most affected by cyanotoxins and an estimated 11 million people rely on Lake Erie for drinking water. As such there is great interest in drinking water treatment strategies to remove cyanotoxins in this region. Other excellent reviews of cyanotoxin removal by drinking water treatment strategies have been published elsewhere (Hitzfeld et al., 2000; Westrick et al., 2010). Accordingly, this topic will only be covered briefly here.

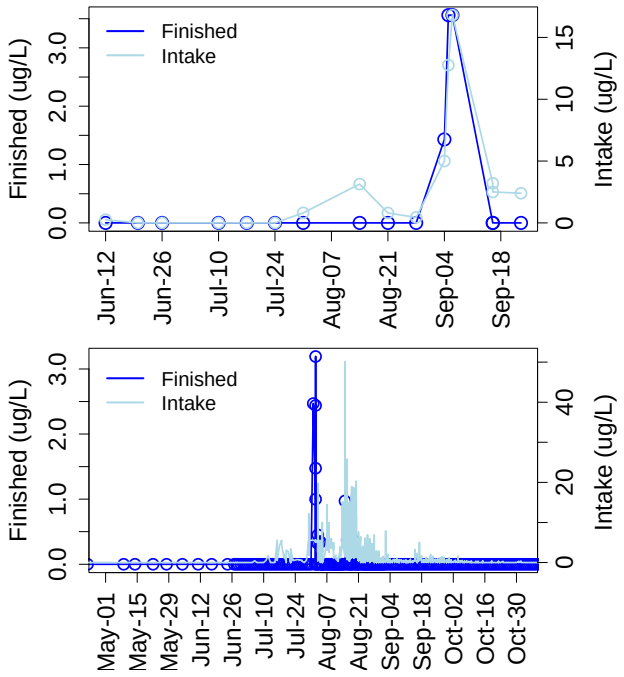

Figure 5. Concentrations of MCs in finished and intake drinking water at Carroll County (Top) and Toledo (bottom), OH plants in 2013 and 2014, respectively.

Cyanotoxins 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 (Table 2 in (Hoeger et al., 2005)). In a recent survey of finished drinking water supplies from 24 plants in the United States, 75% of samples tested positive for MCLR and some samples contained concentrations unacceptable for human consumption (Carmichael, 2000). In 2013 MCs were detected in finished drinking water from the Carroll County Township drinking water facility in Ohio, which draws water from the Western Basin. Levels spiked to just over 3.5 µg/L in finished water coinciding with a large cyanoHAB event that produced over 15 µg/L in raw intake water (Figure 5). Then in 2014 MCs were detected in finished drinking water at the Toledo drinking water treatment plant at nearly 2.5 µg/L, 1.5 µg/L and 1 µg/L on three separate occasions that did not necessarily coincide with spikes in raw water (Figure 5).

The Ohio EPA has released data on concentrations of MCs in intake and finished drinking water from drinking water treatment plants (DWTPs) that draw water from Lake Erie

Table 2. Mean and maximum MC concentrations (µg/L) at drinking water intakes in Lake Erie reported by Ohio EPA.

Intake Site	Mean	Max	N
Put-In-Bay WTP Lake Erie Intake	5.83	340.00	63
Oregon WTP Lake Erie Intake	3.93	37.20	99
Camp Patmos WTP Lake Erie Intake	2.27	28.00	53
Carroll Water & Sewer WTP Lake Erie Intake	2.08	18.20	77
Toledo WTP Lake Erie Intake	1.24	50.00	1377
Painesville WTP Lake Erie Intake 2	1.10	3.90	9
Ottawa County WTP Lake Erie Intake	0.92	12.14	99
Lake Erie Utilities WTP Lake Erie Intake	0.69	4.33	54
Aqua Ohio - Mentor WTP Lake Erie Intake	0.67	2.20	8
Lake Co West WTP Lake Erie Intake	0.65	1.61	14
Kelleys Island WTP Lake Erie Intake	0.57	5.88	66
Fairport Harbor WTP Lake Erie Intake	0.39	1.20	7
Huron WTP Lake Erie Intake	0.34	4.62	31
Marblehead WTP Lake Erie Intake 1	0.33	3.80	61
Sandusky WTP Lake Erie Intake	0.30	2.50	73
Lake Co East WTP Lake Erie Intake	0.23	0.73	14
Avon Lake WTP Lake Erie 54 inch Intake	0.13	0.67	28
Lorain WTP Lake Erie Intake	0.12	0.61	24
Vermilion WTP Lake Erie Intake	0.00	0.00	13
Elyria WTP Lake Erie Intake	0.00	0.00	3
Painesville WTP Lake Erie Intake 1	0.00	0.00	1

and other locations in Ohio and neighboring states. Table 2 shows mean and maximum MC concentrations detected in intake water from Lake Erie (Ohio Environmental Protection Agency, 2016). The overall average and maximum level from all intakes is 1.04 and 340 µg/L, respectively. Nine drinking water treatment plants in the Ohio EPA dataset show detectable levels of MCs in finished drinking water (Table 3). Six of these draw water from Lake Erie and a seventh (Campbell Soup Factory) draws from the nearby Maumee River. Maximum levels in finished water at DWTPs drawing from Lake Erie range from 0.23 to above 3 µg/L, with max levels at the Carroll County and Toledo DWTPs. The Campbell Soup Factory had detects in finished water above 0.3 µg/L. This suggests some processed foods may be another potential human exposure route for MCs (Table 3) (Ohio Environmental Protection Agency, 2016).

Overall, the highest MC levels detected in finished drinking water are from the Celina DWTP. This plant draws water from Grand Lake St. Mary's, which has produced cyanoHABs in recent years that have caused human illnesses, and which drains to the Maumee River and ultimately into Lake Erie.

These data suggest that current treatment strategies are not always effective at removing

Table 3. Mean and maximum MC concentrations (μg/L) in finished drinking water reported by Ohio EPA.

Plant	**Mean**	**Max**	**N**
Celina WTP	0.039	11	295
Carroll Water & Sewer WTP	0.113	3.56	77
Cadiz WTP	0.050	3.4	68
Toledo WTP	0.021	3.19	690
Kelleys Island WTP	0.025	1.68	67
Put-In-Bay WTP	0.016	0.6	60
Camp Patmos WTP	0.009	0.5	54
Campbell Soup Supply Co WTP	0.038	0.35	18
Oregon WTP	0.002	0.23	96

cyanotoxins and occasionally fail to reduce concentrations to below the World Health Organization's safe drinking water level of 1 μg/L MC per day. Studies investigating the efficacy of treatment processes have focused primarily on MCs, while little is known about other cyanotoxins. This is problematic, since future scenarios of climate and land use change suggest the intensity and frequency of cyanobacterial blooms will increase concomitant with changes in community composition (e.g. invasive species)(Paerl and Huisman, 2008; Brookes and Carey, 2011). The most commonly employed drinking water treatment processes in the Great Lakes region include sedimentation (i.e. flocculation and clarification), mechanical filtration, ozonation, activated/granulated carbon filtration, and disinfection with chlorine or other oxidizing agents.

The ability of DWTP processes to remove cyanotoxins depends upon processes employed, cyanotoxin load, and environmental conditions of the source waters (reviewed in (Hitzfeld et al., 2000)). *Sedimentation* alone does not remove dissolved cyanotoxins, nor can it be expected to remove toxins within buoyant cells. However, sedimentation is thought to provide

the best mechanism for removing MCs since studies show that the bulk of MC in lakes is contained within cells and not in the dissolved phase.

Rapid sand filtration is ineffective at removing both cyanobacterial cells and dissolved toxins, and in fact, can even lyse cells releasing toxins into the dissolved fraction (Mohamed, 2016). Other studies have shown that rapid sand filtration may be effective with prefiltration (e.g. gravel) (Pereira et al., 2012), but additional chemicals, such as alum, can also lyse cells (Han et al., 2016). *Slow sand filtration* can be effective at removing cyanobacterial cells and toxins, but the primary mechanism is by biodegradation via heterotrophic bacteria, by physical cell removal or adsorption of dissolved toxins (Ho et al., 2006). Because biodegradation is the primary removal mechanism, and is thus growth dependent, sand filtration may be ineffective at lower temperatures (Grützmacher et al., 2002).

Chlorination is the most widely used process that has been shown to destroy some, but not all cyanotoxins. Removal efficiency by chlorination is dependent upon a large number of factors. For example, MCs react with hypochlorous acid (HOCl) approximately 20 times faster than with hypochlorite (OCl^-) and degradation rates are negligible at pH values above 7.5 (Acero et al., 2005). Similar results have been found for cylindrospermopsin (Hitzfeld et al., 2000). 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 (Nicholson et al., 2003; Rodriguez et al., 2007). As would be expected, the chlorine oxidation rate of cyanotoxins is linear with chlorine dose and temperature where at least 0.5 mg/L residual chlorine is required for a 30 min contact time (reviewed in (Hitzfeld et al., 2000)). Chlorination is the primary process facilitating cell lysis, thereby causing toxin release (Daly et al., 2007). 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* by only 30% (Drikas and Hrudey, 1994). Chlorination of cyanotoxins is also in direct competition with naturally occurring dissolved organic matter (Kull et al., 2006). Pre-chlorination or oxidation of raw water results in cell lysis and toxin release. This may be beneficial since the toxin is exposed to oxidizing agents, but only if sufficient contact time with chlorine or other oxidizing agents can be guaranteed. However, pre-chlorination may increase the probability of MCs in finished drinking water and decrease MC removal by primary sedimentation and flocculation. Thus, it is to be avoided during bloom events, when higher MC concentrations are expected, or when contact time cannot be

guaranteed. Chlorination may not destroy all cyanotoxins (e.g. anatoxin-a) and chlorination by-products of MCs may be produced (Drikas and Hrudey, 1994). 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 (Weinberg, 2002). Pre- treatment with chlorine and other oxidants such as permanganate to control biofouling of intake pipes needs to be carefully balanced with the potential to cause cell lysis and toxin release before primary settlement processes that are effective at removing toxin-containing cells.

Ozonation appears to be slightly more effective than chlorination in removing cyanotoxins, but the rate of degradation is also dependent on a range of factors. Similar to 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 (Orr et al., 2004). In one study, cyanobacterial extracts containing 135-220 pg/L MCLR required 1.0 mg/L ozone over 5 min for complete toxin destruction (Rositano et al., 2001). According to the United States Environmental Protection Agency (USEPA), typical concentrations applied during drinking water treatment range from < 0.1 mg/L to 1.0 mg/L (Agency, 1999). As with chlorination, there is the possibility for ozonation to transform cyanotoxins or other organic matter to toxic by-products (Himberg et al., 1989; Legube, 2003).

Granulated activated carbon (GAC) filtration or powdered activated carbon (PAC) is probably one of the most effective treatments for removal of a larger range of cyanotoxins (reviewed in (Hitzfeld et al., 2000) and (Westrick et al., 2010)). It has shown to be effective in the removal of MCLR and some, but not all, saxitoxins using either adsorption or biodegradation (i.e. microbial growth on GAC) removal mechanisms and in conjunction with ozonation (Orr et al., 2004). A study of four drinking water treatment plants in Wisconsin showed an average 61% reduction in MCs after the pretreatment stage, which involved use of PAC (Karner et al., 2001). It is not clear if GAC and PAC are effective in binding/degrading other toxins such as anatoxin-a and BMAA. In fact, little information exists on the effect of any treatment strategy for the removal of BMAA and its isomers. Anatoxin-a binds most efficiently to activated carbon at a pH above the pKa (pH 9.4) in its deprotonated form. As such, pH plays a critical role in the ability of GAC/PAC to remove polar or charged toxins. 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. Some studies have shown accumulation of algal biomass on the surface of GAC filters, perhaps even due to growth, that may be a significant source of toxin load if those cells are lysed, such as during back flushing of filters leading to toxin breakthrough (Zamyadi et al. 2012).

Other common, but less employed, treatments for cyanotoxin removal include decomposition by ultra violet (UV) light and removal by membrane filtration (e.g. micro-, ultra-, and nanofiltration) and reverse osmosis (RO). MCs are highly stable under natural sunlight and undergo isomerization when exposed to low levels of UV light (Tsuji et al., 1994). However, under high exposure to UV light, rapid decomposition of MCLR, -YR, and -LA was observed (e.g. < 10 min), but required additional oxidative catalysts for the treatment of raw lake water (Shephard et al., 1998). RO and membrane filtration are far more expensive than traditional treatments, and as a function of the process, toxin rich waste water is retained. Still, previous studies have indicated that ultra- and nanofiltration were effective in removing > 95% of MCs (Gijsbertsen-Abrahamse et al., 2006), with hydrophobic membrane filters being the most effective (Lee and Walker, 2008). Fewer studies have been conducted using RO. However, RO is a convenient method in areas treating brackish waters, and > 96% retention of MCs was observed in tap and salt water containing up to 3000 parts per million NaCl (Neumann and Weckesser, 1998).

While a number of studies have been conducted to examine the effect of treatment strategies on MC removal, a dual national program to evaluate and review treatment strategies for the removal of all major secondary metabolites produced by cyanoHABs is lacking. A range of treatment regimes are currently in practice, but no two DWTPs are alike, and each cyanotoxin responds differently to a given treatment strategy. Each DWTP needs to optimize their processes to efficiently remove/destroy cyanotoxins if source waters are suspected to contain them. This requires monitoring of cyanotoxins in source waters and throughout the treatment process. The majority of DWTP in the Great Lakes region that draw from source waters containing cyanoHABs have historically not conducted this monitoring.

G. Current Technology and Monitoring Methods for Cyanotoxins.

Cyanotoxin detection methods have been developed largely in the form of biological or immunological assays and analytical chemistry for laboratory measurements. The major caveats of these methods include inconsistency and unavailability among toxin standards (or suitable substitutes/surrogates), lack of standardization among laboratory measurements, costs of materials or instrument usage, and the capacity of each method to measure all fractions of the total toxin pool within a sample (i.e. intracellular, covalently-bound and dissolved) as well as all toxin variants that may exist in nature. MCs are often found intracellularly within the thylakoid membranes (Young et al., 2005), but MCs and other toxins can also be found dissolved in water after natural cell lysis, or could be actively exported from the cell (e.g. anatoxin-a).

Cell lysis is required to capture the total toxin pool. Preferred methods of cell lysis are freeze- thaws (Selwood et al., 2007) or microtip sonication after a concentration step, such as lyophilization. "Over sonication" could destroy MCs at higher power and duration (Rajasekhar et al., 2012); the ability to process multiple samples simultaneously also makes freeze- thaws a better option compared to sonication. At least one study (Kim et al., 2009) showed that lyophilization followed by freeze- thaw cycles is the most efficient method of cell lysis. However, lyophilization may be too time- consuming (e.g. 48 hours to lyophilize 10-50 ml) for public health monitoring.

