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Freshwater Paralytic Shellfish Poisoning Toxins and other Cyanobacterial Neurotoxins in New York Lakes

by

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A dissertation
submitted in partial fulfillment
of the requirements for the
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# List of Abbreviations and Symbols

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ADDA</td>
<td>[(2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4E,6E-dienoic acid]</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATX</td>
<td>Anatoxin class of neurotoxins including anatoxin-a, homo-anatoxin, and dihydro-anatoxin</td>
</tr>
<tr>
<td>AWS</td>
<td>Average wind speed</td>
</tr>
<tr>
<td>AWD</td>
<td>Average wind direction</td>
</tr>
<tr>
<td>BMAA</td>
<td>β-N-methylamino-l-alanine</td>
</tr>
<tr>
<td>CSLAP</td>
<td>Citizens statewide lake assessment program</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>EFSA</td>
<td>European food safety authority</td>
</tr>
<tr>
<td>EPA</td>
<td>Environmental protection agency</td>
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<tr>
<td>GTX</td>
<td>Gonyautoxin</td>
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<tr>
<td>GTXol</td>
<td>Gonyautoxinol</td>
</tr>
<tr>
<td>HRMS</td>
<td>High resolution mass spectrometry</td>
</tr>
<tr>
<td>HILIC</td>
<td>Hydrophilic interaction liquid chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography tandem mass spectrometry</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>LASSO</td>
<td>Least absolute shrinkage and selection operator</td>
</tr>
<tr>
<td>MC</td>
<td>Microcystin</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption ionization mass spectrometry</td>
</tr>
<tr>
<td>MMPB</td>
<td>2-methyl-3-methoxy-4-phenylbutyric acid (microcystin method)</td>
</tr>
<tr>
<td>NEO</td>
<td>Neosaxitoxin</td>
</tr>
<tr>
<td>nAChR</td>
<td>Nicotinic acetylcholine receptors</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
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</tr>
<tr>
<td>OLS</td>
<td>Ordinary least squares</td>
</tr>
<tr>
<td>PST</td>
<td>Paralytic shellfish poisoning Toxin</td>
</tr>
<tr>
<td>PCOX</td>
<td>Post-column chemical oxidation for saxitoxins</td>
</tr>
<tr>
<td>PAR</td>
<td>Photosynthetically active radiation</td>
</tr>
<tr>
<td>STX</td>
<td>Saxitoxin</td>
</tr>
<tr>
<td>Tukey HSD</td>
<td>Tukey’s honestly significant difference test</td>
</tr>
<tr>
<td>TP</td>
<td>Total phosphorus</td>
</tr>
<tr>
<td>TN</td>
<td>Total nitrogen</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
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ABSTRACT


Cyanobacterial blooms occur worldwide and produce a range of harmful compounds. While most attention had focused on the hepatoxic microcystins, other cyanobacterial toxins such as the neurotoxic paralytic shellfish poisoning toxins (PSTs) and anatoxins (ATXs) may threaten environmental health.

Anatoxins (ATXs) are potent nicotinic agonists that can cause death within minutes. While the distribution of anatoxin-a has been evaluated in lakes, two derivatives, homo-anatoxin and dihydro-anatoxin, have not been evaluated.

Paralytic shellfish poisoning toxins (PSTs) are lethal neurotoxins with more than 60 known variants. While originally identified in marine systems, PSTs are frequently detected in freshwaters. The distribution of PSTs in freshwater systems have not been evaluated. We include the anatoxins and PSTs in the first survey of New York lakes for neurotoxins.

The occurrence of ATXs and PSTs were determined in more than 1,000 blooms sampled across 245 New York lakes. While less prevalent than the microcystins, these neurotoxins were found in ~15% of blooms surveyed. The PSTs occurred at concentrations well above drinking water and recreational guidelines for the toxins, while anatoxin concentrations were below recreational guidelines but above drinking water guidelines.

Two New York lakes were examined in more detail for the presence of toxins. Butterfield Lake primarily contained PSTs produced by benthic Microseira wolfei. These cyanobacteria were found at two sites, where the toxins were unlikely to pose a significant health risk to lake users as PSTs were not detected in open waters. Concern focused on humans or animals being exposed to dislodged benthic material.

Chautauqua Lake experienced numerous planktonic blooms that contained a complex mixture of toxins. The microcystins and PSTs were detected in high concentration, while anatoxin-a concentrations were low. Potential for exposure, and the variability in bloom size and toxin composition, suggests potential recreational human health risks.

This work emphasizes that neurotoxins present a significant health concern within New York State, and that monitoring programs for cyanobacteria toxins should not focus solely on the microcystins to assess human health risk from cyanotoxins. The
potential for exposure to these toxins varies between lakes, and monitoring strategies may need to be adapted to different locations.

Key words: cyanobacterial toxins. paralytic shellfish poisoning toxins, neurotoxins,

New York harmful algal blooms, benthic cyanobacteria, Chautauqua Lake, Butterfield Lake

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Introduction

The Nature of the Problem

1.1 Harmful Cyanobacteria and Neurotoxins

Cyanobacteria are a large and diverse group of photosynthetic prokaryotes with a wide distribution throughout the United States [1]. Cyanobacteria produce toxins, including the hepatotoxic microcystins (MCs), the neurotoxic anatoxins (ATXs) and paralytic shellfish poisoning toxins (PSTs), along with a suite of other bioactive compounds [2,3]. While cyanobacteria were known to produce harmful compounds in the 1930’s through bioassays using crude cyanobacterial extracts [4], cyanotoxins are now known to have a wide range of health effects, both chronic and acute, and have been linked to the deaths of humans and animals worldwide [5,6]. Anthropogenic changes to the climate and an increase in global nutrient load [7] from agriculture and other industries have caused proliferations of cyanobacteria and of their associated toxins [8,9].

Cyanobacterial blooms occur in every state of the United States [10]. Great attention has been placed on the recurring blooms in priority water bodies such as the Great Lakes where blooms have occurred in Lake Erie [11], Green Bay, Lake Michigan [12], Saginaw and Sturgeon Bays, Lake Huron [5] and near shore areas of Lake Ontario [13] and Lake Superior [14]. Blooms have also occurred in hundreds of smaller lakes in
states surrounding the Great Lakes including New York [15], Ohio [16], Pennsylvania [17], and Wisconsin [18].

Most efforts have focused on open-water blooms formed by pelagic cyanobacteria. However freshwater benthic cyanobacteria can also produce cyanotoxins, and the environmental health risks associated with these benthic blooms have only recently been recognized [19]. The presence of benthic “blooms” has been harder to quantify than pelagic cyanobacterial blooms, especially in lentic systems. Often their presence is established only after the death(s) of animals exposed to the water or that consumed the toxic material. Such events have been described worldwide, including but not limited to New Zealand [20], France [21], and Switzerland [22], and the United States [23]. Toxin producing benthic cyanobacteria are now known to be widespread throughout lakes and rivers in the United States [5,24,25].