Extraction of MCs and other cyanobacterial peptides is best achieved with at least 50% methanol (Harada et al., 1988), while extraction of the more polar toxins—cylindrospermopsin, anatoxin-a, and saxitoxin—requires water with formic or acetic acid and gentle heating (Welker et al., 2002; Selwood et al., 2007). Solid phase extraction is routinely used as an alternative or additional step for concentrating and further purification of toxins, whereby amphiphilic peptides (e.g. MCs) are purified on C18 silica material (e.g. Bond Elute cartridge) and polar compounds are purified on activated carbon after a pH adjustment above their pKa for deprotonation. The variability in standards, extraction protocols, and analytical equipment among laboratories makes comparisons between laboratories difficult. Additionally, there are few certified reference standards available compared to the vast number of cyanobacterial toxins produced.

Several analytical techniques have been developed for cyanotoxin quantification and differentiation. The most commonly employed are discussed here. One of the most reliable of these methods is liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). LC-MS/MS satisfies the need for sensitivity and specificity of analysis through compound

molecular weight detection, fragment ion selection, and retention time of analytes. MCs and other cyanobacterial peptides are typically separated using reversed-phase C18 columns (Allis et al., 2007; Lajeunesse et al., 2012; Ballot et al., 2014). Because neurotoxins including the alkaloids (e.g. saxitoxins, anatoxins), amino acids, and cylindrospermopsin are generally more polar, they are better separated using hydrophilic interaction liquid chromatography (HILIC) (Dell'Aversano et al., 2004; Selwood et al., 2007; Faassen et al., 2012; Lajeunesse et al., 2012; Ballot et al., 2014). In some cases, gas chromatography has been coupled with tandem mass spectrometry (GC-MS/MS) to measure both MCs and anatoxin-a (James et al., 1997). Acetylation of anatoxin-a and conversion of MCs to the carboxylic acid 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB) were necessary for GC-MS analysis.

Typical analytical methods screen for a few select congeners, though there may be many potentially present or some structures that have yet to be described. There are some methods to address this analytically by converting all congeners to a common end- product. For example, saxitoxins can be hydrolyzed under alkaline conditions leaving only the common aromatic end-product. Similarly, conversion of MCs to MMPB is a process by which total MCs – all present congeners and free or protein-bound – can be analyzed; this method is achieved by Lemieux oxidation (Sano et al., 1992) or ozonolysis (Harada et al., 1996) of the ADDA amino acid of MCs to MMPB. Detection of MMPB has been used to measure MCs in animal tissues, human serum and water samples (Williams et al., 1997; Hilborn et al., 2007; Neffling et al., 2010; Roy-Lachapelle et al., 2014). Since the ADDA chain is universal to MCs, the MMPB method provides unique capabilities for the measurement of all MCs present, rather than specific congeners for which analytical standards are available. However, in addition to free MCs, the method also captures MCs covalently bound to proteins, and fragmented or transformed MCs that still contain an ADDA group.

Based on column separation properties and MS/MS technology, several unique toxins can be specifically analyzed in a single sample. However, the ability to measure a suite of toxins is dependent on having analytical reference standards. A lack of isotopically labeled surrogate standards hampers the ability to accurately quantify cyananotoxins (e.g. using standard addition). Currently there are no commercial certified reference standards available for anatoxin-a(s).

Analytical methods other than MS/MS have been combined with liquid chromatography for toxin detection, such as fluorescence, ultraviolet (UV), or photodiode array detection. LC

coupled with UV detection can be used for cyanotoxin measurements (Mahakhant et al., 1998; Selwood et al., 2007). UV detection is a useful option because the instrumentation is widely available and inexpensive; however, co- eluting compounds can lead to false positive detection, and sensitivity is poor requiring concentration of large sample volumes and extensive sample cleanup. Photodiode array detection combined with LC separation has been used to detect cylindrospermopsin (Welker et al., 2002), anatoxin-a (Gugger et al., 2005), MCs and nodularin (Spoof et al., 2010). Using this method, absorption spectrum characteristics can be used to confirm compound detection, but sensitivity is no better than UV detection.

Toxins with aromatic ring structures that naturally fluoresce can be quantified using LC with fluorometric detection (FLD). This has been principally used to detect the saxitoxins (Lawrence et al., 2005), anatoxin-a (James et al., 1997) and amino acids after derivatization including BMAA and its isomers (Faassen et al., 2012). Fluorescence detection (FLD) is more specific than UV detection since detection is based both on specific excitation and emission wavelengths given by the target compounds or with proper instrumentation excitation and emission spectra. Like LC with UV detection, some type I (over-detection) errors have been associated with LC- FLD detection due to co- elution of contaminating molecules (Rosen and Hellenas, 2008; Reveillon et al., 2014).

The direct quantification of cyanotoxins using the LC-MS/MS method provides the most accurate result for most target analytes, but comes at a higher cost per sample compared to other screening methods. As an alternative to high costs of equipment use, a number of biological activity- based methods have been developed to indirectly quantify cyanotoxins or cyanotoxin groups based on their molecular targets or mode of action. To measure MCs and nodularins, protein phosphatase inhibition assays have been developed and found comparable to using UV detection, enzyme-linked immunosorbent assay (ELISA) and HPLC-MS/MS (An and Carmichael, 1994; Ward et al., 1997; Bouaicha et al., 2002) . Several samples can be analyzed for MCs simultaneously in a microtiter plate in a matter of a few hours post- extraction with a test kit. Potential interferences with the protein phosphatase inhibition assay include interference due to the presence of heavy metals, turbidity, or other compounds capable of inhibiting PP1/PP2A. Commercially available kits exist for the protein phosphatase inhibition assays for MCs. Similarly kits for an anatoxin-a nicotinic acetylcholine receptor-binding assay (RBA) are available that can quantify a range of 10 to 500 μg/L anatoxin-a. This assay is also susceptible to

type I errors as multiple compounds can bind to acetylcholine receptors including nicotine and some insecticides. Nonetheless, the result is still indicative of the overt toxicity of the water sample.

ELISA kits are commercially available for MCs/nodularin, BMAA, cylindrospermopsin, and saxitoxins. These assays function as "indirect" ELISAs through antibody- binding in three main steps: 1) a toxin- specific detection antibody is added to the sample, 2) a secondary antibody specific to the detection antibody and containing an enzyme conjugate (i.e. horseradish-peroxidase) is added, and 3) a colorimetric substrate that reacts with the enzyme is then added. After a specified time, the enzyme- substrate reaction is halted, and the colorimetric response is directly proportional to the toxin concentration. Conversely, a "direct" ELISA (with the least cross-reactivity among cyanotoxin ELISAs) is used to screen for BMAA. This means that it will most likely bind BMAA only and has only a 0.01- 0.2% chance of binding BMAA's naturally occurring isomers or other toxins. Other ELISA kits detect multiple congeners of toxins, so further analytical testing should be done after ELISA to accurately quantify the toxins present in the water sample. Cross- reactivity has been shown to occur with the MC ELISA assay giving type I errors, which varied according to methanol concentration and pH among other factors (Metcalf et al., 2000).

ELISAs are a cost- effective useful screening tool, but in most circumstances require confirmation by other methods (e.g. LC-MS/MS) (Triantis et al., 2010). Since most ELISA assays target all toxin congeners, results are difficult to interpret when congeners have varying levels of toxicity. For example, ELISAs targeting the ADDA group of MCs will show a false positive result if ADDA is free in solution, unattached to intact MC.

The numerous methods available for the determination of cyanobacterial toxins all have advantages and disadvantages, and importantly, many of them are fundamentally different in what they actually measure (e.g. biological versus analytical assays). Therefore, as a matter of public health practice it would be difficult and inappropriate to assign a single method as the "gold standard" for cyanotoxin determination. Figure 6 suggests one possible workflow for the application of methods to confirm the presence of toxic MCs, which, with some modifications would likely be sufficient for saxitoxin, cylindrospermopsin, and anatoxin-a. Optimally 3X freeze/thaw extractions should be completed to homogenize intra- and extracellular toxins.

Biological assays are high-throughput, sensitive, and relatively inexpensive. In addition, in theory most target a wide range of toxin structural variants. Therefore, MC, saxitoxin, and cylindrospermopsin should all be screened initially using ELISA, and anatoxin-a should be screened using the nicotinic RBA (Figure 6). Since ELISAs are subject to type I errors, in routine monitoring programs samples giving positive results by ELISA should be analyzed by LC-MS/MS. Because anatoxin-a, freshwater saxitoxins, and cylindrospermopsins appear to have fewer congeners than MCs, and standards are available for most or all of those congeners, additional assays may not be required after confirmation with LC-MS/MS. An exception may be some unique saxitoxin variants, such as those produced by *Lyngbya* species.

There are a variety of scenarios under which the MC ELISA would not match results by LC-MS/MS. For instance, LC-MS/MS may produce negative results if all or a proportion of the MC variants in the sample are unusually rare, and not targeted by the LC-MS/MS method.

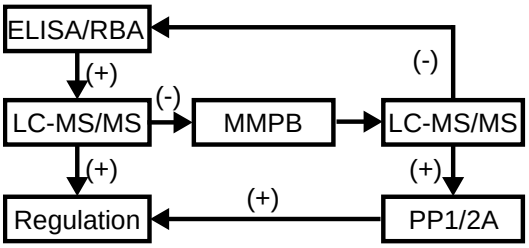

Figure 6. Starting with ELISA/RBA step, suggested methods to confirm a sample contains toxic MC, saxitoxin, and/or cylindrospermopsin, and anatoxins.

In addition, the LC-MS/MS result may not match the ELISA result if much of the MCs present in the sample are not toxic and covalently bound to proteins, or if ADDA detected by ELISA is cleaved from the MC structure and free in solution. All ADDA groups in the solution can be converted to a common end product, MMPB, via Lemieux oxidation or ozonolysis, and the resulting concentration of MMPB measured via LC-MS/MS. In theory, the concentration of MMPB should confirm the ELISA result since both assays are targeting the same structure. It does not, however, indicate the amount of MC present in the sample that is toxic because both the MMPB method and ELISA target non- toxic (protein- bound, free ADDA) as well as toxic forms of MCs. The PP1/2A inhibition assay would indicate the proportion of MCs in the sample that are toxic, or retain the ability to inhibit this enzyme. Using this three- tiered approach should provide a more reliable assessment of the actual toxicity of a water source in shortest amount of time possible and avoid costly errors in public health decision- making.

It should be noted that for recreational and source drinking water, pretreatment of samples may be needed prior to ELISA/RBA analysis. For example, high amounts of dissolved organic carbon can interfere with these assays, which are also often pH dependent. For finished drinking water, chlorine in the sample must be quenched, often by sodium thiosulfate, before an ELISA can be performed as chlorine may destroy antibodies.

A number of methods are available to measure cyanotoxins directly or indirectly. No method is currently available that targets all congeners and all toxic forms. The tiered approach presented in figure 6 provides one workflow for achieving this. However, the capacity for cyanotoxin testing in the Great Lakes region has not been assessed. It is not clear what laboratories are available and able to contribute testing. Furthermore, procedures for regular cross-laboratory testing are needed to ensure uniform testing procedures across the region. Finally, as discussed previously, few or no suitable proven surrogate standards exist for assessing extraction efficiency of cyanotoxins. As the problem of cyanoHABs is increasing, there is a great need to establish uniform, accurate methods and practices across laboratories that target all possible toxin variants. Such a task would have been nearly impossible just a few decades ago. However, recent developments in analytical instrumentation (including cost of instrumentation), biological assays, and our understanding of cyanotoxin diversity and toxicology have improved greatly since the first cyanotoxins were discovered.

H. Health Risks, Mechanisms of toxicity and potential for chronic disease

Cyanotoxins associated with cyanoHABs in lakes are generally divided into three groups: liver toxins, neurotoxins, and dermatoxins. However, some cyanotoxins may not fit into these categories or have properties of more than one category.

Cyanotoxins may remain in the bacterial cells (intracellular), be actively excreted, or be released by cell lysis, into the environment (extracellular). Toxins can also exist bound to other substances such as membranes and proteins. Human activities, including drinking water treatment, may affect these states. Another challenge is establishing human health standards (given the state of current toxicological knowledge) for these toxins to allow for environmental assessment. Such caveats are important for monitoring strategies and determining toxin potencies which will be discussed further in the sections below.

Liver Toxins

In the Great Lakes region, the most commonly observed or targeted cyanobacterial liver toxins are the microcystins (MCs) (Vanderploeg et al., 2001; Boyer, 2007; Rinta-Kanto et al., 2009). They are cyclic heptapeptides with five non-protein amino acids and two variable protein amino acids. Methylations, hydroxylations, epimerizations and amino acid replacements lead to structural diversity of MCs where at least 85 different variants have been detected in lakes or cell cultures (Sivonen and Jones, 1999). The number of different combinations of amino acid substitutions and structural alterations suggests at least 300,000 different congeners are theoretically possible (personal communication C.O. Miles, Norwegian Veterinary Institute). MCs covalently bind to and inhibit protein phosphatases type 1 and 2A in eukaryotic cells, though other proteins and enzymes may also be inhibited (Honkanen et al., 1990). MCs are specifically transported into hepatocytes and across the blood-brain barrier by organic anion transporter polypeptides (OATPs) (Fischer et al., 2005). In nature, MC with leucine and arginine at the variable protein amino acid positions (MCLR) and MC with two arginine residues (MCRR) are often reported to be the dominant variants detected, or at least targeted in environmental studies (Briand et al., 2005; Boyer, 2007; Ballot et al., 2014). *Microcystis* is the dominant MC producer, but *Planktothrix*, and/or *Anabaena* taxa are also commonly associated with MC production (Ernst et al., 2001; Rinta-Kanto and Wilhelm, 2006). A variety of other genera have been found to produce MCs including *Planktothrix*, *Oscillatoria*, *Nostoc*, and *Gloeotrichia* (Luukkainen et al., 1993; Carey et al., 2007). With the recent identification of MC synthetase genes, the diversity of MC producers in nature is only beginning to be realized (Rinta-Kanto and Wilhelm, 2006).

While MCs appear to be the most prevalent cyanotoxins, cyanobacteria also produce other liver toxins. Nodularin is a hepatotoxic cyclic pentapeptide with structural similarity to MCs. As such, it also inhibits protein phosphatases, but is primarily produced by *Nodularia spumigena* in brackish waters and may also occur in freshwaters (Beattie et al., 2000). Cylindrospermopsin and its analogs (e.g. deoxycylindrospermopsin) is a sulfate ester of a tricyclic guanidine substituted with a hydroxymethyluracil. It is produced by *Cylindrospermopsis raciborskii* and some other genera (e.g. *Aphanizomenon*) causes liver and kidney toxicity by inhibiting the synthesis of protein and glutathione with other toxic effects (see below and (Runnegar et al., 2002)). This compound also displays genotoxic effects *in vitro* and *in vivo*

(Humpage et al., 2005). Although *C. raciborskii* is normally associated with (sub)tropical habitats, it is now considered an invasive species in temperate regions, including the Great Lakes (Hong et al., 2006; Conroy et al., 2007).

Neurotoxins

Anatoxin-a and homoanatoxin-a are produced by species of *Anabaena*, *Oscillitoria* and *Aphanizomenon* among others (Devlin et al., 1977; Edwards et al., 1992). Anatoxin-a(s) is a naturally occurring organophosphate containing a methylphosphate ester attached to cyclic guanidine, structurally unrelated to anatoxin-a. Less is known about the distribution of anatoxin-a(s), but *Anabaena* species, particularly *Anabaena flos-aquae*, have been shown to produce anatoxin-a(s). (Carmichael et al., 1979). Anatoxin-a and homoanatoxin-a are nicotinic acetylcholine agonists and Anatoxin-a(s) is an organophosphate that irreversibly binds to acetylcholinesterase in peripheral nerve cells (Cook et al., 1988; Cook et al., 1989b; Cook et al., 1991; Hyde and Carmichael, 1991; Thomas et al., 1993). The net effect of all anatoxins is uncontrolled activation of nicotinic and muscarinic acetylcholine receptors resulting in respiratory paralysis.

Several other cyanobacterial neurotoxins are an emerging concern in North American recreational lakes. Recently, the possible chronic neurotoxin beta-N-methylamino-L-alanine (BMAA) was found in diverse species of cyanobacteria (Cox et al., 2005). It is normally associated with root symbionts, soil cyanobacteria (*Nostoc* species), of the Cycad tree and has been linked to amyotrophic lateral sclerosis/parkinsonism–dementia complex (ALS/PDC) in Guam and other human populations (Cox et al., 2003; Johnson et al., 2008; Pablo et al., 2009; Field et al., 2013; Murch et al., 2004). Thus, BMAA is associated with chronic illnesses with no evidence that is acutely toxic. Cox et al. recently found that BMAA is also produced by every major order of cyanobacteria including common freshwater bloom forming species such as *Microcystis, Anabaena* and *Planktothrix* species (Cox et al., 2005). Various other research groups have confirmed this finding (Jiang et al., 2012), while others either failed to detect BMAA, found much lower concentrations, or detected isomers of BMAA instead (Rosen and Hellenas, 2008; Li et al., 2010; Faassen et al., 2012). Other reports indicate that BMAA and its isomers are present in some lakes and/or algal bloom material and dispersed in various organisms throughout the aquatic food web (Field et al., 2013).

Saxitoxin and its more than 50 analogs are tricyclic alkaloid neurotoxins that permanently block voltage-gated sodium channels in nerve cells causing paralysis. They are widely distributed in nature occurring in both fresh and saltwater and found in evolutionarily disparate organisms including cyanobacteria, dinoflagellates, and fish (Nakashima et al., 2004; Halme et al., 2012). Historically this toxin is rarely found in northern temperate lakes, but the apparent spread of tropical or subtropical cyanobacterial species (i.e. *C. raciborskii*) capable of producing saxitoxin, into northern lakes including the Great Lakes suggests this toxin may become more prevalent (Sinha et al., 2012). In addition, genera common to temperate lakes (e.g. *Aphanizomenon*) have been found recently to produce saxitoxin in northern lakes (Ballot et al., 2010). Similarly, historically, *Lyngbya wollei* was associated with lakes in the Southwestern United States, but has now invaded some parts of the Great Lakes, especially the Western Basin of Lake Erie (Bridgeman and Penamon, 2010). This organism blooms in thick mats at the sediment surface in shallow zones, and it produces two common saxitoxin variants as well as six that are unique to this species (Carmichael et al., 1997; Onodera et al., 1997).

Dermatoxins

Rash and contact dermatitis are reported anecdotally in cases of human exposure to cyanoHABs (Stewart et al., 2006e). This antigenic substance recruits an immune response resulting in inflammation and exacerbates pathogenicity, including atopic dermatitis. It has been suggested that the lipopolysaccharides (LPS) of cyanobacteria may contribute to human illness, particularly epidermal allergic reactions. However, there is little evidence in the scientific literature that skin contact with the LPS of cyanobacteria causes a skin rash (Stewart et al., 2006d; Stewart et al., 2006a). Rather, it may be that skin rashes after cyanoHAB exposure are caused by one of hundreds of other bioactive metabolites produced by cyanobacteria. For example, cylindrospermopsin was shown to cause hypersensitivity reactions in the mouse ear-swelling test (Stewart et al., 2006b). In addition, the marine cyanobacterium *Lyngbya mujuscula* produces tumor promoters and skin irritants including the indole alkaloid lyngbyatoxin A, and the polyacetates, aplysiatoxin and debromoaplysiatoxin (as reviewed by (Osborne et al., 2001)). These molecules bind to phorbol ester receptors activating protein kinase C inducing excess phosphorylation of cellular proteins leading to disruption in cell cycle regulation and tumor formation with the initial symptoms being acute skin lesions (Fujiki et al., 1981). Repeat

exposure to these molecules is thought to cause skin cancer (Fujiki et al., 1984). While *L. majuscula* does not occur in freshwater lakes, anecdotal evidence suggests that exposure to the freshwater species, *L. wollei* in freshwaters may induce dermatitis (Pittman, 2006; Fossa et al., 2012). Whether *L. wollei* produces compounds similar to lyngbyatoxin A and the aplysiatoxins is unknown.

While health care providers understand many of the human health hazards associated with cyanotoxins, the ability to associate environmental cyanotoxin exposure with individual cases and illness diagnoses remains a public health challenge. Recently, 228 hospital visit records in New York State were linked to environmental exposure to harmful algal blooms using the recorded World Health Organization's International Classification of Disease (ICD) (Figgat et al, 2016). The visits, recorded from 2008 - 2014, resulted in multiple principal diagnoses and occurred throughout all four seasons.

The US Centers for Disease Control and Prevention launched a monitoring framework in 2016, the One Health Harmful Algal Bloom System (OHHABS, https://www.cdc.gov/habs/ohhabs.html), to provide for long-term monitoring and reporting of potential public health events due to cyanotoxin exposure and support public advisories related to harmful algal blooms in the United States. Relying on data sharing from participating states and territories, the system enables reporting on human and animal illness from exposure to cyanoHAB events in marine, brackish and freshwater environments.

During operations from 2007-2011, the program (then known as the Harmful Algal Bloom-Related Illness Surveillance System (HABISS)) reported cases of illnesses associated with exposures to cyanobacteria or algae (Backer et al, 2015). The percentage of food vs. recreational water as the human exposure source was 60% and 40 % of reports, respectively. For water exposures, human cases from freshwaters were the most common, with much lower numbers of cases from marine waters and brackish water. This summary of HABISS data discussed animal cases for exposure by water ingestion, though none were reported for humans. Comparisons of human exposure to cyanotoxins from drinking water and recreational waters sources would be supported under the new OHHABS initiative. Systems such as these will provide an opportunity to leverage ongoing human and animal surveillance of HABs exposure to support understanding and prevention of HABs and HABs related illness.

Toxicity of CyanoHABs

In 1878 George Francis, of Adelaide, South Australia published his observations of mysterious animal deaths (horses, sheep, dog, pig) after they drank from Lake Alexandrina (Francis, 1878). He observed that animal deaths occurred when blooms of cyanobacteria, perhaps *Nodularia*, were blown by the wind toward the shore. Francis explained that the animals died within hours after drinking lake water exhibiting a range of symptoms consistent with neurotoxicity including stupor and unconsciousness, convulsions with head drawn back, and rigid spasm. Interestingly, he noted that the animals did not drink from "*puddles where scum has collected*," but avoided those areas and drank from "fresh" waters. As such, he wrote, "… *the poisoning is not caused by drinking a putrescent fluid full of bacteria as at first supposed*." These statements are widely cited as the first scientific reports implicating cyanobacteria in the production of one or more potent toxic substances.

Following this report, others followed in the United States, Canada, and elsewhere during the 1880's and early 1900's as summarized by Fitch and colleagues in 1934 (Fitch et al., 1934). These reports confirmed Francis' observations suggesting that poisonous compounds were associated with blooms of cyanobacteria.

Early experiments with laboratory animals involved intraperitoneal injection (i.p.) of raw or crude extracts of bloom material, cultured cells, or crude extracts of both. Results from these early studies formed the recognition that cyanobacteria (or other organisms associated with them) were likely responsible for toxin production; however, procedures for cyanobacterial isolation/culturing and toxin purification were unknown until the 1970's. Therefore, studies before this time are difficult to interpret and will only be considered briefly here. It should be noted that even current methods for growing cyanobacteria free of contaminating organisms (e.g. heterotrophic bacteria) remain difficult. Nonetheless, modern toxicological studies have at least used purified toxin material.

Commonly found Cyanotoxins

The most frequently occurring and/or detected cyanotoxins in the Great Lakes region are MCs, anatoxins, cylindrospermopsin, and saxitoxins. Evidence for their mechanism of human and animal toxicity is discussed below.

Microcystins

Early Studies

It had been observed by 1930 that various species of *Microcystis* were often the dominant organism present in lake water associated with animal deaths. Fitch et al. (Fitch et al., 1934) were among the first to describe the effects of *Microcystis* bloom material on laboratory animals through i.p. injection. Guinea pigs, rabbits, and pigeons died rapidly (minutes to hours) producing similar symptoms including restlessness, urination, defecation, deep breathing, hind-quarter weakness, coughing, salivation, lachrymation, and clonic spasms. They observed that toxicity of bloom samples decreased when stored in a refrigerator, probably due to degradation of the toxins by associated heterotrophic bacteria. In addition, it is likely that multiple substances were acting on the animals to cause the illness since raw bloom material was used. Thus, efforts were made to isolate and culture *Microcystis aeruginosa* in the laboratory for toxicological studies (Hughes et al., 1958; Gorham, 1962).

In 1946 (just after the end of WWII), Ashworth and Mason (Ashworth and Mason, 1946) made detailed observations of pathological changes in rats after injection with a chloroform, acetone, ether extract of *M. aeruginosa* culture. This study as well as others (e.g. (Wheeler et al., 1942)) established that gross pathological changes associated with *M. aeruginosa* are primarily observed in the liver. This study also showed that the effects were similar to other hepatotoxic agents involving cytolysis of liver cells. They reported that after injection with a sub- lethal dose of *M. aeruginosa* culture, the liver goes through defined stages. Approximately 30 min after exposure the liver becomes slightly enlarged, red, and tense. At three to six hours, liver weight is 25% above the controls, there is increased blood content of parenchyma, and the tissue becomes soft and friable with all lobes affected. At 2 – 3 days, the liver has shrunk by 2/3, it is yellow and mottled, and blood coagulation is slow. At five days after the maximal sub-lethal dose, the liver returns to a normal state. This is the result of a one- time exposure to a high, but sub- lethal dose.

It wasn't until 1959 that a MC toxin (presumably) was partially purified and characterized from *M. aeruginosa*. This was largely enabled by the isolation and mass culturing of *M. aeruginosa* (Hughes et al., 1958). Bishop et al. (Bishop et al., 1959) purified a toxic fraction (i.e. by mouse bioassay) from *M. aeruginosa* NRC-1 using Soxhlet extraction with methanol. Five peptides were detected upon electrophoresis of the crude extract at pH 7, and one was found to be acutely toxic in the mouse bioassay (i.p. LD50 = 460 µg/kg b.w.). This peptide (or mixture thereof) was likely an MC, but the amino acid content given was not consistent with the MC base structure. This fraction was identified as the "fast death factor" earlier described by Hughes et al. (Hughes et al., 1958). It should be noted that Paul R. Gorham's laboratory was behind much of the early work showing that cyanobacteria produce toxins harmful to a variety of organisms in controlled settings, as well as some of the initial characterizations of cyanotoxin properties.

In 1965 Konst et al. (Konst et al., 1965) conducted animal trials with guinea pig, rabbit, mice, duck, chicken, lamb, and calf. As the starting material, they used freeze-dried *M. aeruginosa* and administered it by oral or the i.p. route. Again the liver was found to be the most affected organ in both exposures with minor abnormalities in heart and lungs. A significant finding of this early study was that the oral route was much less toxic (40x) than i.p. route. In addition, as had been observed by others (e.g. Ashworth and Mason), blood coagulation was slow in dosed animals likely due to liver damage and depression of prothrombin levels.

While these early studies did not use pure toxin, the analysis of whole cell toxicity is somewhat enlightening. For example, Konst et al. as well as Hughs et al. observed rapid death and supposed neurotoxic effects in mice injected with *M. aeruginosa* including convulsions, dragging of hind legs, and loss of equilibrium. Were these symptoms due to acute liver failure, neurotoxicity of MC, or other neurotoxins associated with *M. aeruginosa*? This was unknown at that time. It is important information as it corroborates recent findings that MC is a neurotoxin (see below), and that at least some strains of *M. aeruginosa* are capable of producing other non-ribosomally synthesized peptides that show neurotoxicity (e.g. cyanopeptolin)(Gademann et al., 2010).

Pathological Studies Using Pure MC Toxin

A description of the definitive structure of MC was given by Botes et al. (Botes et al., 1982) and Rinehart et al. (Rinehart et al., 1988). These studies and prior work established that *M. aeruginosa* does indeed produce cyclical toxic peptides as reported by Bishop et al (1959). Structural elucidation paved the way for controlled toxicological studies using purified toxin, rather than bloom material or cultured cells potentially containing a mixture of toxins of unknown quantity. Culturing and toxin isolation studies were primarily performed by Gorham and Carmichael, which were critical to the elucidation of cyanotoxin properties. In addition, analytical methods were developed soon after to quantify the amount of toxin used (Dierstein et al., 1988), as well as appropriate extraction methods for MCs (Harada et al., 1988).

In 1989, Hooser et al. (Hooser et al., 1989) performed one of the first studies of acute toxicity of MCLR (i.e. MC with leucine and arginine in the two variable amino acid positions) using purified toxin (by preparative high-pressure liquid chromatography) in rats and mice via i.p. exposure. The lowest one time dose producing death in rats and mice within 24 hours and 90 min, respectively, was 160 µg/kg b.w. in rats and 100 µg/kg b.w. in mice by i.p injection. At i.p. doses of 80 and 40 µg/kg b.w., male and female rats, respectively, had no clinical signs of toxicity, or gross or microscopic lesions. At 120 and 80 µg/kg b.w. a portion of male and female rats, respectively, showed clinical signs of toxicity. All animals showing clinical signs eventually died. Similar results were found by Runnegar et al. (Runnegar et al., 1993) using both MCLR and MCYM where 84 µg/kg b.w. resulted in nearly complete inhibition of liver protein phosphatase followed by death.

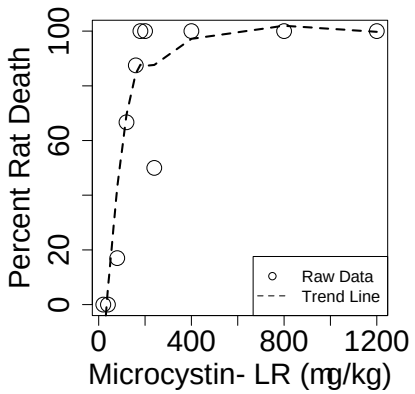

Figure 7. Dose- to- death curve for MCLR in the rat. Data from Hooser et al. 1989.

These results show that MCs display an extremely steep dose to death curve (Figure 7). The study by Hooser et al. shows that the difference between a dose that causes death and complete recovery (with i.p. exposure) is less than a factor of 2. Similarly, Lovell et al. (Lovell et al., 1989) showed that 25 µg/kg b.w. is the max dose resulting in no death in mice while the LD50 is just 7.5 µg/kg b.w. higher at 32.5 µg/kg b.w.