Our knowledge regarding the distribution, ecology, biology, and health effects of the hepatotoxic MCs dwarfs our understanding of the neurotoxic cyanotoxins. Information about the distribution of the neurotoxins has been limited by: 1) lack of analytical methodologies to detect these toxins in the natural environment, including knowledge about important variants and their prevalence; 2) poor understanding of the cyanobacterial source(s) of these toxins are poorly understood; and 3) uncertainty in their importance regarding human health and whether they should be a priority as emerging contaminants. Anatoxin-a, the parent compound of the ATX class, has been
the best studied of the neurotoxins group. Even for this compound, multiple agencies including the US-EPA did not have sufficient toxicological information to issue a guidance value [3,26,27]. There is even less information regarding the anatoxin derivatives homo-anatoxin-a and dihydro-anatoxin. While assessment of the regional distributions of anatoxin-a in multiple water bodies have been performed with some frequency, the occurrence and distribution of homo-anatoxin or dihydro-anatoxin throughout multiple water bodies are rarely evaluated.

Similarly, most studies on the freshwater PSTs are limited to the detection of these toxins in specific locations and/or their production by specific genera. There are limited studies on PST toxin production in vitro [28] or the environmental drivers that control PST production in situ [29,30], while there are even fewer studies evaluating the analytical methodologies for freshwater PST toxins [31]. In the most cases where PST monitoring was performed, the primary analytical tool was a saxitoxin ELISA. This assay was developed for use with marine PSTs, and ELISAs are known to have poor cross-reactivity with congeners other than STX [32]. The same can be said for use of liquid chromatography tandem mass spectrometry (LC-MS/MS), a technique developed for use with marine PSTs and generally targeting these same PST variants [33]. Freshwater PSTs may be structurally different from the more common marine toxins, as exemplified by the discovery and isolation of novel PSTs, the lyngbyatoxins [34], from Microseira wolfei (basionym Lyngbya wolfei) [35]. Choosing the appropriate analytical tool
for the measurement of freshwater PSTs is critical for properly evaluating their occurrence and potential health risks.

1.2 Hypothesis and Objectives

The hypothesis for this work is that cyanobacteria neurotoxins are produced by both planktonic and benthic cyanobacteria in New York State waters, and that the concentrations of these toxins exceed recreational guidelines set to protect humans from contact exposure. After a literature review (Chapter 2), The application and effectiveness of four different analytical methods for the detection of freshwater PSTs are evaluated in Chapter 3. These methods are applied to measure the occurrence and distribution of the neurotoxic PSTs and ATXs in samples collected by the New York Citizen Science Lake Assessment Program between 2014-2018 are outlined in Chapter 4. A detailed investigation into the distribution and environmental factors associated with the toxins was performed in two lakes. The production of neuro by benthic cyanobacteria in Butterfield Lake is described in Chapter 6. In contrast, toxin production in Chautauqua Lake was associated with pelagic cyanobacteria, as described in Chapter 6. The implications of this work and the suggested management decisions are presented in Chapter 7.
1.3. References


16. Ohio Algae Information for Recreational Waters Available online:

   http://epa.ohio.gov/habalgae.aspx#147744471-publications-and-helpful-links

   (accessed on Nov 20, 2015).


Background and Context

2.1 Eutrophication, Climate Change, and the Expansion of Harmful Algal Blooms

2.1.1 Global Distribution of Harmful Algae and some Factors Related to their Proliferation

Harmful algal blooms (HABs) are large proliferations of algae that have well-known ecological, environmental and animal health, and economic impacts [1]. HAB-forming organisms include both eukaryotic algae and prokaryotic cyanobacteria, with the latter frequently referred to as blue-green algae due to purple-red pigments produced by these organisms. Several HAB forming algae and cyanobacteria have global distributions including the marine dinoflagellate Gymnodinium catenatum [2] and Gonyaulax species, diatoms in the genus Pseudo-nitzchia [3], and species in the freshwater cyanobacterial genera Microcystis [4] and Dolichospermum [5]. HABs formed by these and other algae have proliferated around the globe due to influxes of nutrients from a variety of anthropogenic sources [6]. Changes in climate have also been linked to HAB formation and longevity in both marine and freshwater environments [7]. Climate change stressors have significant effects on freshwater and marine HABs, and the ecosystems in which they occur, through direct and indirect processes, including but not limited to changes in temperature, acidity, nutrient influx, weather, hydrology, hypoxia, light availability, and acidity [8–10]. Complex interactions between climate
change and planet-scale aquatic cycles have caused major HAB events. A marine heat wave in the Northeastern Pacific Ocean in 2014-2015, widely referred to as “the blob” by the scientific community and others, provoked a persistent bloom of *Pseudo-nitzschia* off the western coast of the United States [11–13]. A second heat wave occurred in a similar location in 2019, with water temperatures up to 5 degrees above normal [14]. These heat waves occur globally [15] and their presence and intensity are linked to climate change [16], and will likely produce further HAB events.

### 2.1.2 Public Perception and Economic Impact of HABs

Annual costs due to marine and freshwater HABs for the US and EU between 1987-2000 and 1989-1998 were estimated to be 82 and 813 million dollars, respectively [17]. The costs today would likely be much higher, as HAB research has expanded the number of known toxins, their distribution, and their health effects. There are increasing costs associated with HAB monitoring, as the size and impact of these blooms has grown in recent decades. The cost of an individual HAB event has been difficult to quantify, but these costs can be significant [18]. HABs do not distinguish between locales with or without adequate economic resources to combat the problem. HABs can disproportionately affect poor towns and cities that rely on tourism for income and economic revival [19–22].
A portion of economic harm associated with HABs is associated with the fear of illness from exposure to unknown toxic vectors, where in the United States HAB events have led potentially affected residents to contact poison control centers or seek urgent or emergency health care [18,23,24]. A household survey in the aftermath of a do-not-drink warning in Toledo, Ohio caused by microcystin contamination of drinking water [18] identified that 16% of the households assessed had one or more residents reporting an illness associated with the drinking water advisory [25]. The maximum concentrations of microcystin in the finished water [18] was above the 10-day (1.6 µg/L) and lifetime (1.0 µg/L) exposure limits for only two days, meaning the hepatotoxins were unlikely to induce symptoms. During this event, a quarter of affected residents used the internet to find information [26], a source that may propagate misinformation. In a survey of Swedish residents’ personal experiences with HABs, 17.4% reported experiencing recreational danger from exposure to HABs, with 4% reporting a health effect from exposure to HABs (n = 1,997) [27]. While HABs and their toxins can be dangerous, the documented cases of human illness that can be directly attributed to cyanotoxin exposure are limited. Communication from water authorities about the risks associated with HABs is important to manage public perception during a bloom event.
2.2 Cyanobacterial Toxin Review

Cyanobacteria produce a number of bioactive compounds including toxins and antifeedants [28–30]. Toxins are generally divided into five families (Figure 2.1), the hepatotoxic microcystins, the neurotoxic anatoxins, the neurotoxic paralytic shellfish toxins (PSTs) (Figure 2.2), the neurotoxin anatoxin-a(S), which is structurally unique from the other anatoxins, and the cytotoxic cylindrospermopsin family. A potential sixth group, the neuroexcitatory molecule β-methyl amino alanine, will not be discussed as part of this review, but has been extensively reviewed elsewhere [18,30–32].