In the Hooser et al. study, time to death with MCLR exposure via i.p. injection ranged from 20 - 32 hours (120 - 240 µg/kg b.w.) or 6 - 8 hours (400 - 1200 µg/kg b.w.) and symptoms were largely lethargy and

ruffling of fur. Liver weight was significantly higher for all dosage levels. Alanine aminotransferase, alkaline phosphatase, urea, and creatine serum levels increased over time in all treated animals suggesting liver damage at all dosages. There was also a decrease in serum glucose levels. Upon pathological examination, it was found that a breakdown in sinusoidal liver endothelial cells and hepatocyte dissociation resulted in the presence of hepaotycytes and cellular debris in the pulmonary artery and lungs. Hepatocyte damage preceded the presence of these cells in lungs thus disproving the earlier theory that liver damage is due to pulmonary thrombosis.

Repeat MC Oral Dose Studies

By comparison, MCs are 30 - 100 fold less toxic by oral exposure in rats and mice. Fawell et al. (Fawell et al., 1999a) conducted tests of MCLR (pure toxin from Calbiochem-Novabiochem) toxicity in mouse and rat through both i.p. injection and oral gavage. Experiments were carried out to examine acute, developmental, and long-term (13 weeks) effects. As in previous studies, symptoms of acute exposure included convulsions, hypoactivity, prostration, and slow respiration. Death by oral route in mice occurred with a single dose of 1,580 µg/kg b.w. while no death occurred at 500 µg/kg b.w.. The authors concluded a no observed adverse effect level (NOAEL) in the mouse through oral doses of 40 µg/kg b.w. per day over the 13-week period. This was the lowest concentration tested, so it is not clear if lower doses would produce adverse health effects. Criteria for the effect level included gross and microscopic liver pathology and blood chemistry. At the next highest level of 200 µg/kg b.w. per day, slight changes in blood chemistry were noted, but there was some uncertainty about these results. Fetal development was assessed, but the mice were not followed after birth (i.e. for learning, memory, or overall cognitive deficits). Interspecies differences were noted as MCLR was somewhat less toxic to rats. The results of this study were similar to those of Falconer et al. (Falconer et al., 1994) using pigs in an oral exposure route study. These two studies are currently the basis for the WHO maximum allowable concentration for MCLR in drinking water (1 µg/L) that exists today 16 years later (WHO, 1999; WHO, 2003).

R. Heinze (Heinze, 1999) also performed a repeat oral dose study of MC toxicity in rats. This study provided male adult rats MCLR in drinking water for 28 days at dosages of 50, or 150

µg/kg b.w./day. Body weights were measured weekly. After the exposure, endpoints were determined including body and organ weights, liver serum enzyme activity, blood cell counts, differential counts of leucocytes, hematocrit and hemoglobin, and histopathology of liver and kidney specimens. At both dosage levels liver weight was increased as was serum liver enzymes alkaline phosphatase and lactate dehydrogenase. In addition, histopathology showed evidence of liver damage or lesions in both groups. Thus, the lowest dose showing toxicity by these endpoints was 50 µg/kg/day.

Effects on other tissues from oral exposure to MCs

In addition to liver damage MCs have also been shown to affect brain and reproductive tissues (Maidana et al., 2006; Zhang et al., 2011; Kist et al., 2012; Zhao et al., 2012; Zhou et al., 2012; Wang et al., 2013; Zhang et al., 2013; Li et al., 2014a; Zhou et al., 2014; Zhao et al., 2015). Of note for risk analysis are those studies that examine effects from repeat oral dose exposures. In a recent study, Li et al. (Li et al., 2014b) exposed rats orally to MCLR (0.2, 1.0, and 5.0 µg/kg b.w.) by intragastric gavage for 8 weeks every two days and measured liver serum enzymes and effects on neurobehavior, or learning using the Morris water maze test. This test measures how quickly the animals learn to find a platform in a circular pool to escape the water. At 5 µg/kg b.w. there was a significant increase in serum cholinesterase levels and escape latency in the water maze test after the 8 weeks of oral exposure to MCLR. Furthermore, post-mortem analysis demonstrated accumulation of nitric oxide and nitric oxide producing cells in regions of the hippocampus. Nitric oxide acts as a neurotransmitter in the brain and is associated with learning and memory (reviewed in (Paul and Ekambaram, 2011)). Based on this study, central nervous system toxicity of MCLR begins to occur at 5 µg/kg b.w. delivered every 2 days.

Li et al. (Li et al., 2015b) examined the neuro-developmental effects of MCLR in a repeat maternal oral dose study. In this study Sprague-Dawley female rats (28 days old) were exposed to 1.0, 5.0, or 20 µg/kg b.w. MCLR by gavage every 48 hours for 8 weeks. After the 8 weeks the mice were mated and the offspring examined for adverse effects including body weight, morphological aberrations (external malformations, hair appearance, incisor eruption, and bilateral eye opening), deficiencies in motor development, learning and memory delays (i.e. Morris water maze test), histopathological analysis of hippocampus CA1 regions, and lipid peroxidation and antioxidant indices in the hippocampus. Cliff avoidance time decreased in pups

seven days postnatal at all exposure levels. In addition, performance in the Morris water maze test at postnatal day 60 was diminished. Specifically, the frequency of entering the platform of all exposed male offspring and female offspring from the 5 and 20 µg/L exposed group was significantly lower compared to controls. In addition, swimming speed of female offspring from mothers treated with 20 µg/kg b.w. MCLR was significantly decreased. Malondialdehyde contents and superoxide dismutase activity were significantly higher in the highest exposure groups.

Effects of MCLR on reproductive tissues have also been reported in repeat dose oral exposure studies. Using mice, Chen et al. (Chen et al., 2011) examined the effects of oral exposure to MCLR in drinking water on sperm count and motility, body and testis weights, serum testosterone, and apoptosis in testicular tissue. For the exposure, male mice were given sterile water *ad libitum* containing MCLR at 1, 3.2, and 10 µg/L for three or six months. At 3.2 and 10 µg/L levels sperm counts and motility were significantly decreased after both three and six months. In addition, serum testosterone and luteinizing hormone levels were decreased in the 3.2 and 10 µg/L exposure levels after 3 and 6 months. At 6 months there was a clear dose response relationship between exposure level and apoptosis of testicular cells.

The reproductive toxicity of MCLR reported by Chen et al. is corroborated by a variety of other in vivo studies using i.p. injection and in vitro studies using cultured cells or isolated reproductive tissue. Ding et al. exposed male 8- week old mice to 3.3 – 6.7 µg/kg b.w. MCLR in cell extracts daily for 14 days by i.p. injection and then measured toxic effects on reproductive organs. There was a significant decrease in body weight, sperm viability, rapid sperm motility, and an increase in percent sperm immobility. Li et al. (2008) observed that in mice exposed to 5 µg/kg b.w. /day via i.p. injection for 28 days sperm motility significantly decreased while at 15 µg/kg b.w./ day there was a decrease in testis weight, sperm concentration, serum testosterone, human luteinizing hormone, and follicle stimulating hormone. In other studies chromatin condensation, nuclei fragmentation and DNA fragmentation in testes cells has also been reported as a result of exposure to MCLR (Zhang et al., 2011). Increases in p53, Bax, and caspases in testis tissue have also been observed as signs of programmed cell death (Li and Han, 2012; Xiong et al., 2009; Zhang et al., 2011).

Molecular Mechanism of MC Toxicity

These important studies described above established criteria for protecting human populations from injury due to acute MC exposure. They examine the acute and overall observable pathological effects after consumption of water containing MCs at high concentrations (> 1,000 µg/kg b.w.). These concentrations are unlikely to occur in treated drinking water with even minimal primary treatment. In recent years, the effects of chronic low- dose exposure to MCs as well as toxicity to tissues other than the liver have been examined. These studies are based on the known mode of action of MCs. The molecular mechanism of MC toxicity resembles that of other biomolecules. The list of naturally occurring molecules that inhibit phosphatases in nature includes dinophysistoxins, calyculin, dragmacidins, tautomycin, tautomycetin, cytostatins, phospholine, leustroducsins, phoslactomycins, fostriecin, cantharidin, okadaic acid as well as MCs (Swingle et al., 2007). In particular, okadaic acid is associated with chronic diseases such as tumor production and cancer. Okadaic acid is a marine biotoxin produced by dinoflagellates and it accumulates in various host tissues including shellfish and sponges (van den Top et al., 2011; Armi et al., 2012; Li et al., 2012). It is a tumor promoter and potent inhibitor of type 1 and 2A protein phosphatases (PP1/2A). Yoshizawa et al. (Yoshizawa et al., 1990) discovered that in cytosolic fractions of mouse liver, MCLR inhibited the binding of okadaic acid to protein phosphatases, increasing protein phosphorylation and decreasing phosphatase activity in 50% of controls using nanomolar levels of MCLR. Structural studies show that the methylene carbon of the methyl-dehydroalanine residue of MCs covalently binds to a cysteine residue on the C subunit of PP1/2A phosphatases leading to enzyme inhibition (MacKintosh et al., 1995). This also has the effect of preventing the detection of MCs in exposed individuals by most approaches and accumulation of the bound product in host tissues, primarily the liver.

The consequences of PP1/2A inhibition by MCs are of significance for both acute and chronic toxicity. The PP1/2A phosphatases are essential for cellular survival. They help maintain homeostasis by controlling the activity of signal transduction pathways through the dephosphorylation of effector molecules. They consist of three subunits (A, B, and C) where C is catalytic, A is structural, and B determines substrate specificity. Multiple types of each subunit are present in human cells providing functional diversity. Phosphatase subunits bind forming an active structure only when the C subunit is methylated at the terminal Leu^{309}. PP2A is one of the most abundant proteins in eukaryotic cells and the most common phosphatase. Along with other proteins, active PP2A down regulates pathways involved in instigating cell proliferation and

growth, protein synthesis, and resistance to cell death or apoptosis (as reviewed by (Seshacharyulu et al., 2013)). As such, it is a tumor suppressor and a new target for anti- cancer therapies (Perrotti and Neviani, 2008, 2013). Indeed, PP2A is mutated or altered in many types of cancer cells (Wang et al., 1998; Takagi et al., 2000; Zhu et al., 2001). The activity of PP2A in response to stressors including DNA damage is controlled by its methylation state. Both MCLR and okadaic acid have been shown to directly inhibit the methylation of PP2A preventing the formation of an active PP2A holoenzyme. Thus, MCs and okadaic acid are tumor-promoters through kinase driven malignancy (Guergnon et al., 2011; Perrotti and Neviani, 2013; Liu and Sun, 2015). In 2010, MCLR was classified as being "possibly carcinogenic to humans (Group 2B)" by the International Agency for Research on Cancer (Agudo et al., 2006).

This mechanism suggests a dualistic response to MCs by mammalian cells. At a high dose, MCs cause massive changes in cell morphology through cytoskeletal rearrangements and oxidative stress, leading to loss of cell-to-cell adhesion and cell death (Falconer and Yeung, 1992; Huang et al., 2015; Zhou et al., 2015a). In a chronic model of low level exposure, constant mild PP2A inhibition leads to reprogramming of the cell, runaway cell growth, and tumor production, analogous to the effects of endogenous human protein CIP2A (cancerous inhibitor of PP2A) associated with breast and lung cancer (Junttila et al., 2007; Come et al., 2009; Lucas et al., 2011; Rincon et al., 2015). The level of MC required to cause complete liver failure and death is known (> 1000 µg/kg b.w. via oral dose), but the level required over time in multiple exposures to cause cellular reprogramming of liver cells (or other tissues) and tumor growth is unknown (Gehringer, 2004).

MCs also inhibit PP1/2A in brain cells (Fischer et al., 2005). In the central nervous system, PP1/2A control long term potentiation and long- term depression through dephosphorylation of the AMPA receptor at glutamatergic synapses. These processes facilitate learning and memory. There is evidence that MCs migrate to brain tissues following exposure (Papadimitriou et al., 2010) and are able to cross the blood- brain barrier (Meriluoto et al., 1990; Fischer and Dietrich, 2000; Cazenave et al., 2005). Inhibition of PP1/2A by MCs in rat brain has been shown to block long- term potentiation induction suggesting that MC exposure may induce cognitive delays (Wang et al., 2013). Indeed, direct injection of MC to rat hippocampus at femtogram levels has been shown to affect learning and memory (Maidana et al., 2006; Li et al., 2014a; Li et al., 2015b). MCs have also been shown to alter fish behavior and learning as well as

increase acetylcholinesterase activity in fish brain (Kist et al., 2012). In the well- described human MC intoxication event of dialysis patients in Caruaru, Brazil, patients experienced symptoms of neurotoxicity including deafness, tinnitus, and intermittent blindness (Pouria et al., 1998). Thus, it appears that MCs are central nervous system toxins; however, there is currently a lack of information concerning effects of long- term chronic exposure to low levels of MC that may affect processes such as learning and memory, particularly in children whose development is dependent on these activities. Such issues were not addressed in developing the WHO 1 µg/L recommendation for drinking water (WHO, 1999; WHO, 2003).

Health outcomes and target organs affected by MCs are highly dependent on OATPs (organic anion transporting polypeptides) that transport MCs into Eukaryotic cells. There are 11 OATPs in human cells. OATPs 1A2, 1B1, and 1B3 have been shown to transport MCs (Meier-Abt et al., 2007). OATP 1A2 is expressed in the blood- brain barrier (Gao et al., 2000; Lee et al., 2005; Ose et al., 2010; Zhou et al., 2015b), kidney (Lee et al., 2005), cholangiocytes (bile duct epithelial cells) (Franke et al., 2009; Zhou et al., 2011), testes, and enterocytes (Glaeser et al., 2007). OATP 1B1 and 1B3 are restricted to the liver under normal conditions. Thus, in addition to liver and brain, the kidney, bile duct, testes, and intestines are all additional potential targets of MC toxicity. Furthermore, other OATPs may be involved in MC transport. For example, OATPs 3A1 and 4A1 have been implicated in the uptake of MCs and these OATPs are distributed ubiquitously in human tissues (Zeller et al., 2011; Obaidat et al., 2012).

OATPs play a large role in determining one's sensitivity to xenobiotics, pharmaceuticals, and biotoxins, including MCs as well as other amphiphilic algal metabolites (e.g. peptides)(Hagenbuch and Gui, 2008). Genetic variations in OATP genes can increase or decrease OATP protein transport activity resulting in altered pharmacokinetics (Niemi et al., 2005; Niemi et al., 2006; Takane et al., 2007; Niemi et al., 2011). For example, healthy individuals carrying the common thymidine to cytosine single-nucleotide polymorphism (SNP) at base pair 521 of the OATP 1B1 gene (that transports MCs) are more sensitive to statins and other important medications (and potentially MCs) (Iwai et al., 2004). OATP 1B1 alleles with this SNP have decreased transport activity toward some statins, significantly increasing statin serum concentrations. On the other hand, this allele has increased activity towards other drugs and toxins (as reviewed by (Niemi et al., 2011)). Genetic alterations in OATPs are currently a robust field of inquiry. Over 200 SNPs have been identified in human OATP genes, which may be more

common in Asian populations (Iida et al., 2001). The rate of OATP 1B1 and 1B3 expression shows significant inter-individual variability and the expression levels of OATP 1B1 is correlated with SNPs in OATP 1B1 genes (Nies et al., 2013). It is therefore likely that metabolism, clearance rate of MCs, and overall human health outcomes associated with MC exposure are highly dependent upon an individual's genetics, particularly with respect to OATP transporters. In addition, it is not clear whether rodent and human OATPs share the same tissue distribution, activity/substrate interactions, and overall phenotypic response. Therefore, rodent studies of MC toxicity (discussed above) may not reflect true toxicity in humans. As such, the 1,000- fold safety factor employed in calculating the WHO maximum concentration guideline value for drinking water is clearly warranted (WHO, 1999; WHO, 2003).

Anatoxin-a

In early reports, blooms of other cyanobacteria, including *Anabaena flos-aquae* and *Aphanizomenon flos-aquae*, were implicated in the death of water fowl and domesticated animals (Fitch et al., 1934). Extracts of both *Anabaena* and *Aphanizomenon* produced very fast deaths (within 7 minutes) through i.p. injection with symptoms of neurotoxic poisoning including convulsions, limb twitching, eventual paralysis, and death (Gorham, 1960; Gorham, 1962, 1964; Carmichael et al., 1975). The previously defined "aphantoxins" from *Aph. flos-aquae* have now been determined to be saxitoxins and gonyautoxins (see below). Carmichael et al. (Carmichael et al., 1975) determined that the toxin from *Ana*. *flos-aquae* strain NRC-44-1 (isolated from Burton Lake, Saskatchewan Canada) was a neuromuscular depolarizing agent and specifically a cholinergic agonist acting upon nicotinic acetycholine receptors with high affinity and muscarinic receptors with low affinity (Carmichael et al., 1979; Aronstam and Witkop, 1981). The toxin was named anatoxin-a or *Anabaena* toxin "A" to discriminate it from other toxins in *Anabaena* species. The toxin structure was proposed by O. E. Edwards to C. S. Huber in personal communications and the crystal structure was determined by Huber in 1972 (Huber, 1972), further characterized by Devlin in 1977 (Devlin et al., 1977), and synthesized from L-cocaine in the same year providing a pure and abundant source of the toxin for toxicological studies (Gampbell et al., 1977).

Anatoxin-a binds to nicotinic acetylcholine receptors with the same affinity or greater as acetylcholine. Structurally, anatoxin-a is a bicyclic amine alkaloid, similar to that of epibatidine,

but lacking the peperidine motif (Sharples et al., 2000). Upon binding irreversibly to acetylcholine receptors, anatoxin-a causes depolarization of postsynaptic neuronal cells, or efflux of Ca^+ and Na^+ ions, generating an action potential. Acetylcholinesterase does not degrade the anatoxin-a - acetylcholine receptor complex. Thus, the depolarized state becomes permanent and the nerve is desensitized. Anatoxin-a also apparently affects the presynaptic nerve reducing the frequency and quantal content of miniature end plate potentials (Biggs and Dryden, 1977). Symptoms begin with convulsions eventually leading to paralysis and death due to suffocation by respiratory arrest. The positive optical isomer (+)-anatoxin-a acts as an agonist of the acetylcholine receptor at concentrations orders of magnitude below that of (-)-anatoxin-a and is five times more lethal than a racemic mixture of (+/-)- anatoxin-a (Kofuji et al., 1990; Adeyemo and Siren, 1992).

The reported dose of anatoxin-a causing lethality via i.p. injection in mice varies widely. Fawell et al. (Fawell et al., 1999b) report that 100% of animals died receiving anatoxin-a at 100 µg/kg b.w. body weight (b.w.)(within 1 min) and at 60 µg/kg b.w. during Erwin and rotarod tests, respectively. Carmichael et al. (Carmichael et al., 1975) reported 300 µg/kg b.w. as the minimum lethal dose. Rogers et al. reported that 300 µg/kg b.w. caused all animals to die, and 250 µg/kg b.w. was the 50% lethal dose. In any case, the LD50 via oral gavage is orders of magnitude higher at > 10,000 µg/kg b.w. (Stevens and Krieger, 1991b; RB et al., 1994; Fawell et al., 1999b). Recovery from exposure to sub- lethal concentrations is reported to be quick with no lasting, long- term effects in mice given sub- lethal concentrations (Astrachan et al., 1980; Fawell et al., 1999b). A methylated variant of anatoxin-a, homoanatoxin-a was synthesized as a homolog for structure- activity studies (Wonnacott et al., 1992), then subsequently found to be produced naturally in an *Oscillitoria* species (Skulberg et al., 1992). Homoanatoxin-a has an identical mode of action and similar potency as anatoxin-a (Skulberg et al., 1992). The 4-hydroxyanatoxin-a analog is an oxygenated variant of homoanatoxin-a produced by *Raphidiopsis* (or probably *Cylindrospermopsis* (Moustaka-Gouni et al., 2009)) and is apparently non- toxic (Namikoshi et al., 2004; Araoz et al., 2010).

Carmichael et al. (Carmichael et al., 1975) observed that different strains of *Ana. Flos-aquae* produce different symptoms in the mouse. Some strains caused toxicity similar to that of anatoxin-a, but with the added symptom of salivation. Thus, Mahmood et al. (Mahmood and Carmichael, 1986, 1987) described another neurotoxin from *Ana. Flos-aquae* NRC 525-17,

previously isolated from Buffalo Pound Lake Saskatchewan, Canada in 1965. The toxin was named anatoxin-a(s) where the "s" indicates salivation. Upon injecting mice with an extract of *Ana. flos-aquae,* mice died of respiratory arrest within minutes following convulsions. However, the toxin did not mimic acetylcholine. Instead, anatoxin-a(s) was found to cause acetylcholine accumulation by inhibiting acetylcholinesterase in both muscarinic and nicotinic junctions. Thus, infusion with anatoxin-a(s) results in marked declines in heart rate and blood pressure, prior to decreases in respiratory volume (Cook et al., 1989a). The inhibition of acetylcholinesterase by anatoxin-a(s) is irreversible. Mice that do not die given 290 µg/kg b.w. anatoxin-a(s) by i.p. injection show inhibition of red- blood cell cholinesterase for at least 8 days accompanied by twitching and fasciculations (Cook et al., 1991). Thus, symptoms at sub-lethal concentrations may be prolonged.

The structure of anatoxin-a(s) is unrelated to anatoxin-a and was found to be a unique phosphate ester of a cyclic N-hydroxyguanidine (Mahmood and Carmichael, 1987). The structure was in agreement with previous studies showing that similar structures, namely esters of N-hydroxysuccinimide, are effective in inactivating acetylcholinesterase (Blumberg and Silman, 1978). The LD50 of anatoxin-a(s) via i.p. injection in mice is considerably lower than that of anatoxin-a at 20 µg/kg b.w.; while in rats, a 9 µg/kg b.w. dose has been shown to be consistently lethal within 1 hour (Mahmood and Carmichael, 1987; Cook et al., 1989b). Overt clinical signs including behavioral deficits are observed at 1.5 µg/kg b.w. (Mahmood and Carmichael, 1986, 1987; Mahmood et al., 1988; Cook et al., 1990; Cook et al., 1991). By comparison, another acetylcholinesterase inhibitor, paraoxone insecticide, caused clinical symptoms at 800 µg/kg b.w. in rats (Cook et al., 1989b). Anantoxin-a(s) is a direct agonist at muscarinic sites with indirect neuromuscular blockade (Cook et al., 1989a). Thus, the effects of anatoxin-a(s) can be blocked at least temporarily with atropine (Cook et al., 1989a).

For the most part, the anatoxins are associated with only acute illness with no connections to long- term neurotoxic illness. However, some recent studies have suggested possible developmental effects of anatoxin-a or effects of anatoxin-a at lower concentrations in repeat doses in mammalian cells, amphibians, and fish. Rogers et al. show perturbations in mouse yolk sac in an *in vitro* assay at ~165 µg/L and above and dose-dependent transient narcosis, edema, and loss of equilibrium in toad embryos. Rymuszka et al. (Rymuszka and Sieroslawska, 2011) show immune cell cytotoxicity in carp exposed to 25 µg/L by immersion.

Despite these studies, there appears to be little evidence for chronic effects of anatoxin-a at sub-lethal/low- dose concentrations or developmental effects in utero. However, there are few studies that have examined more subtle pathological changes (e.g. epigenetics). In addition, there are few or no studies that have examined the contribution of anatoxin-a(s) to chronic disease, and it should be noted that commercial or academic sources of anatoxin-a(s) disappeared after the 1990s, at least within the U.S. Thus modern toxicological studies of anatoxin-a(s) are currently non- existent.

At least three studies on the oral toxicity of anatoxin-a have been conducted. Stevens and Krieger (Stevens and Krieger, 1991a) determined that the oral LD50 of synthetic anatoxin-a in mice using a single oral dose is 16,200 µg/kg b.w. However, the method of delivery is not given and no other endpoints were measured. In a study by Astrachan and Archer (Astrachan et al., 1980; Astrachan and Archer, 1981) rats were exposed to a partially purified extract of anatoxin-a producing *A. flos-aquae* for seven weeks at dosages of 500 and 5000 µg/kg b.w. No changes in body weight, food consumption, serum liver enzymes and gross pathology were observed. Increased white blood cell counts were observed for 5 weeks. Based on this the authors indicated a NOAEL of 50 µg/kg b.w./day. However, since a partially purified extract was used it is not clear if the effects are due to anatoxin-a or other cyanobacterial metabolites.

Fawell et al. (Fawell et al., 1999b) exposed male and female mice to anatoxin-a for 28 days via oral gavage. Ten male and ten female mice in each exposure group were given doses of 0, 98, 490, and 2,460 µg/kg b.w./day anatoxin-a. Mice were examined daily for clinical signs of illness, body weights were measured weekly and in the final week of the study blood hematology and serum chemistry were characterized. In addition, tissues from the control and high dose group were examined microscopically. There were no dose- related, statistically significant changes in any of the parameters measured. Three animals died during the study, but without any symptoms and one death occurred due to fighting among mice in the same cage. A NOAEL of 98 µg/kg b.w./ day is suggested, but it is not clear what endpoint that NOAEL is based upon.

Cylindrospermopsin

Cylindrospermopsin has been implicated in several human poisoning events (Carmichael et al., 2001). In 1979, an outbreak of severe gastrointestinal illness with symptoms of acute liver failure occurred on Palm Island, Northern Queensland, Australia (Byth, 1980; Griffiths and

Saker, 2003). The outbreak was associated with the local water supply at Solomon Dam, which had been treated with copper sulfate known to lyse cyanobacterial cells releasing toxins and other cellular constituents. *Anabaena* and *Cylindrospermopsis* were the two major genera present in source waters at the time illnesses occurred (Hawkins and Griffiths, 1983). Strains of these two genera were isolated and tested for toxicity (Hawkins et al., 1985). The *Anabaena* strain proved to be non-toxic while i.p. injection of mice with lyophilized culture of the *Cylindrospermopsis* strain caused death within hours (LD50 = 64 mg/kg)(Hawkins et al., 1985). The liver was primarily affected with massive hepatocyte necrosis. In addition, lesions were produced in kidneys, adrenal glands, lungs, and intestines. Prior to this event, *Cylindrospermopsis* species were presumed to be non- toxic.

Ohtani et al. (Ohtani et al., 1992) purified a toxin from *Cylindrospermopsis* and characterized its structure in 1992. Subsequently, cylindrospermopsin was also detected in cultures of the cyanobacterium *Umezakia natans* (Nostocaceae)(Harada et al., 1994). Norris et al. (Norris et al., 2002) suggested that cylindrospermopsin alone does not account for the toxicity of *Cylindrospermopsis* extracts. In attempts to resolve this, they identified and characterized an analog of cylindrospermopsin in *Cylindrospermosis* extracts, deoxycylindrospermopsin. This compound is produced in various quantities by *Cylindrospermopsis* alongside cylindrospermopsin. It appears this analog is non- toxic in mice via i.p. up to 800 µg/kg b.w. On the other hand, recently, Neumann et al. (Neumann et al., 2007) show that deoxycylindrospermopsin inhibits protein synthesis at the same potency as cylindrospermopsin in an *in vitro* model. In addition, 7-epicylindrospermopsin is another analog of cylindrospermopsin that is toxic, but has so far only been found in *Aphanizomenon* species as a minor metabolite (Banker et al., 2000). Breakdown products and analogs of cylindrospermopsin have been found in drinking water systems including cylindrospermic acid and chlorocylindrospermopsin. Based on a study by Looper et al. (Looper and Williams, 2004) it appears that the oxygenation at the C7 position of this toxin is not required for protein synthesis inhibition. Thus, the native molecule and all analogs detected so far including breakdown products detected during drinking water purification inhibit protein synthesis.

The LD50 in mice of cylindrospermopsin over a 24-hour time period via i.p. is 2000 µg/kg b.w., but over 5 days the LD50 is 200 µg/kg b.w. (similar to anatoxin-a)(Ohtani et al., 1992). Cylindrospermosin toxicity is still under investigation, but likely occurs by multiple

mechanisms. In hepatocytes, cylindrospermopsin has been shown to cause a decrease in glutathione levels, inhibition of protein synthesis, membrane proliferation, fat droplet accumulation, and decreased levels of P450 enzyme (Runnegar et al., 1994; Terao et al., 1994). Overall, the toxicity of cylindrospermopsin follows three mechanisms, which may be interacting: 1) cytochrome P450-metabolism dependent reactive oxygen species (ROS) production, 2) inhibition of protein synthesis, and 3) genotoxicity.

In the presence of cytochrome P450 inhibitors (piperonyl butoxide), mice are completely protected from death given cylindrospermopsin up to 800 µg/kg b.w. (Norris et al., 2002). Thus, P450 plays a critical role in the toxicity of cylindrospermopsin. Cytochrome P450 enzymes are oxygenases responsible for metabolism of xenobiotic substances. The activities of these enzymes with certain substrates have been shown to contribute to the production of ROS, mutagenicity, and cellular toxicity, particularly in liver disease. Treatment of human hepatoma cells with less than 10 µg/L cylindrospermopsin induces ROS production. ROS production may also be induced by metabolites of cylindrospermopsin. In mice administered 200 µg/kg b.w. cylindrospermopsin, 23% of cylindrospermopsin is retained in the liver for up to 2 days. In addition, both methanol extractable and non- methanol extractable metabolites of cylindrospermopsin are retained in the liver. ROS production accompanied with a reduction in reduced glutathione is problematic since glutathione has a central role in protection against ROS (Lushchak, 2012). However, Humpage et al. (Humpage and Falconer, 2003) report that malondialdehyde, a marker of oxidative stress, did not increase in the presence of cylindrospermopsin, suggesting that ROS may not be the mediator of cylindrospermopsin cytotoxicity. They also show that cylindrospermopsin, rather than phase 1 products of cylindrospermopsin metabolism by P450, is the primary acutely acting cytotoxin and not cylindrospermopsin breakdown products.