2.2.1 Microcystins

2.2.1.1 Background

Microcystins (MCs) have been extensively discussed elsewhere [4,18,33–35] and will be described only briefly here. MCs are a class of hepatotoxic peptides that are the primary focus for most cyanotoxin testing programs. There are currently over 250 known variants of MCs [36]. Microcystins are characterized by a seven member cyclic-peptide ring containing the unusual amino acid ADDA ([2S,3S,8S,9S]-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4E,6E-dienoic acid]) [37]. The ring has two variable positions, which form the basis of the generic nomenclature. The most well-known microcystin variant is MC-LR, where two variable amino acids in the MC ring
are leucine (L) and arginine (R) (Figure 2.1). Although congener profiles vary spatially and temporally, MC-LR is one of the most abundant variants in the United States. A survey of seven MCs in lakes across the lower 48 states in United States found the most common MC variants to be MC-LR, -LA, -RR, -YR, and -LY [38], while in New York -LR, -RR, and -YR were the most common congeners [39]. MCs are produced by selected species in many different cyanobacterial genera, including *Anabaenopsis*, *Aphanocapsa*, *Arthrospira*, *Dolichospermum* (basionym *Anabaena*), *Hapalosiphon*, *Microcystis*, *Nostoc*, *Oscillatoria*, *Planktothrix*, *Snowella*, *Synechocystis*, and *Woronichinia* [18].

### 2.2.2.2 Methods for Detection

Microcystins (MCs) can be assayed or analyzed by a number of methods. While bioassays have and can be used for MC analysis [40], alternative analytical tools have largely replaced this method. Microcystins (MCs) are protein phosphatase inhibitors, and the protein phosphatase inhabitation assay (PPIA) was developed to detect MCs [41,42]. The PPIA quantifies MCs based on their biological activity, rather than their molar abundance. ELISA assays with antibodies directed against the ADDA amino acid have been developed [43], with the EPA ELISA method 546 certified for the analysis of MCs in freshwater [44]. Other analytical methods for MCs include high pressure liquid chromatography (HPLC) coupled with mass spectrometry (EPA method 544) [45], oxidation to liberate 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB) from the
ADDA functional group followed by analysis with GC with flame ionization or mass spectrometric detection, or fluorescence detection [46,47], HPLC with diode array detection [40], and matrix-assisted laser desorption ionization mass spectrometry (MALDI) [48].

2.2.2.3 Toxicity and Health Effects from Exposure to Microcystins

Microcystins (MCs) are protein phosphatase inhibitors that primarily impact the liver in higher organisms, although they can impact other organs that contain the necessary receptors [49], such as the kidney, reproductive organs, colon, and brain [50]. The most famous case of cyanotoxin poisoning was when MC-contaminated water was used in a Brazilian dialysis clinic, where 70 of 130 patients died following infusion of the contaminated water, and an additional 30 experienced liver failure [51,52]. Acute MC exposure in humans has been reported in Argentina [53] and China [54,55], while MCs have been implicated in the deaths of organisms such as flamingos in Tanzania [56], cows in Switzerland [57], dogs [58], sheep, horses, pigs, ducks, and other wild animals [35,59]. Microcystins (MCs) are a tumor promotor [59–62], with epidemiological evidence of elevated cancer incidence in China [63] and Serbia [64].
Figure 2.1. Structures of several of the common cyanobacterial toxins.
Figure 2.2. Saxitoxin (STX) and functional group modifications for a selection of common marine and freshwater paralytic shellfish toxins (PSTs). Not all STX derivatives are shown. Many of the other derivatives share functional groups with STX.
2.2.2 Cylindrospermopsin

2.2.2.1 Background

Cylindrospermopsin (CYL) (Figure 2.1) is a tricyclic guanidine alkaloid that produces hepato and renal toxicity [65]. Named after *Cylindrospermopsis raciborskii*, from which it was first isolated and characterized [66], CYL is produced by *Cuspidothrix* (basionym *Aphanizomenon*), *Dolichospermum* (basionym *Anabaena*), *Raphidiopsis*, and *Umezakia*. Two other variants of CYL include epi-cylindrospermopsin and deoxy-cylindrospermopsin, characterized by a change in stereochemistry or the removal of the hydroxy functional group at the 7-position. In the United States, CYL has been detected in eutrophic lakes in Florida [67], and in North Carolina [68]. Cylindrospermopsin is exceedingly rare in New York, having been undetected in more than 10,000 samples collected between 2014-2019 [69], and in less than 5% of samples collected between 2000-2004 [70].

2.2.2.2 Methods for Detection

Methods for the detection of CYL include HPLC with diode array detection (λ_max at 262) [65]. More recent developments included mass spectrometric detection (EPA method 545) [71]. Dell’Aversano et al. used hydrophilic interaction liquid chromatography (HILIC) with mass spectrometric detection of CYL with microcystins, paralytic shellfish poisoning toxins and anatoxins [72]. Other methods for detecting
CYL include capillary zone electrophoresis, bioassays using mouse, crustacean
(*Thamnocephalus platyurus*), or brine shrimp (*Artemia salina*), cell lines, protein synthesis
inhabitation assay, and ELISA [65].

2.2.2.3 Toxicity and Health Effects from Exposure to Cylindrospermopsin

Cylindrospermopsin (CYL) is a cytotoxin that inhibits the synthesis of protein
and of glutathione, causing cell death [30]. Damage to the liver, kidneys,
gastrointestinal tract, endocrine organs, immune system, vascular system, and muscles
was expressed when CYL was tested on rodents [73]. Epi-cylindrospermopsin had a
toxicity similar to cylindrospermopsin as measured by intraperitoneal injection in the
mouse bioassay [74], while deoxy-cylindrospermopsin had $1/10^{th}$ the toxicity of CYL in
the same assay. Deoxy-cylindrospermopsin was still highly cytotoxic as it expressed
significant inhibition of protein synthesis and cell division [75,76].

The most famous case of human CYL poisonings comes from Australia in 1979,
where 138 children and young adults were sickened, suffering from malaise, vomiting,
anorexia, and enlarged tender livers. The event was termed the Palm Island mystery
disease [77]. Cylindrospermopsin (and potentially its derivatives) have been implicated
in the deaths of cows in Australia [78] and of other animals in the region [73].

The United States EPA used the available information to develop drinking water
guidelines for CYL of 0.7 µg/L over 10-days for pre-school aged children based on a no
observed adverse effect level of 30 µg/kg/day [79]. Similar toxin thresholds have been proposed throughout the world [80]. Some regulatory agencies used a lower reference dose of 20 µg/kg/day as a lowest observed adverse effect level, but for both reference doses the final regulatory guidelines were similar [73]. Health effects from consumption of contaminated drinking water occurred as described above and in other drinking water sources, where high concentrations of dissolved CYL have been found in Australia [74]. While lakes and drinking water sources around the globe may also contain CYL [81], CYL is not a dominant toxin in New York, and global detections of CYL have been limited.

2.2.3 Anatoxin-a, homo-anatoxin, and dihydro-anatoxin

2.2.3.1 Background

ATXs (Figure 2.1) are a class of lethal neurotoxins known for their acute toxicity [82]. The structure of anatoxin-a was elucidated in 1977 by Devlin et al. [83]. Since that time, a number of other variants have also been discovered in cyanobacteria including homo-anatoxin [84] dihydro-anatoxin [85], epoxy-anatoxin [86], epoxy-homoanatoxin [87], dihydro-homoanatoxin [88], and 4-hydroxyhomo-anatoxin [89]. ATXs are produced by members of the cyanobacterial genera *Arthrospira, Cuspidothrix* (basionym *Aphanizomenon*), *Cylindrospermum, Dolichospermum* (basionym *Anabaena*), *Microcystis,*
Oscillatoria, Phormidium, Planktothrix, and Raphidiopsis (basionym Cylindrospermopsis) [18].

### 2.2.3.2 Methods for Detection

Due to anatoxin-a’s poor UV and visible absorbance ($\lambda_{\text{max}}$ at 227), absorbance detection is not a viable approach for trace analysis of the compound in natural environments [30]. Derivatization of anatoxin-a followed by detection by GC-MS was reported by Smith and Lewis [85], while derivatization with 4-fluoro-7-nitro-2,1,3-benzoazadiazole followed by HPLC with fluorescence detection at 470 nm and 530 nm excitation and emission was reported by James et al. [87]. Both of these methods have been superseded by the advent of LC-MS and LC-MS/MS, as these mass spectroscopy-based methods do not require derivatization and have better limits of detection. Initial studies using mass spectroscopy focused on the parent molecular ion at $m/z$ 166. This molecular ion is the same as for the amino acid phenylalanine, leading to the potential for mis-identification of anatoxin-a if the chromatography did not resolve these two compounds [90]. EPA method 545 for anatoxin-a is a liquid chromatography tandem mass spectrometry method (LC-MS/MS) where anatoxin-a is detected using the 165.8 → 148.8 transition [71]. Modifications of this method can include other transitions, with the ratio of the confirmation fragments versus quantification fragment used for compound verification. While phenylalanine fragments similarly to anatoxin-a, there
are different ratios of the confirmation and quantification ions, allowing one to
differentiate anatoxin-a from phenylalanine by both retention time and fragmentation
[90]. There are no EPA-certified methods for the ATX derivatives, however these
compounds are commonly detected using mass spectrometry [89,91–93]. Homo-
anatoxin-a fragments similarly to anatoxin-a in LC-MS/MS [94], thus one can quantify
homo-anatoxin using an anatoxin-a standard. However, the hydrogenated double-bond
in the dihydro-derivatives of anatoxin-a and homo-anatoxin leads to a different
fragmentation pathway, making direct quantification using anatoxin-a standards
difficult. Dihydro-anatoxin can be easily synthesized from anatoxin-a [95], making the
preparation of synthetic dihydro-anatoxin- standards possible. While mass
spectrometry has been the primary method for the detection of anatoxin-a, antibodies to
anatoxin-a have recently been produced, allowing for detection of anatoxin-a through
the use of an ELISA [96].

2.2.3.3 Risks from Exposure to Anatoxin-a and Derivatives

ATX toxicity occurs through binding of the toxin to nicotinic acetylcholine
receptors (nAChR) [97] in both muscle and neuronal type receptors [30], where it is a
potent post-synaptic depolarizing neuromuscular blocking agent. Symptoms of ATX
poisoning include muscle fasciculations, decreased movement, abdominal breathing,
cyanosis, convulsions, and death [18]. ATX exposure has been linked to the deaths of
cows in Canada [98], and dogs in Scotland [99], France [93], and New Zealand [91,92]. While homo-anatoxin was implicated in the deaths of some of these animals, there have been few animal studies investigating the toxicity of this derivative, or of dihydro-anatoxin.

In binding assays at two neuronal nAChR sites and as a nicotinic agonist, homo-anatoxin had the same potency as anatoxin-a at the receptors, but it was a ~10-fold weaker nicotinic agonist than anatoxin-a [100]. Anatoxin-a and homo-anatoxin had similar effects on phrenic nerve-diaphragm preparations [84] and similar LD50s as measured by intraperitoneal injection [101]. The combinatorial evidence from these studies suggests that homo-anatoxin toxicity is similar to anatoxin-a.

Dihydro-anatoxin by comparison was found to be non-lethal in cyanobacterial extracts evaluated by bioassay [85,87]. In contrast, synthetic dihydro-anatoxin was also highly potent as measured by the mouse bioassay by Bates and Rapoport, although it was 12.5 fold less toxic than anatoxin-a [101]. Comparable to the mouse bioassay, an assay of dihydro-anatoxins’ contracture potency by Swanson et al. found 10-fold reduced effect compared to anatoxin-a [102]. Although these studies suggest dihydro-anatoxin is less toxic than anatoxin-a, there is significant uncertainty regarding the toxicity of this derivative relative to anatoxin-a, and whether dihydro-anatoxin can harm humans or animals that are exposed to this toxin.
There is a lack of information about the chronic toxicity of anatoxin-a, however acute anatoxin-a exposure induces conformation changes in nAChR receptors, changing the binding affinity of other ligands to the channel [97]. There is no evidence that conformational changes in these channels lead to chronic effects in addition to their acute effects. Relationships between exposure to chronic or sub-lethal anatoxin-a concentrations and chemical and/or behavioral changes in various organisms have been inconclusive [103].

2.2.3.4 Anatoxin Regulatory Guidelines

Many of the toxicological assessments for anatoxin-a were performed following its original isolation and structural identification, leaving gaps in the toxicology needed for development of exposure guidelines for ATXs. While several guidelines for drinking water and recreational exposure to anatoxin-a have been proposed, missing information on the toxicological effects of acute and long-term exposure to anatoxin-a has prevented national authorities from establishing reference doses [104–106], forcing regional authorities to create their own guidelines and resulting in a patchwork of regulations (Table 2.1).
2.2.4 Anatoxin-a(s)

Anatoxin-a(s) is an organophosphorus-containing natural product (Figure 2.1), that irreversibly inhibits acetylcholinesterase [30]. While structurally unrelated to anatoxin-a, anatoxin-a(s) produce salivation in intoxicated animals, hence the addition of the (s) to its name [18]. Originally elucidated from Anabaena flos-aquae, the compound has only been identified a limited number of times in the environment as it is unstable in alkaline pH and at temperatures above 40 °C [107].

Detection of anatoxin-a(s) using UV is ineffective as the $\lambda_{max}$ for the compound is at 220 nm. Anatoxin-a(s) can be detected using LC-MS techniques [108] and the cholinesterase assay [109]. Analytical methods for anatoxin-a(s) have been hampered by the lack of analytical standards and the instability of the compound in alkaline conditions that might be encountered in bloom events [18]. Other tests for anatoxin-a(s) include the Ellman test, and an electrochemical biosensor [30].

Anatoxin-a(s) biological effects are similar to organophosphate and carbamate pesticides [30]. Signs of exposure include hypersalivation, lacrimation, mucoid nasal discharge, tremors, ataxia, diarrhea, recumbency and seizures prior to death [110]. Death associated with anatoxin-a(s) exposure has occurred in dogs, pigs and geese [18], and waterbirds [73]. No known cases of human exposure have been reported [18].
Table 2.1. Drinking water and recreational exposure guidelines for anatoxins. All values are reported in µg/L.