Cylindrospermopsin has been shown to inhibit protein synthesis at the elongation step in both plant and mammalian cell extracts (Froscio et al., 2008). Furthermore, the toxin has been shown to bind non- covalently to components of the translational machinery that does not include the ribosomes (Harada et al., 1994; Froscio et al., 2008). This suggests cylindrospermopsin binds to Eukaryotic initiation factors. While the exact mechanism of protein synthesis inhibition is not completely understood it appears to be the primary mode of toxicity at lower doses of cylindrospermopsin, while at high doses cytochrome P450 metabolism of

cylindrospermopsin producing ROS induces cell death (Runnegar et al., 1994; Runnegar et al., 2002).

Cylindrospermopsin is associated with genotoxicity. Falconer and Humpage (Falconer and Humpage, 2001) show that mice treated with cylindrospermopsin by oral gavage showed 5 tumors out of 53 treated animals compared to none in the control group. Treatment of immortalized human cell lines with 1 – 10 µg/L cylindrospermopsin has been shown to cause the production of micronuclei and DNA damage (Humpage et al., 2000). Furthermore, comet assays to detect DNA breaks were positive for cylindrospermopsin treatment in a lymphoblastoid cell line, where inhibitors of P450 (omeprazole and SKF525) were protective against genotoxicity. Straser et al. (Straser et al., 2011) show that cylindrospermopsin caused the up- regulation of genes affecting the P53 tumor suppressor protein. P53 is involved in cell cycle arrest particularly during the course of DNA damage. MDM2 and CDKN1A have been shown to negatively regulate P53 (Colman et al., 2000; Broude et al., 2007). In cylindrospermopsin treated cells, expression of both of these proteins is increased (Straser et al., 2011) suggesting lack of cell cycle arrest during DNA damage by cylindrospermopsin.

Based on 10 and 11-week oral exposure studies in mice, a NOAEL of 30 µg/kg b.w. per day was indicated by Humpage and Falconer (Humpage and Falconer, 2003) (Humpage and Falconer, 2002) suggesting a maximum acceptable concentration for drinking water of 1 µg/L. In this study, two experiments were carried out, but only the second experiment used purified toxin. Male Swiss albino mice were given purified cylindrospermopsin by oral gavage daily at 0 – 240 µg/kg b.w. The cylindrospermopsin was purified from cell lysate by solid phase extraction on a C18 cartridge followed by size exclusion using Sephadex- G10, and preparative HPLC purification on a C18 column. The purified product was characterized by time-of-flight mass spectrometry and nuclear magnetic resonance spectroscopy. These analyses showed that the final yield of cylindrospermopsin was 10.4 mg in a total of 22 mg, and that at least one of the major contaminants was phenylalanine. Thus, the purity of cylindrospermopsin used was 47%. An elemental analysis could have shown that these were the only two compounds in the preparation. The solvent used to dissolve the purified cylindrospermopsin before dosing (e.g. methanol or water) was not reported.

Clinical examinations of the mice were performed throughout the exposure period. This included examination of skin, fur, eyes, mucous membranes, respiration, pupil size, gait,

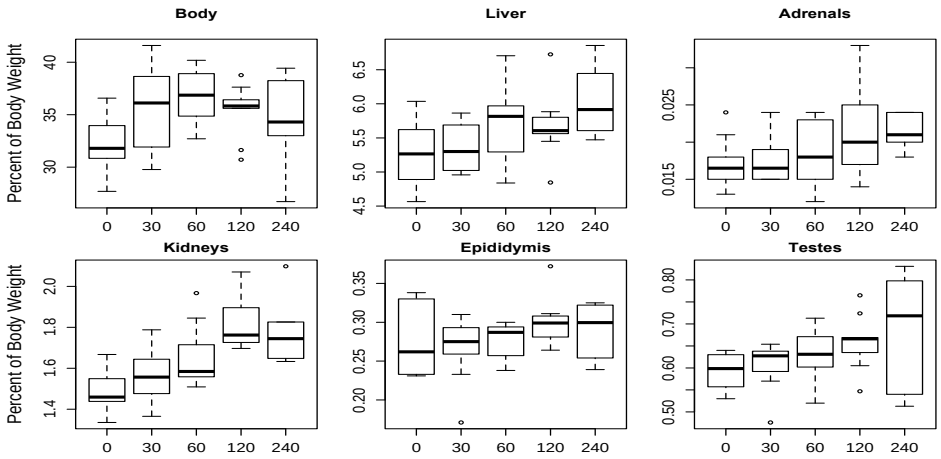

Figure 8. Increase in organ and body weights due to oral exposure to 0 – 240 µg/kg b.w. cylindrospermopsin. Data from Humpage and Falconer 2002.

posture, grooming behavior pattern, noise response, visual response, touch response, grip strength, motor activity, and evidence of lachrymation or abnormal excretions. Following the trial, serum and urine chemistry were characterized, hematology was performed, body and organ weights were determined, and a complete histological examination of all organs was performed.

No clinical changes in appearance or behavior were observed at any exposure level, except for a reduction in water consumption at all levels at 53 – 72% of the control in the last four weeks of the experiment. There was also no visible organ or tissue damage upon post-mortem examination and no significant changes in serum liver enzymes. The most significant changes were seen in body and organ weights expressed as a percentage of body weight. There was a significant increase in body weights in the 30 and 60 µg/kg b.w. doses (Figure 9), but this trend did not continue in the higher doses. Organs that followed a positive increasing trend with dose were liver, adrenal glands, kidneys, epididymis, and testes. Kidney weight was significantly increased at all doses at and above 60 µg/kg b.w. Liver weight was significantly increased in the 240 µg/kg b.w. dose and if not for two outliers was also significantly increased at the 120 µg/kg b.w. dose. A dose- dependent increase in mitotic figures, inflammatory foci and necrotic cells was observed in liver sections, but not in other organs.

Since kidney weight was increased in a dose- dependent manner at 60 µg/kg b.w. and higher the NOAEL for cylindrospermopsin kidney toxicity was determined to be the next lowest dose at 30 µg/kg b.w. where no adverse dose dependent effects were observed given the

endpoints measured. Humpage and Falconer calculated a drinking water guideline of 1 µg/L. To do this a tolerable daily intake level of 0.03 µg/kg b.w./ day was estimated by dividing the NOAEL by an uncertainty factor of 1000. The uncertainty factor included a multiplier of 10 for lack of sufficient data on cylindrospermopsin toxicity, and 100 for inter- and intraspecies variability for a product of 1000. A drinking water guideline value was calculated by multiplying the tolerable daily intake level of 0.03 µg/kg/day by the weight of an adult (60 kg) and the proportion of drinking water from tap water (0.9) divided by tap water intake (2 Liters/day).

Saxitoxin

Voltage- gated ion channels are found in neuronal and muscle tissue and are responsible for the rising phase and propagation of action potentials in excitatory cells (Catterall, 2000). A variety of organisms in nature produce molecules that block, activate, or modulate these ion channels (Stevens et al., Frontiers in Pharmacology). Among these, saxitoxins and structurally similar compounds (gonyautoxins) bind to voltage gated sodium channels preventing the flow of sodium ions and action potentials (Fingerman et al., 1953; Evans, 1964; Kao and Nishiyama, 1965). Saxitoxin also modifies the voltage gating processes in potassium channels and blocks L-type calcium channels (J et al., 2003; Su et al., 2004). Thus early symptoms of saxitoxin poisoning are tingling or burning of the lips, tongue and throat. Later symptoms include complete numbness of the face followed by paralysis. Death usually occurs due to suffocation or cardiac arrest.

Saxitoxins and its analogs are collectively known as paralytic shellfish poisons (PSPs) and are widely distributed in nature occurring in bacteria, cyanobacteria, marine algae (dinoflagellates), shellfish, fish, worms, Echinoderms, and crustaceans among others (reviewed in (Llewellyn, 2006)). Some of the earliest investigations of PSPs were focused on identifying the source of toxicity in shellfish, primarily in mussels due to outbreaks of PSP poisoning in California affecting hundreds of people (Sommer and Meyer, 1935). In 1937 Sommer et al. (Sommer et al., 1937) presented evidence that the source of toxicity in mussels was associated with algal cells and that toxicity was highest when large numbers of certain algae, dinoflagellates, were present in the water column. Furthermore they purified paralytic toxins from field collections of *Gonyaulax catenella*. Genes for saxitoxin synthesis have now been discovered in the dinoflagellate *Alexandrium* and several genera of cyanobacteria. This

definitively proves that these organisms synthesize saxitoxin *de novo*. In other organisms it is thought that saxitoxin and other PSPs accumulate within animal tissues, either due to the presence of dinoflagellate/cyanobacterial symbionts, or due to the consumption of these PSP producers, rather than synthesizing the toxin themselves. In fact, some PSP containing animals contain saxitoxin binding proteins or transferrins known as saxiphillin (Llewellyn, 2006). The saxiphillin allows saxitoxin to accumulate to high levels within animal tissues without causing toxicity since the saxiphillin competes with saxitoxin binding to sodium channels (Llewellyn and Moczydlowski, 1994).

This is a potential human health hazard. For example, puffer fish contain saxiphillins allowing them to accumulate high levels of saxitoxin in certain organs, primarily the liver (Yotsu-Yamashita et al., 2013). Consumption of puffer fish has led to human illness and fatalities (Noguchia and Ebesub, 2001). Saxiphillins are widely distributed in nature occurring in ecologically diverse species including fish, reptiles, and amphibians including the American bullfrog (Llewellyn and Moczydlowski, 1994). Logically, given that saxitoxins have been found in the Great Lakes region it is possible that bullfrogs or other animals possessing saxiphillins from this area may accumulate saxitoxins produced cyanobacteria. Bullfrog legs are a major commodity exported from the US.

Saxitoxin potency in mice is higher than that of most other cyanotoxins discussed above. Using a purified preparation from shellfish, Wiberg and Stevenson (Wiberg and Stephenson, 1960) show that the oral, intraperitoneal, and intravenous LD50 for saxitoxin in mice is 236 µg/kg, 10 µg/kg, and 3.4 µg/kg, respectively. Furthermore, the dose response curve was steep, as the difference between the dose killing 50% vs 99% of animals was only 6 µg/kg. Using purified toxin, Kao and Nishiyama (Kao and Nishiyama, 1965) found that i.p. injection of 2 µg/kg resulted in severe neuromuscular paralysis and hypotension while only 0.75 µg/kg produced paralysis without hypotension. In addition, the authors noted that placing droplets of toxin at 0.01 µg/ml (10 µg/L) on their lips and hands resulted in paraesthesia and numbness. Based on reports of known human illness due to saxitoxin or PSP exposure in ~500 individuals the European Food Safety Authority, Panel on Contaminants in the Food Chain (CONTAM) estimated a NOAEL for saxitoxin of 0.5 µg saxitoxin equivalents per kg b.w., translating to a 30 µg dose for a 60 kg individual (Alexander et al., 2009).

Synthesis and transformation of saxitoxins and PSPs leads to structural diversity and therefore a range of toxicities. PSPs are broadly defined as hydrophobic or hydrophilic, and may contain side chains including sulfate, acetate, carbamoyl, hydroxyl, or hydroxybenzoate groups. Those PSPs with carbamoyl side chains appear to have the greatest affinity for their ligands whether ion channels or saxiphillins (Llewellyn and Moczydlowski, 1994). Once released from dinoflagellates or cyanobacteria the PSPs may be transformed by associated heterotrophic bacteria. For example, bacteria isolated from mollusk tissue have been shown to transform gonyautoxins types 1 and 4 to types 2 and 3. Furthermore, other isolates were able to decarbamoylate or sulfate gonyautoxins (Smith et al., 2001). In addition, Kotaki et al. (Kotaki et al., 1985) show that *Vibrio* and *Pseudoalteromonas* species isolated from crabs and a grastropod converted gonyautoxins to saxitoxin and neosaxitoxin.

In the Great Lakes region *L. wollei* has been shown to produce decarbamoylsaxitoxin and decarbamoylgonyautoxin-2 and -3 as well as six novel saxitoxins. According to mouse bioassays these appear to be at least 10 fold less toxic than saxitoxin (Llewellyn and Moczydlowski, 1994). However, as mentioned previously there is the possibility that *L. wollei* saxitoxins could be converted to other more toxic PSPs by bacteria. In addition, the potential saxitoxin producer *Cylindrospermopsis raciborskii* has been detected in the Great Lakes region (Conroy et al., 2007). While no *Aphanizomenon* species from the Great Lakes region have been shown to produce saxitoxins (or aphantoxins), blooms of *Aphanizomenon* species in other north temperate regions of the world have been shown to produce saxitoxins (Liu et al., 2006) and *Aphanizomenon* is a common bloom forming species in the Great Lakes region (Boyer, 2008; Davis et al., 2015). Thus there is considerable concern that with increased eutrophication saxitoxins could become a greater human health concern in the Great Lakes region.

I. Numerical Limits

In order to protect public health it is necessary to establish the maximum cyanotoxin concentration and duration of exposure, below which no adverse health outcome is expected. In 2007 and again in 2012 Chorus (Chorus, 2012) reviewed cyanotoxin risk management plans and regulations developed by various countries. At least six countries including Canada have developed regulations establishing maximum acceptable concentrations, or provisional guideline values for select cyanotoxins and/or cyanobacterial biomass in either recreational and/or finished

drinking water systems. In the United States there are no federal regulations limiting cyanotoxin concentrations in finished drinking water, however, some individual states, including Oklahoma have developed their own regulations for recreational waters. The United States only recently released its draft *Health Recreational Ambient Water Quality Criteria and/or Swimming Advisories for Microcystins and Cylindrospermopsin* (EPA, 2016b) (https://www.epa.gov/wqc/microbial-pathogenrecreational-water-quality-criteria#swimming). The public comment period for these criteria will move forward in 2017, and states will be able to reconsider their swimming advisory posting and water quality standards upon release of the final criteria.

A lack of regulation is due in part to uncertainty over what cyanotoxin concentration and duration of exposure is considered safe. As a first step, the U.S. EPA placed cyanobacteria biomass (draft 1) or cyanotoxins (anatoxin-a, MCLR, and cylindrospermopsin) on their Contaminant Candidate Lists since 1998 (draft 1 and 2) and 2009 (draft 3), respectively. Draft 4 to be released soon is limited to these three cyanotoxins (e.g. saxitoxin is not listed). In some cases, it may be possible to derive numerical limits based on the most current literature.

Microcystin

The majority of countries with some form of regulation or established health advisories follow closely to the WHO provisional guideline value of 1 µg/L/day MC in drinking water (WHO, 1999; WHO, 2003).This limit is based on the tolerable daily intake level of 40 µg/kg b.w. per day determined by Fawell et al. (Fawell et al., 1999a) and Falconer et al. (Falconer et al., 1994), a mean body weight of 60 kg, and a 1000 fold uncertainty factor. The uncertainty factor includes a factor of 100 for intra- and interspecies variation in toxicity as well as a factor of 10 for lack of information on MC toxicity, particularly chronic exposures. It was also assumed adults drink 2 L of water per day and that 80% of this is from tap water. Thus, this guideline is primarily directed at adults.