<table>
<thead>
<tr>
<th>Source</th>
<th>Drinking Water Exposure</th>
<th>Recreational (Short Term) Exposure</th>
<th>Recreational (Subchronic) Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>California (anatoxin-a) [111]</td>
<td>-</td>
<td>90</td>
<td>-</td>
</tr>
<tr>
<td>Minnesota (anatoxin-a) [112]</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>New Zealand (anatoxin-a) [113]</td>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>New Zealand (homo-anatoxin) [113]</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ohio (anatoxin-a) [114]</td>
<td>20</td>
<td>300</td>
<td>-</td>
</tr>
<tr>
<td>Oregon (anatoxin-a) [115]</td>
<td>3</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>Quebec (anatoxin-a) [116]</td>
<td>3.7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Washington State (anatoxin-a) [117]</td>
<td>1</td>
<td>450</td>
<td>75</td>
</tr>
<tr>
<td>Australia (PSTs) [118]</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Brazil (PSTs) [119]</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>New Zealand (PSTs) [113]</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Oregon (PSTs) [115]</td>
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<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Ohio (PSTs) [114]</td>
<td>0.3</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Washington State (PSTs) [120]</td>
<td>3</td>
<td>75</td>
<td>-</td>
</tr>
</tbody>
</table>
2.2.5 Paralytic Shellfish Poisoning Toxins

2.2.5.1 Background

Paralytic shellfish poisoning toxins (PSTs) are a class of algal toxins which inhibit the sodium channel of higher organisms [121–123]. Produced by a variety of marine dinoflagellates in addition to cyanobacteria, these toxins create significant human health problems in regions where they occur. Shellfish feed on toxic dinoflagellates and accumulate the toxins, where they can cause illness if consumed. Human consumption of these now toxic shellfish leads to a clinical condition known as paralytic shellfish poisoning. Some shellfish, such as the Alaskan butter clam (Saxidomus gigantea) depurate the toxin very slowly and hence may remain toxic year-round after a single exposure [124].

Saxitoxin (STX), the parent compound in the PST group (Figure 2.3), is extremely toxic and thus shellfish are closely monitored for PSTs where exposure to toxic dinoflagellates might occur. In addition to STX, the PST family contains more than 60 known analogs of STX [125–127]. These STX analogs can be more common than the parent STX in both freshwater and marine systems, but are generally less toxic than STX as measured by the mouse bioassay [128,129]. In addition to their occurrence in marine systems, PST are being increasingly found in freshwater systems, where PSTs have been
Figure 2.3. Saxitoxin (STX) and functional group modifications for a selection of common marine and freshwater paralytic shellfish toxins (PSTs). Not all STX derivatives are shown. Many of the other derivatives share functional groups with STX.
detected in Australia [130,131], Brazil [132], United States (USA) [38,129,133–137], Canada [138], Germany [139], France [140], Portugal [141], Russia [142], and New Zealand [143]. Freshwater PSTs in these countries are produced by cyanobacteria belonging to the genera Cuspidothrix (basionym Aphanizomenon), Dolichospermum (basionym Anabaena), Microseira (basionym Lyngbya), Planktothrix, Raphidiopsis (basionym Cylindrospermopsis), and Scytonema. The Lyngbya wolfei toxins (LWTXs) are acetylated PST derivatives and are believed to be unique to freshwater environments, and have been found throughout the United States.

2.2.5.2 Methods for Detection

Paralytic shellfish poisoning toxins (PSTs) are an important focus of this thesis, and their analysis is generally considered to be more difficult than for many of the other cyanobacteria toxins. The earliest certified method for analysis of PST was the mouse bioassay (AOAC method 959.08). This method has garnered considerable protest from animal rights groups over the years and has greatly fallen out of favor due to both cost and ethical issues [144]. Current alternative techniques for the detection of PSTs include ELISA assays, HPLC coupled with mass spectrometry, HPLC coupled with fluorescence detection, and receptor-binding assays. Each technique will be discussed individually in the ensuing sections.
2.2.5.2.1 Receptor Binding Assay

The receptor-binding assay detects PSTs by their ability to compete with the binding of tritiated STX to rat brain sodium channels [145]. PST concentrations determined by this assay are expressed in saxitoxin binding equivalents and are thus dependent on the biological activity of different PST congeners, reflecting the specific toxicity of PST mixtures [146]. The receptor-binding assay is an effective option for detecting an integrated value of a mixture of PSTs using a single STX standard. The receptor-binding assay is AOAC certified for the detection of PSTs in shellfish [147].

2.2.5.2.2 Saxitoxin and Neosaxitoxin Enzyme Linked Immunosorbent Assay (ELISA)

Key to the preparation of a successful immunoassay for STX and other PSTs is the development of antibodies against the saxitoxin ring system. PSTs are too small to elicit an immune response directly, therefore the hapten must be conjugated to an appropriate carrier molecule to generate anti-toxin antibodies. These carrier molecules can be coupled to PSTs using a variety of approaches, including formadehyde condensation by the Mannich reaction, reductive alkylation with periodic acid, or the glutaraldehyde reaction [148]. The anti-toxin antibodies were produced using a toxin-immunogen conjugate (e.g. glucose oxidase [148] or keyhole limpet hemocyanin [149] coupled to STX or NEO) injected into mice [150] or rabbits [151,152]. Antibodies can be
purified using affinity chromatography [153,154] gel electrophoresis [151], or used directly for analysis.

Saxitoxin (STX) is highly decorated around the core ring system in the different PST congeners (Figure 2.3). The cross-reactivity of these antibodies to the individual PST variants depends on the enzyme-STX conjugate used to make those antibodies [155–157]. Different linkages of STX to the immunogen change the epitope of the PST antigen available for binding to antibodies, leading to highly variable cross-reactivity between congeners. Monoclonal antibodies prepared using STX as the hapten have poor cross reactivity with the PST NEO derivatives (e.g. NEO, GTX 1 and 4) that are hydroxylated at N-1 position (Figure 2.3) [158]. Some PST ELISA assays use polyclonal mixtures of antibodies to increase the cross-reactivity of assays with multiple PST variants [158]. Paralytic shellfish toxin ELISAs have been developed in indirect [151,153,155,159] or competitive [153,156,159] formats, although the commonly used and commercially available PST ELISAs are competitive, using the binding of free toxin versus a toxin-enzyme conjugate with STX or NEO to an anti-toxin antibody [148]. The Abraxis STX-ELISA allows free toxins to compete with a STX-horseradish peroxidase (STX-HRP) conjugate for a rabbit anti-toxin antibody [152]. These STX-HRP rabbit antibody complexes are captured by an anti-rabbit antibody fixed to the ELISA plate. Addition of HRP substrate reacts with the captured HRP conjugate to give a product whose absorbance is quantified at 450 nm.
2.2.5.2.3 Oxidation and Fluorescence Detection with High Performance Liquid Chromatography

PSTs with their polar zwitterionic nature are difficult to resolve using traditional reverse phase HPLC. The ring system by itself lacks a suitable chromophore for direction detection by UV absorbance. For this reason, most early HPLC methods coupled ion-pair chromatography with post-column oxidation (PCOX). This chemical oxidation converted the PST backbone to a fluorescent derivative which could be detected using a grating fluorometer [160]. Early versions of the PCOX system required the use of three isocratic solvents and three injections for the resolution of all toxins [161]. This significantly reduced the efficiency of the method for high-throughput purposes. Newer iterations of PCOX have solvent systems that can resolve most toxins in one analytical run, with the exception of the C-toxins [160,162]. Other oxidation methods used pre-column oxidation to convert the PSTs into fluorescent derivatives followed by separation with HPLC. This allowed for the separation and detection of PSTs without the use of ion-pair reagents [163]. Both the pre- and post-column methods are AOAC certified for the analysis of PSTs in shellfish [162,163]. Conversion of the PST backbone to a fluorescent derivative is not specific to an individual congener. This allows these techniques to detect toxins without individual standards, including the detection and quantification of the LWTXs 2-6 [136] and other unelucidated PSTs [164].
The fluorescent conversion of STX and related derivatives occurs through a Bayer-Villiger oxidation of the bond between the tetrahedral carbon at C-4 and the hydrated ketone at C-12, breaking the ring while the remaining rings become fluorescent through aromatization (Figure 2.4) [101,165]. Hydrolysis of the carbamoyl functional group from C-13 does occur, but was not found to be necessary for the ring conversion [166].

Different functional groups heavily impact how well the reaction proceeds to produce fluorescent products. A sulfate at C-11 increases the response 3 to 5-fold over STX due to electron withdrawal from C-12, while hydroxylation at N-1 reduces reaction 2-fold [136]. Saxitoxin derivatives containing an alcohol at the C-12 position, rather than a hydrated ketone have a conversion efficiency lower than their diol counterparts, with LWTX 1 and dc-saxitoxinol converting to fluorescent derivatives 10-times less efficiently than STX [126,136]. The oxidant used during early PCOX trials was hydrogen peroxide. This was unable to oxidize the alcohol to a ketone, and so it was originally proposed that the saxitoxinol derivatives could not be converted to fluorescent derivatives [167]. However, replacement of hydrogen peroxide with periodic acid, currently the most widely used oxidant for PCOX, allows for the detection of these compounds as periodic acid can oxidize the alcohol back to a ketone [168].

The presence of the ketone/diol at the C-12 position as a requirement for the PST ring system to be oxidized into a fluorescent derivative, which is an important
consideration for the use of this technique with the LWTXs 1-6. Changes in other functional groups increase or decrease fluorescent conversion, where the C-11 sulfate on LWTX 1 produced no increase in conversion relative to dc-saxitoxinol, unlike GTX 3 which oxidizes more efficiently than STX. [126]. The efficiency of the fluorescent conversion was limited by the oxidation of the C-12 alcohol back to a ketone, which was not affected by the electron withdrawing from the 11-sulfate moiety.

2.2.5.2.4 Mass Spectrometry

Traditional reverse phase HPLC methods for PSTs used ion pair reagents for retention due to the polar nature of the PSTs backbone. These ion pair reagents are incompatible with mass spectrometry as they lead to ion suppression and source contamination. In recent decades, the development of columns using hydrophilic liquid interaction chromatography (HILIC) technology have improved the separations for highly charged and polar compounds that are difficult to retain and separate with reverse phase chromatography [169].

Recent improvements in analytical methodology for the detection of PSTs have revolved around improved detection by mass spectrometry utilizing advances in HILIC. Coupled with the new generation of mass spectrometers, this has made methods
Figure 2.4. Scheme showing the conversion of saxitoxin to a fluorescent compound (a).

Compound a converts to a second non-fluorescent compound b during isolation [165–167].
more sensitive, selective, and flexible. Analytical methods for PSTs utilizing this newer HILIC-MS/MS technology include Dell’Aversano et al. [170], Boundy et al. [171], Diener et al. [172], Turrel et al. [173], Cho et al. [174], Ciminiello et al. [175], Thomas et al. [176], Blay et al. [177], Halme et al. [178], Bragg et al. [179], Lajeunesse et al. [138], Ballot et al. [139], Foss et al. [135], and Armstrong et al. [180]. Single and inter-laboratory validation of a HILIC-MS/MS method for the detection/quantitation of PSTs in shellfish were recently successful [181,182]. These mass spectrometry methods for PSTs have the ability to detect the full suite of marine PSTs simultaneously in a single analysis, often with analysis times under 10 minutes utilizing UPLC technology. Exemplifying the power of the new instrumentation and the reduced detection limits, the toxin profiles for six PST producing cyanobacteria cultures were recently reevaluated identifying a number of PSTs that had not previously been detected using older analytical techniques (Table 2.2) [127].

While mass spectrometric identification of PSTs has shown tremendous promise for the identification of marine PSTs, the availability of analytical standards has not kept up with the identification of new compounds [126,129,183–185]. For the freshwater PSTs, the discovery of the *Lyngbya wolfei* toxins (LWTXs) exemplifies this issue, as the lack of commercially available standards limits both the ability to detect these compounds by mass spectrometry as well as accurate quantification. The LWTXs have been detected in *Microseira* in Guntersville Reservoir, Alabama, United States [129], Blue
Hole Springs and Silver Glen Springs in Florida, [135], Butterfield Lake in New York [136], Lake Erie (unpublished), and in Canada in the St. Lawrence River near Montreal [138].

Acute health effects and risks from marine PSTs through consumption of contaminated shellfish have been well established [186–189]. To prevent human illness from exposure to toxins, total PSTs are limited to 80 µg STX eq./100g of shellfish tissue in the European Union, Australia, Canada, and the United States, where this guideline was derived based on bioassays and cases of human exposure [190]. While these limits are applicable in reducing the incidence of illness caused by the consumption of contaminated shellfish, there has of yet been no universal approach toward designating exposure limits in freshwater used for drinking or recreation.

There was a wide range in toxicity between the different toxin variants as measured by the mouse bioassay [125], and therefore toxicity and risks from exposure are generally expressed in STX equivalents, converting the concentrations of a PST mixture into STX toxicity using toxicity equivalency factors (Table 2.3) [128]. Three of the freshwater PST variants showed no toxicity by the mouse bioassay [129]. However, it is unclear if these toxins still pose a health risk in humans. PSTs may bioaccumulate in a number of organisms other than shellfish, including fish, which may be a further vector for toxin exposure [191].
Table 2.2. PST variants quantified (ng/mg of lyophilized material) by hydrophilic interaction liquid chromatography in cyanobacterial cultures. Table reproduced from D’agostino et al. [127].

<table>
<thead>
<tr>
<th>PST</th>
<th>Scytonema cf. crispum</th>
<th>Scytonema cf. crispum</th>
<th>Microseira wollei</th>
<th>Raphidiopsis</th>
<th>Aphanizomenon sp. NH-5</th>
<th>Dolichospermum circinale</th>
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<td>ND</td>
<td>ND</td>
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<td>790</td>
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<td>ND</td>
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</tr>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
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<td>C4</td>
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<td>Trace</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
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<td>8.8</td>
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<td>ND</td>
<td>110</td>
</tr>
<tr>
<td>dcGTX3</td>
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<td>50</td>
</tr>
<tr>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>Trace</td>
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<td>1</td>
<td>5.6</td>
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<td>0.013</td>
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<td>10</td>
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<td>790</td>
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<td>ND</td>
<td>110</td>
<td>720</td>
<td>ND</td>
</tr>
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<td>ND</td>
<td>Trace</td>
<td>ND</td>
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</tr>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.29</td>
</tr>
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<td>ND</td>
<td>ND</td>
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<td>ND</td>
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<td>ND</td>
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<td>ND</td>
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<td>LWTX-6</td>
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<td>ND</td>
<td>28</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Total</td>
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<td>12.1</td>
<td>222</td>
<td>174</td>
<td>852</td>
<td>3020</td>
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</table>