Children as opposed to adults have most often been the victims in human poisoning events (Stewart et al., 2006c; Hilborn et al., 2014) and are more at risk for illness as a result of cyanotoxin exposure given a relatively smaller body size proportional to the volume of water they consume and the potential for developmental toxicity (Weirich and Miller, 2014; D'Anglada et al., 2015b, a, c). Weirich and Miller (Weirich and Miller, 2014) recalculated the

WHO guideline value adjusting the mean body weight to that of children aged 9 months, 5.5, or 9.5 years. In addition, daily drinking water intake was adjusted to 1 L for all ages. Based on this calculation for children aged 5.5 years and 9 months the guideline value was reduced to 0.6 and 0.3 µg/L, respectively.

The USEPA recently published a health advisory for MCs in drinking water (D'Anglada and Strong, 2015a). They established provisional guideline values for two different age groups, 1) 0.3 µg/L for bottle- fed infants and pre-school aged children under 6 years of age, and 2) 1.6 µg/L for children older than 6 years of age and adults. Both are ten- day guideline values, meaning that exposure for ten days below these levels is considered protective of public health, but effects of exposure at any level for longer than 10 days is unknown. The guideline values were calculated by dividing a lowest-observed-adverse effect level (LOAEL) of 50 µg/kg b.w./day by the product of an uncertainty factor (1,000) multiplied by the drinking water intake rate (L/day) normalized to mean body weight of each age group.

Reference dosages such as the LOAEL/NOAEL are typically taken from animal exposure studies. While a number of animal studies have described the toxicity of MCs, relatively few studies have quantified toxicity of MC in a rodent model using pure compound and in well-described, controlled repeat oral dose trials at multiple exposure levels. Oral exposure compared to other exposure routes, such i.p. injection in animal models, mimics human exposure to cyanotoxins in drinking water, as well as ingestion recreationally. In addition, use of pure compound as opposed to mixtures or crude cell extracts is necessary in order to establish health effects of each compound independently, though more research is needed on the effects of defined cyanotoxin mixtures.

A NOAEL of 40 µg/kg b.w./day derived from Fawell et al. (Fawell et al., 1999a) and supported by a separate study by Falconer et al. (Falconer et al., 1994) was used by the WHO in calculating their provisional guideline level. The LOAEL for calculating the EPA guideline value for MCs was based on the study by R. Heinze in 1999 (Heinze, 1999). The USEPA advisory for MCs used this study to determine that the LOAEL for MCLR is 50 µg/kg b.w./day via the oral route with the major end point being liver lesions. The study by Heinze did not test lower dosages to determine if they would also cause liver damage, so based on that study alone it is not known if 50 µg/kg b.w./day is the lowest dose causing liver lesions. Modeling the dose-response relationship might provide evidence that lower doses would produce no or minimal

adverse effects, but with only two exposure groups it would be difficult to establish a statistically significant dose- response curve. As such, the USEPA applied an uncertainty factor of 3 to account for extrapolating from a LOAEL to a NOAEL, meaning that the NOAEL could be as low as 16.7 µg/kg b.w. / day. This may not be sufficient for other adverse effects reported for MCs including reproductive and neurotoxic effects (see below).

A LOAEL of 50 µg/kg b.w./day is at least partially corroborated by the Fawell et al. study, which found no significant pathological changes in the liver at 40 µg/kg b.w./day MCLR. Yet, the Fawell and Heinze studies are not directly comparable because the Fawell et al. study used mice, not rats, in their 13- week repeat oral dose study. In addition, mice were given MCLR by oral gavage whereas the Heinze study provided five rats per cage with 150 ml/day of drinking water containing MCLR, 93 - 97% of which was consumed by the animals. The Heinze study was also for a shorter duration (28 days), which might be more realistic for calculating a 10- day health advisory.

The EPA guideline value takes into account the drinking water intake rate by mean body weight ratio (DWI/BW) of each age group. These values were derived from existing data. For children < 3 months old the data used by EPA were from the USDA Continuing Survey of Food Intakes by Individuals program 1994-1996, and 1998 (Kahn and Stralka, 2008), whereas data for all other age groups were from the National Health and Nutrition Examination Survey 2003-2006 (Centers for Disease Control and Prevention, 2003-2006). Young children as opposed to teens and adults have a higher daily water intake rate per kg of body weight. For example, infants < 3 months old have a DWI/BW over ten times that of 16 to 18 year olds (Figure 9). Unless there is some reason to expect that infants and children will not be consuming municipal drinking water, the guideline values for cyanotoxin exposure need to be directed at the youngest age groups. The USEPA advisory uses DWI/BW data at the 90^{th} percentile of usage to calculate the guideline values. The resulting values for various age groups then ranges from 0.2 µg/L for infants to 2.2 µg/L for 3 – 6 year olds at this 90^{th} percentile level. A guideline value of 0.3 µg/L was chosen instead of 0.2 µg/L for children < 6 years of age.

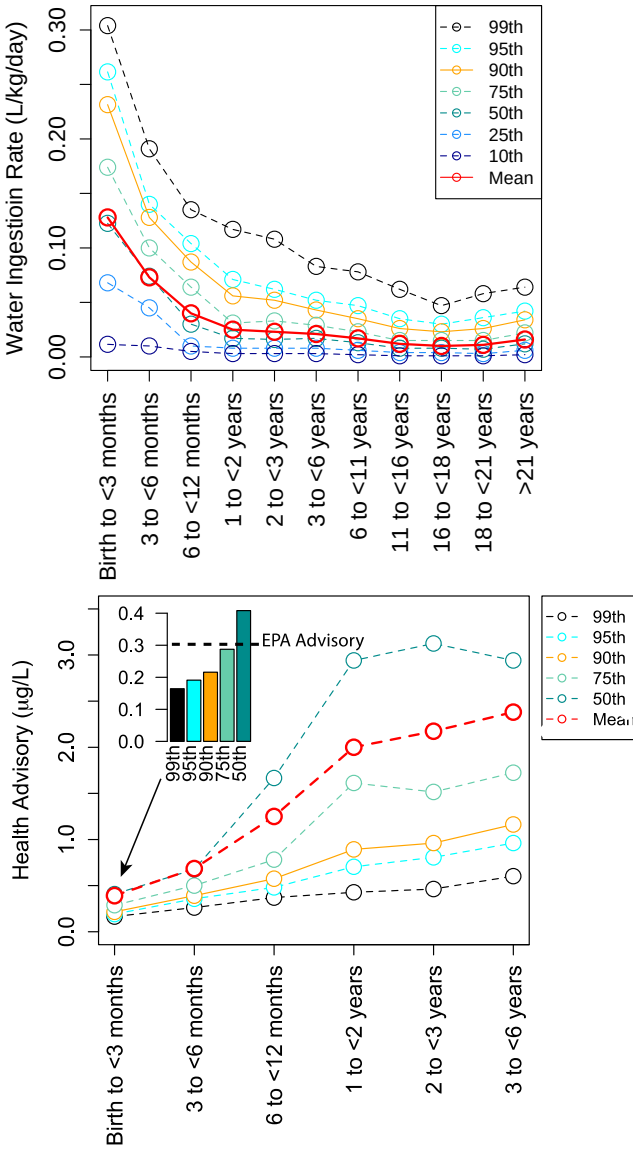

Figure 9. Drinking water ingestion rate across age groups and percentiles (top) and health advisories for children under six calculated using 50th to 99th percentile drinking water ingestion rates (bottom). Resulting health advisories for infants < 3 months calculated with 50th to 99th percentiles (inset).

The EPA advisory states that the uncertainty factor of 10 for intraspecies variation adequately accounted for this difference and reflects the average DWI/BW ratio of children under 6. That uncertainty factor included 10 for inter- and 10 for intraspecies variability, 3 for lack of data, and 3 for extrapolating from a LOAEL to NOAEL.

The percentile category of DWI/BW data used affects the calculated guideline value considerably. For example, for children under 3 months of age, arguably the most sensitive population, the calculated guideline value ranges from 0.16 to 4.34 µg/L depending on if DWI/BW data are taken from the 99th or 10th percentile respectively (Figure 8). Thus, using the EPA formula, the most conservative guideline value would be 0.16 µg/L for bottle- fed infants.

There is some question as to whether a provisional guideline value can be applied to children and infants when the LOAEL used to calculate that guideline value was derived from a study of adult male rats. There is some evidence in the literature for potential developmental effects of MCs (Zhang et al., 2008; Wang et al., 2012). As such it is possible that individuals of a younger age could develop adverse effects later in life due to an exposure at levels lower than 50 µg/kg b.w./day, during critical periods of development.

Liver damage may not be the only adverse health effect end point to consider when developing numerical limits for MCs. While the liver is clearly one of the most affected organs, MCs also affect brain and reproductive tissues (Maidana et al., 2006; Zhang et al., 2011; Kist et al., 2012; Zhao et al., 2012; Zhou et al., 2012; Wang et al., 2013; Zhang et al., 2013; Li et al., 2014a; Zhou et al., 2014; Zhao et al., 2015).

The study by Li et al. (Li et al., 2014a;) reported central nervous system toxicity after repeat oral dose exposures of MCLR in rats. The EPA health advisory does not consider this study in its formulation of the drinking water guideline value for MCs or neurotoxicity of MCLR as an end point. One problem with the Li et al. study (Li et al., 2014a) is that the MCLR stock (1 mg) was dissolved in 1 ml methanol to a concentration of 1,000 µg/ml, then diluted ten- fold with pure water to a working stock concentration of 100 µg/ml. At that point the working stock was 10% methanol. This working stock was then diluted with water to produce drinking water at 0.2, 1.0, and 5.0 µg/ml or 0.02, 0.1, and 0.5% methanol. Thus in addition to varying MCLR dosage, rats received varying levels of methanol, a known neurotoxin (Murray et al., 1991; Weiss et al., 1996). By comparison, the Heinze study dissolved 20 mg MCLR in absolute ethanol (10,000 µg/ml) to make a stock solution, this was diluted in pure water to 1,000 µg/ml working stock (10% ethanol) and diluted further by an unknown amount to produce 150 ml of MCLR laden drinking water for 5 rats. The use of ethanol over methanol as a solvent is beneficial as ethanol is much less toxic than methanol (Kraut and Kurtz, 2008).

Li et al. (Li et al., 2015b) also examined the developmental effects of MCLR in a repeat maternal oral dose study in rats showing deficiencies in the Morris water maze test of offspring due to MCLR exposure in utero. As with Li et al (2014a), the study by Li et al. (2015b) used methanol to make the stock solution of MCLR resulting in trace levels of methanol in all exposures. However, in the Li et al. (2015b) study methanol was included in the negative control and was normalized to 0.002% (i.e. 1.6 mg/kg b.w.) in all treatments including the control. The EPA health advisory for MCs does not consider this study in calculating the guideline value for MCs in drinking water. The EPA health effects support document (D'Anglada et al., 2015b) indicates that the study is confounded because there may be synergy between methanol exposure and MCLR.

Reproductive toxicity is another endpoint to be considered in formulating guideline values. Chen et al. (Chen et al., 2011) show effects of MCLR on male reproductive tissues (e.g.

sperm motility/counts, testis weight) in repeat dose oral exposure studies in mice. The EPA health advisory considered this study in deciding whether to include reproductive effects as an endpoint, but determined that the study design was lacking in several ways. The EPA indicates that no testis weights were given, no methods were given for how sperm was handled, or how percent sperm motility was determined, the purity of MCLR, species and ages of mice, body weight, amount of water consumed, and dosage levels.

Chen et al. indicate that sperm counts and motility were determined using the HTM-TOX IVOS semen analyzer and that MCLR was purchased from Alexis Biochemicals (Enzo Life Sciences), which sells MCLR at >95% purity. The species of mouse is not given, but their body weights are given (15 – 25 g) which would allow one to estimate the exposure level. Water intake across 28 mouse strains ranges from 4 – 8 ml per day (Bachmanov et al., 2002). The lowest level at which effects were observed was at the 3.2 µg/L level. Therefore, at this exposure level the dosages were likely between 0.9 and 1.7 µg/kg b.w./day for a 15 g mouse consuming between 4 and 8 ml per day, or between 0.5 and 1.0 µg/kg b.w./day for a 25 g mouse consuming between 4 and 8 ml per day, an overall mean estimated dosage of 1.0 µg/kg b.w./day at the 3.2 µg/L level. The EPA Health Support Document for MCs calculates a LOAEL and NOAEL of 0.79 and 0.25 µg/kg b.w./day for reproductive toxicity based on this study. This suggests that adverse effects caused by MCLR on reproductive tissues occurs at doses below the LOAEL for liver toxicity of 50 µg/kg b.w./day.

If the guideline values were calculated for endpoints of reproductive and neurotoxicity using the aforementioned studies then they would be lower than guideline values based on liver toxicity. Using the Chen et al. (Chen et al., 2011) study for a reproductive toxicity LOAEL (1.0 µg/kg b.w./day), and the Li et al. study for a neurotoxicity LOAEL (5 µg/kg b.w./day) the guideline value at the 90^{th} percentile DWI/BW ratio ranges from 0.004 – 0.029 µg/L/day for reproductive toxicity and 0.02 – 0.15 µg/L/day for neurotoxicity across age groups from < 3 months to adults. The mean guideline values for children <6 years old would be 0.01 µg/L and 0.07 µg/L for reproductive and neurotoxicity, respectively (Table 4). These are 4- and 30- fold lower than the EPA guideline value for liver toxicity in children < 6 years old. Given these large differences it is critical that future studies confirm the level of MC that displays reproductive and neurotoxicity in a repeat oral dose study design.

Monitoring and analytical methods for MC detection will necessarily be influenced by the drinking water guideline value. The EPA advisory suggests MCLR is a suitable surrogate for the toxicity of all MCs because it is one of the most commonly occurring or monitored in the environment and is one of the most toxic congeners. Furthermore, the EPA advisory indicates that the guideline values apply to total MCs in a given sample. As mentioned previously MCs can exist bound to proteins or other molecules with thiol groups (e.g. free cysteine), transformed, or otherwise in a non- toxic state. The guideline value should be applied to the total toxic MCs (TTMCs), which would include non- transformed, non- protein bound total MCs capable of covalently binding to and inhibiting protein phosphatases. Methods for specifically quantitating the TTMC pool in drinking water have not been demonstrated. The ELISA measures non- toxic protein bound as well as toxic, non-protein bound MCs. The EPA advisory suggests using EPA Method 544 for quantification of intracellular and extracellular MCs using LC-MS/MS in drinking water. However, this method doesn't target total MCs and the surrogate standard indicated for use in that method is no longer available. The PP1/2A inhibition assay might be useful as an activity assay in quantifying TTMCs, but it would also detect other PP1/2A inhibitors mentioned above). If the guideline value is for TTMCs then method development for detecting these compounds is critical.