39
Table 2.3. Toxicity of different PST congeners relative to STX as measured by the mouse bioassay.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Relative Toxicity [128]</th>
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<td>STX</td>
<td>1</td>
</tr>
<tr>
<td>NEO</td>
<td>0.5-1.2</td>
</tr>
<tr>
<td>GTX1</td>
<td>0.8-1</td>
</tr>
<tr>
<td>GTX2</td>
<td>0.4</td>
</tr>
<tr>
<td>GTX3</td>
<td>0.6-1.1</td>
</tr>
<tr>
<td>GTX4</td>
<td>0.3-0.7</td>
</tr>
<tr>
<td>GTX5</td>
<td>0.1-0.2</td>
</tr>
<tr>
<td>GTX6</td>
<td>0.1</td>
</tr>
<tr>
<td>dcSTX</td>
<td>0.4-1.02</td>
</tr>
<tr>
<td>dcNEO</td>
<td>0.02-0.4</td>
</tr>
<tr>
<td>dcGTX1</td>
<td>0.5</td>
</tr>
<tr>
<td>dcGTX2</td>
<td>0.2-0.3</td>
</tr>
<tr>
<td>dcGTX3</td>
<td>0.2-0.5</td>
</tr>
<tr>
<td>dcGTX4</td>
<td>0.5</td>
</tr>
<tr>
<td>LWTX1</td>
<td>0 [129]</td>
</tr>
<tr>
<td>LWTX2</td>
<td>0.11 [129]</td>
</tr>
<tr>
<td>LWTX3</td>
<td>0.06 [129]</td>
</tr>
<tr>
<td>LWTX4</td>
<td>0 [129]</td>
</tr>
<tr>
<td>LWTX5</td>
<td>0.14 [129]</td>
</tr>
<tr>
<td>LWTX6</td>
<td>0 [129]</td>
</tr>
<tr>
<td>C1+C2</td>
<td>0-0.2</td>
</tr>
</tbody>
</table>
2.3.5.3 Risks from Exposure to Paralytic Shellfish Poisoning Toxins

Unlike acute poisonings of PSTs, effects from long-term exposure to PSTs are unclear, as in cases of acute poisoning symptoms are believed to resolve within a short time frame [192] and the majority of nerve function appears to return within weeks [193,194]. It is unknown if lower concentrations of PSTs expected in freshwater can lead to chronic health problems.

2.3.5.4 Paralytic Shellfish Poisoning Toxin Regulatory Guidelines

Due to limited information on distributions of PSTs in freshwater, regulatory limits for the PSTs in drinking water or for recreation are not common, however several regulatory limits for PSTs in freshwater have been established (Table 2.1). While many of the drinking water regulations are similar, recreational thresholds in Ohio are far lower than in Oregon or Washington.

2.4 Historical Evaluation of Paralytic Shellfish Poisoning Toxin and Anatoxin-a Distributions

2.4.1 Paralytic Shellfish Poisoning Toxins

While PSTs have been included in several monitoring programs, relative to MCs these compounds were detected far less frequently than MCs. Freshwater PSTs have been detected worldwide (see section 2.3.1), but most large multi-lake surveys generally
did not include PSTs in their analyses. A list studies that investigated the distribution of PSTs in multiple waterbodies is shown in Table 2.4. Relative to the concentrations of PSTs produced by cyanobacteria in culture, PST concentrations measured in lakes have been low [127].

2.4.2 Anatoxins

Anatoxin-a was evaluated in two surveys of 29 and 80 German lakes, with toxin detected in 26% [195] and 25% [196] of lakes, respectively, with the highest concentration of anatoxin-a being 13.1 µg/L, although most concentrations of anatoxin-a were less than 1.0 µg/L. A study in the midwestern United States lakes found anatoxin-a in 7 of the 23 lakes, with mean and median concentrations of 1.6 and 0.16 µg/L respectively [197]. In samples collected across 11 states in the United States, 7% of samples contained anatoxin-a with median concentrations of 0.75 µg/L [198]. Anatoxin-a was surveyed in five New York lakes, as well Lake Ontario, and Lake Erie (Table 2.5). While anatoxin-a was relatively common in several of these water bodies, the concentrations were generally low. Anatoxin-a was detected in 54 of 369 (15%) of lakes from 26 European countries [81]. Recently, anatoxin-a and other variants including homo-anatoxin and dihydro-anatoxin have been detected in streams in California, USA [199] and in New Zealand [200,201]. These toxins are produced by benthic cyanobacteria and have been implicated in the deaths of dogs in New Zealand [202],
Table 2.4. Summary of literature with large scale surveys of paralytic shellfish poisoning toxins in lakes.

<table>
<thead>
<tr>
<th>Country</th>
<th>Lakes with PSTs/ Lakes Surveyed (%)</th>
<th>PST Concentration Range (µg STX eq./L)</th>
<th>Primary Analytical Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>France [186]</td>
<td>10 (14% of samples)***</td>
<td>&lt; 0.05</td>
<td>LC-MS/MS</td>
</tr>
<tr>
<td>Germany [128]</td>
<td>2/5 (40%)</td>
<td>NA</td>
<td>STX ELISA/LC-MS/MS</td>
</tr>
<tr>
<td>Germany [185]</td>
<td>10/29 (34%)</td>
<td>NA</td>
<td>STX ELISA/LC-MS/MS</td>
</tr>
<tr>
<td>New Zealand* [187]</td>
<td>38/42 (90%)</td>
<td>0.001-0.99</td>
<td>ELISA/Receptor Assay</td>
</tr>
<tr>
<td>Ohio** [122]</td>
<td>25/105 (24%)</td>
<td>0.022- 0.880</td>
<td>STX ELISA/LC-MS/MS</td>
</tr>
<tr>
<td>United States [38]</td>
<td>6/1161 (&lt;1%)</td>
<td>&gt; 0.2</td>
<td>STX ELISA</td>
</tr>
<tr>
<td>United States [184]</td>
<td>4/23 (17%)</td>
<td>0.02-0.2</td>
<td>STX ELISA</td>
</tr>
</tbody>
</table>

*This study selected only samples from lakes containing cyanobacterial genera known to produce PSTs.

**Includes water treatment facilities

***Not reported which of the 10 lakes contained PSTs
Table 2.5. Summary of anatoxin-a in large scale surveys of several New York lakes, in Lake Ontario, and Lake Erie [95].

<table>
<thead>
<tr>
<th>Country</th>
<th>Years Surveyed</th>
<th>N samples (percent ATX Occurrence) with concentrations &gt;0.001 µg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onondaga Lake</td>
<td>2000-2003</td>
<td>96 (27%)</td>
</tr>
<tr>
<td>Oneida Lake</td>
<td>2000-2005</td>
<td>366 (&lt;1%)</td>
</tr>
<tr>
<td>Lake Neatahwanta</td>
<td>2004-2005</td>
<td>33 (29%)</td>
</tr>
<tr>
<td>Lake Agawam</td>
<td>2003-2004</td>
<td>20 (15%)</td>
</tr>
<tr>
<td>Lake Champlain</td>
<td>2000-2005</td>
<td>788 (6%)</td>
</tr>
<tr>
<td>Lake Ontario</td>
<td>2001-2005</td>
<td>597 (14%)</td>
</tr>
<tr>
<td>Lake Erie</td>
<td>2002-2005</td>
<td>936 (3%)</td>
</tr>
</tbody>
</table>
France [93] and the United States [203]. Constant release of ATXs into streams used for drinking water may pose a chronic risk to consumers [88].

2.5 Addressing Neurotoxin Distributions and Analytical Techniques for Paralytic Shellfish Poisoning Toxins in New York

While MC distributions in freshwater have been well-evaluated, the distribution of the ATX derivatives and PSTs are limited. While there are many known freshwater neurotoxins, whether they pose an environmental or human health risk has yet to be demonstrated. Resources for cyanotoxins are limited, and currently there has been limited evidence for advancing neurotoxin research in areas such as improved monitoring and analytical tools, toxicological evaluations, or neurotoxin biosynthesis. Additionally, while the potential for exposure to MCs in recreation and drinking water have been established, the same is not true for the ATXs and PSTs.