Table 4. Numerical limits for cyanotoxins in drinking water based on varying critical studies and toxic endpoints discussed in this report

Toxin	**Microcystins**					**Cylindrospermopsins**			**Saxitoxins**
Source	WHO	EPA (U.S. and Canada)		This Report	This Report	EPA (U.S. and Canada)		This Report	This Report
Critical study	Fawell et al. 1999a	Heinze 1999		Li et al. 2015	Chen et al. 2011	Humpage and Falconer 2002,2003		Humpage and Falconer 2002,2003	CONTAM
LOAEL/NOAEL (µg/kg/day)	40	50		5	1	30		30	0.5
End point	Liver Toxicity	Liver Toxicity		Central Nervous System Toxicity	Male Reproductive Toxicity	Kidney Toxicity		Kidney Toxicity	Peripheral Nervous System Toxicity
Age of Exposed	Adult	<6 yrs	>6 yrs	<6 yrs	<6 yrs	<6 yrs	>6 yrs	<6 yrs	<6 yrs
DWI/BW/day (L/kg/d)	0.03	0.15	0.03	0.15	0.15	0.15	0.15	0.15	0.15
Uncertainty Factor	1000	1000	1000	1000	1000	300	300	1000	3
Guideline Value (µg/L)	1	0.3	1.6	0.03	0.01	0.7	3	0.2	0.3

Cylindrospermopsin

Some countries have developed regulations and/or advisories for cylindrospermopsin in drinking water including Brazil (Burch, 2008). The USEPA recently developed cylindrospermopsin guideline values for drinking water (D'Anglada and Strong, 2015b). The 10-day guideline values are directed at two age groups, 1) 0.7 µg/L for pre- schooled aged children < 6 years old and 2) 3 µg/L for children over 6 years of age and adults. As with MCs, unless it can be guaranteed that children under six years old will not be consuming the water then drinking water plant operators would need to follow the lower value.

The EPA guideline value for cylindrospermopsin was calculated using the same formula as for MCs and a NOAEL of 30 µg/L/day from Humpage and Falconer (2003) .The calculation of the EPA cylindrospermopsin guideline value differs from the Humpage and Falconer calculation in the use of a lower uncertainty factor and use of drinking water intake rate normalized to body weight of a specific age group (over the first year of life). The uncertainty multiplier used for inter- and intraspecies variation was the same as Humpage and Falconer at 100, but the uncertainty multiplier for lack of data on cylindrospermopsin toxicity was lowered from 10 to 3. This resulted in an overall uncertainty factor for the cylindrospermopsin guideline value of 300. It could be argued that the more conventional multiplier of 10 for lack of data should be applied. There have been no other oral exposure studies of cylindrospermopsin toxicity similar to the Humpage and Falconer study since 2003 and as stated in the EPA support document for cylindrospermopsin (D'Anglada et al., 2015a) "no oral reproductive or developmental and chronic toxicity studies are available for cylindrospermopsin." In addition, the advisory states that there is a lack of data on any potential neurological effects. Finally, an uncertainty factor of 1000 is warranted for cylindrospermopsin because the guideline value is essentially based on one experiment and no replicate experiments. With an uncertainty factor of 1000 the guideline value changes to 0.1 – 0.9 µg/L for age groups from < 3 months to adults over 21 years old, respectively at the 90^{th} percentile of DWI/BW ratio (Figure 10), or 0.2 µg/L using the ingestion rate applied by EPA (Table 4). At the 99^{th} percentile the calculated guideline value is 0.1 – 0.5 µg/L across all age groups. Thus, arguably, a guideline based on more commonly accepted uncertainty values would be 0.1 µg/L for the most sensitive groups.

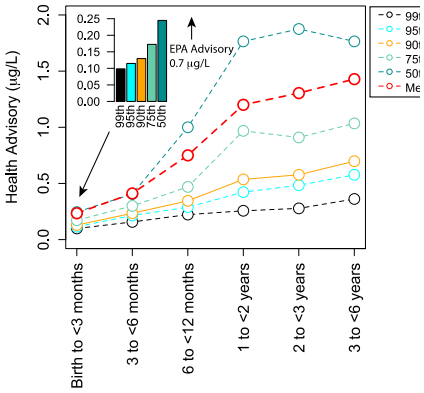

Figure10. Cylindrospermopsin drinking water guideline values for children under six calculated using mean, or 50th to 99th percentile drinking water ingestion rates. Values for infants < 3 months calculated with 50th to 99th percentiles (inset).

Anatoxin-a and anatoxin-a(s)

While anatoxin-a and anatoxin-a(s) act at the same neuronal synapse, their molecular targets and toxicity profiles are different. The LD50 for anatoxin-a via the oral route is 100 and 10- fold greater than that of anatoxin-a(s) and MCLR. Much uncertainty exists on the oral toxicity of anatoxin-a(s) given that few or no studies have been conducted on its toxicity via the oral route and in general toxicology studies of anatoxin-a(s) essentially ceased in the late 90's due to a lack of material or available toxic strains. There is also none or limited data on its occurrence in the Great Lakes region.

Given the lack of repeat dose oral exposure studies it is impossible to establish a reference dose, NOAEL, or LOAEL at this time for anatoxin-a preventing the establishment of any kind of numerical limits for this toxin. A similar conclusion was recently reached by the USEPA (D'Anglada et al., 2015c). The LD50 via oral gavage in mice is >10,000 µg/kg b.w. and mice exposed to sub-lethal doses apparently recover without any lingering symptoms. Effects on reproductive tissue have been reported with repeat doses using i.p. injection, but not by the oral route making it difficult to establish a drinking water guideline for anatoxin-a using reproductive toxicity as an endpoint.

Saxitoxin

According to Munday and Reeve (Munday and Reeve, 2013) currently no sub-acute repeat oral dosing studies of saxitoxin in animals have been reported using approved protocols. In contrast, the LD50 for saxitoxin via the oral route is well known at approximately 200 µg/kg b.w. in the mouse (Wiberg and Stevenson, 1960) and ranges from 91 µg/kg b.w. in pigeons to 800 µg/kg b.w. in monkeys (Table 2 in (Mons et al., 1998)).

A LOAEL for saxitoxins of 1.5 µg/kg b.w. has been reported by the European CONTAM on marine biotoxins based on epidemiological data (Alexander et al., 2009). Unlike other cyanotoxins, human poisonings with saxitoxins or PSPs have been well documented due to frequent human consumption of shellfish containing hazardous levels of saxitoxins (Gessner et al., 1997; Egmond et al., 2004; García et al., 2004). CONTAM reviewed approximately 500 reports of human illness and death from eating shellfish contaminated with saxitoxins. In all cases concentrations of saxitoxins in the shellfish consumed were determined by the mouse bioassay. As such, the congeners of saxitoxins consumed are unknown and data are provided in mouse units. Mouse units were converted to mass per volume of saxitoxin equivalents by multiplying the mouse units by 0.18 µg saxitoxin equivalents per kg, which is widely used as conversion factor (Schantz, 1986).

The use of case reports of human illness often requires a number of assumptions in order to relate dosage to the human health outcome. The reports of human intoxication events used by CONTAM required several such assumptions. For example, in many cases the weight of victims was unknown and assumed to be 60 kg if an adult or average age adjusted weight (Meyer, 1953; Tennant et al., 1955). In one case, the amount of shellfish consumed was estimated based on the number of empty shells found after the meal (Meyer, 1953). In another case, the amount of toxin present in the shellfish consumed was estimated by interpolating concentration in shellfish collected the day before and after the poisoning event (Tennant et al., 1955). In addition, details of how the mouse bioassay was performed were often not available making it unclear if the assay was performed in agreement with the FDA approved method, 959.08 from the Association of Official Analytical Chemists.

These assumptions contribute uncertainty in the calculation of the LOAEL of 1.5 µg/kg b.w. To account for this, an uncertainty factor of 10 could be used in calculating a guideline value for saxitoxins. On the other hand, since the LOAEL was determined based on human cases of illness an uncertainty multiplier for interspecies differences is not required. In addition, considering a population size of 500 human cases of saxitoxin related illness there is less uncertainty variation in toxicity based on intraspecies differences.

The CONTAM panel on saxitoxins divided the LOAEL by a factor of 3 to derive a NOAEL of 0.5 µg/kg b.w. A guideline value can be calculated for saxitoxins using the same formula as for MCs, and this LOAEL of 0.5 µg/kg b.w., as well as the DWI/BW ratio at the 90th percentile, and an uncertainty factor of 10. This produces a calculated guideline value for saxitoxins ranging from 0.2 to 1.5 µg/L across all age group categories (Figure 11) and 0.3 µg/L using the same ingestion rate applied by the EPA of 0.15 L/day for the first year of life (Table 4). On the other hand, for infants < 3 months old at the 99th percentile of the DWI/BW ratio, the most sensitive group, the guideline value is 0.16 µg/L.

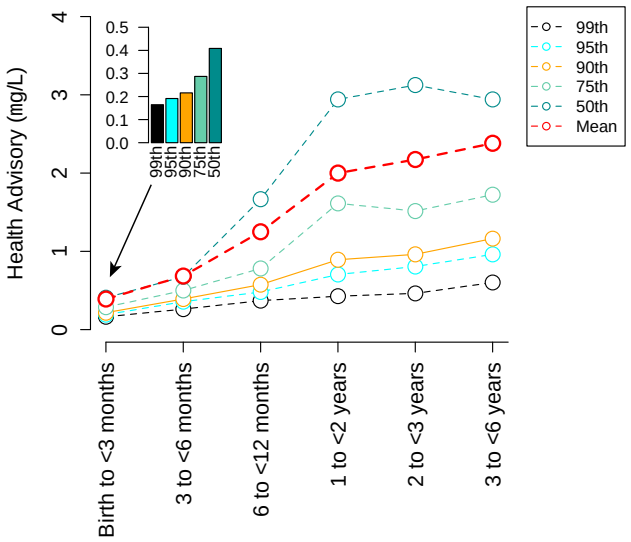

Figure 1. Saxitoxin drinking water guideline values for children under six calculated using mean, or 50th to 99th percentile drinking water ingestion rates. Values for infants < 3 months calculated with 50th to 99th percentiles (inset).

Issues and Considerations in Developing Numerical Limits for Cyanotoxins

The numerical limits discussed above are based on our current understanding of cyanotoxin toxicology. There are various aspects of cyanotoxins that are still relatively under studied, and thus not incorporated into the calculation of numerical limits. For example, for many cyanotoxins other endpoints have not been explored including epigenetic changes, developmental toxicity, behavioral, and cognitive deficits as well as reproductive effects in repeat oral dose studies. In addition, the contribution of cyanotoxins to the burden of chronic diseases in humans is difficult to assess, but has been explored in some epidemiological studies, particularly for MCs. These studies have not been considered in establishing numerical limits due to a lack of sufficient data. In particular, there are no diagnostic tests to assess cyanotoxin exposure in humans and as a result the exposure rate for most cyanotoxins in human populations is unknown. Finally, cyanotoxins rarely occur in isolation, but rather as a complex mixture. For

example, many cyanobacteria genera are capable of producing multiple classes of toxins. Few or no studies have examined antagonistic or inhibitory effects of known cyanotoxin mixtures, although crude extracts containing unknown cyanotoxin mixtures have been used in animal studies. Given these uncertainties, the guideline values established as numerical limits are currently still provisionary.

A variety of issues are apparent with regards to the difficulty in establishing numerical limits for individual cyanotoxins. These include:

1. There have been few repeat oral dose animal studies using purified cyanotoxin. These studies have traditionally served as the basis for developing numerical limits since ingestion is the primary route of cyanotoxin exposure (EPA, 2016).
2. The contribution of cyanotoxins to chronic effects such as tumor promotion and cancer have not been considered in developing numerical limits for cyanotoxins, primarily due to a lack of data.
3. Guideline values should be matched closely with monitoring capabilities. At present it is not clear if this is the case. For example, there is currently no known method that targets TTMCs.
4. It is not clear whether the most sensitive individuals are protected at all levels of water ingestion rate and age group categories if anything less than the 99^{th} percentile of water ingestion rate is selected to calculate a guideline value. In addition, other sensitive groups may not be protected such as those with underlying conditions that make them particularly sensitive to the effects of cyanotoxins.

J. Concluding Remarks

Cyanobacteria are ancient organisms that have developed a number of adaptations that allow them to dominate nutrient rich lakes globally. CyanoHABs are a natural occurrence exacerbated by human activities including increased nutrient runoff, changes in land use, and climate. In the Great Lakes region cyanoHABs most often occur in water bodies that maintain water temperatures above 20 °C for an appreciable period of time and that receive a large amount of nutrient input. The timing of cyanoHAB events at weekly or even monthly scales is difficult to predict and despite decades of research efforts the ability to predict cyanotoxin levels and the

exact environmental conditions under which cyanotoxin production occurs remains elusive. As such, regular monitoring for cyanoHABs and their toxins is necessary.

Cyanobacteria that occur in the Great Lakes region produce hundreds, or perhaps even thousands of toxic or otherwise bioactive substances. Among the most commonly reported are MCs, anatoxins, saxitoxins, and cylindrospermopsins as well as a variety of bioactive peptides. With the exception of cylindrospermopsin, the molecular mechanisms of toxicity and acute pathogenesis of these cyanotoxins are well known. For some cyanotoxins (i.e. MCs and cylindrospermopsins) the molecular mechanism of toxicity and/or pathological effects indicates they are possible carcinogens, and indeed, tumor promotion has been demonstrated in animal studies. However, for purposes of developing numerical limits on cyanotoxin exposure more repeat oral dosing studies are needed. In addition, the development of numerical limits may require the use of epidemiological data to account for the possible contribution of some cyanotoxins to chronic diseases.

MCs are clearly the most often detected, or targeted cyanotoxins in the Great Lakes region. However, historically there have been no regular monitoring programs for cyanotoxins in the Great Lakes. At present our understanding of the variability in cyanotoxin diversity across spatial and temporal scales in the Great Lakes region is relatively unknown, and the rate at which humans are exposed to these toxins has not been adequately addressed due to a lack of monitoring tools. Biomarkers of cyanotoxin exposure are needed in order to develop diagnostic tests and establish rates of human exposure to cyanotoxins. Such information will be useful in determining whether there are associations between cyanotoxin exposure and the development of chronic diseases.

In conclusion, cyanoHABs and their toxins are increasing across the Great Lakes region as a result of increased nutrient pollution of waterways and climate change. In some waterways it is likely that nutrient inputs and the availability of internal nutrients (e.g. in sediments) cannot be reduced low enough to completely halt the development of cyanoHABs and toxin production anytime soon. For this reason long term strategies are needed for managing risk to human health from cyanotoxins. A thorough examination of these management strategies is beyond the scope of this report, but should include regular and improved monitoring of cyanotoxins in lakes or recreational environments and biota, reporting of such results to the public in a timely fashion, continued development of predictive models for forecasting cyanoHABs and their toxins, and

improvements in drinking water treatment technologies or management of existing modern technologies to ensure cyanotoxins are efficiently removed without the production of toxic byproducts. Ultimately the problem of reducing cyanoHABs and their toxins in the Great Lakes will be addressed through intensive nutrient abatement programs in the watershed.

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