There are several analytical methods for PSTs, however comparisons of these methods are largely done using the marine PSTs, rather than freshwater PSTs. Most studies assessing the distributions of paralytic shellfish toxins have used an STX-ELISA, and it is an open question how different PST methods compare for use in detecting freshwater PSTs. The methods for PST analysis and distributions of both the PSTs as well as ATXs in New York lakes are assessed in Chapters 3 and 4, while the distribution of PSTs and other neurotoxins in two individual lakes are evaluated in Chapters 5 and
6, with an emphasis on the human and environmental health risks posed by the
neurotoxins.

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Comparison of Four Analytical Methods for the Analysis of Freshwater Paralytic Shellfish Toxins

3.1 Abstract

Paralytic shellfish toxins (PSTs) are of increasing concern in freshwater ecosystems. While there are a number of analytical methods developed for the analysis of PSTs in marine systems, the methods primarily used to quantify PSTs produced by freshwater cyanobacteria are the STX-ELISA and/or LC-MS/MS. These methods were originally intended for the detection of the marine congeners. Freshwater cyanobacteria can have different congener profiles than their marine dinoflagellate counterparts. The ability of ELISA or LC-MS/MS methods to detect freshwater cyanobacteria toxins, such as the *Lyngbya wollei* toxins or other novel cyanobacterial PSTs, has not been adequately evaluated. Samples from 200 lakes in New York State were initially screened for PSTs by HPLC fluorescence with post column chemical oxidation (PCOX). A subset of samples with high levels of PSTs measured by PCOX were reanalyzed by three LC-MS/MS methods, STX-ELISA, and the STX receptor-binding assay. We applied a combination of these methods for the detection of PSTs from 2017 and 2018 blooms in New York. Agreement between the methods was poor, with some samples negative by ELISA exhibiting a strong response in the PCOX method and some samples positive by

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1To be submitted to *Toxins* as Smith, Z.J.; Smith, J.L.; McCarron, P.; Turner, A.D.; Leighfield, T.A.; Sanderson, M.M.; Doucette, G.J.; Boyer, G.L. Comparison of four analytical methods for the analysis of freshwater paralytic shellfish toxins.
ELISA showing little to no response in the PCOX and LC-MS/MS methods. LC-MS/MS methods from different laboratories often gave conflicting results for the presence or absence of particular or novel PSTs. Analysis of freshwater PSTs clearly presents a significant analytical challenge. We recommend using a combination of methods for detecting freshwater PSTs to reduce the likelihood of reporting falsely negative or positive results and to better protect human health.

3.2 Introduction

Freshwater cyanobacterial toxins have garnered attention in recent years as bloom events have impacted the use of water resources for recreation and/or drinking [1–4]. While the scarcity of potable water has been identified as a primary threat to global security [5], cyanobacterial blooms and their toxins increase the costs and reduce accessibility when resources are strained. Cyanobacteria blooms can lead to unpalatable water by producing taste and odor compounds [6]. They also produce harmful compounds. Toxins produced by cyanobacteria fall into several categories, including hepatotoxins such as the microcystins and nodularin, cytotoxins such as cylindrospermopsins, and neurotoxins including the anatoxins, β-N-methyl amino alanine, and the paralytic shellfish toxins (PSTs or saxitoxins) [7]. Microcystins have garnered the most attention globally and are the primary focus of most large-scale algal monitoring programs [8,9]. While less common than the microcystins, neurotoxic
anatoxins and PSTs are of increasing concern due to their lethality and their impacts on domestic animals and livestock [10,11].

PSTs are sodium channel inhibitors with more than 60 known variants [12]. The PST backbone and functional changes for some of the common PSTs are shown in Figure 3.1. PSTs were originally identified from, and associated with, marine dinoflagellate red tides [13,14]. The parent compound of the class, saxitoxin (STX), is extremely toxic [15,16], and has led to illness in humans from the consumption of contaminated shellfish worldwide [17–22]. Although identified as a marine toxin, PSTs are found in freshwater systems in Australia [23,24], Brazil [25], United States [9,26–31], Canada [32], Portugal [33], France [34], Germany [35], Russia [36], and New Zealand [37]. As reports of freshwater PSTs have increased, including the detection of novel PSTs produced by Microseira wolleii (basionym Lyngbya wolleii) [38] in 1997 [29,30,39], there is a need for PST analytical methods that better target the freshwater PST congeners.

Recent improvements in the detection of PSTs have revolved around improved detection by mass spectrometry utilizing advances in hydrophilic interaction liquid chromatography (HILIC). Coupled with the new generation of mass spectrometers, this has made newer methods more sensitive, selective, and flexible. PSTs methods taking advantage of this newer HILIC-MS/MS technology include Dell’Aversano et al. [40], Boundy et al. [41], Diener et al. [42], Turrel et al. [43], Cho et al. [44], Ciminiello et al.
[45], Thomas et al. [46], Blay et al. [47], Halme et al. [48], Bragg et al. [49], Lajeunesse et al. [32], Ballot et al. [35], Foss et al. [27], and Armstrong et al. [50]. Inter-laboratory validation of a HILIC-MS/MS method for the detection/quantitation of PSTs in shellfish is ongoing [51]. These mass spectrometry methods simultaneously detect the full suite of marine PSTs in a single run, often with run times under 10 min utilizing UPLC technology. Exemplifying the power of the new instrumentation and the reduced detection limits, the toxin profiles for six PST-producing cyanobacteria cultures were reevaluated identifying a number of PSTs that had not previously been detected using older analytical techniques [52].

While mass spectrometric identification of PSTs has shown great promise for the detection of marine PSTs, the availability of analytical standards has not kept up with the identification of new compounds [29,53–55]. For the freshwater PSTs, the discovery of the Lyngbya wolfei toxins (LWTXs) exemplifies this issue, as only one (LWTX 1) of the six LWTXs is currently commercially available. The LWTXs have been detected in the US in Microseira in the Guntersville Reservoir in Alabama [29], Blue Hole Springs and Silver Glen Springs in Florida [27], Butterfield Lake in New York [28], Lake Erie (unpublished), and in Canada in the St. Lawrence River near Montreal [32]. One approach to detect the LWTXs and other freshwater PSTs is to use untargeted methods such as the STX-ELISA. ELISAs detect a wide range of PSTs based on structure [31].
Alternative untargeted analyses include the receptor-binding assay or HPLC with fluorescence detection using pre- or post-column oxidation.

In the receptor-binding assay, PSTs concentrations are determined by evaluating the equilibrium ratio of tritiated STX versus PSTs bound to rat brain sodium channels as the target [56]. PST concentrations determined by this assay correspond with the toxicity of PST mixtures and the binding affinity of different PST congeners toward their biological target [57]. The majority of PSTs bind to sodium channels, and can be detected by the assay. The receptor-binding assay is a valuable replacement for the mouse bioassay for assessing toxicity, where toxin concentrations in freshwaters are generally low. Foregoing the mouse bioassay also avoids ethical and economic concerns [58]. The receptor-binding assay is AOAC certified for the detection of PSTs in shellfish [59].

Conversely, commercially available STX-ELISAs are generally based on the competitive binding of free toxin versus a toxin-enzyme conjugate toward an STX antibody [60]. An ELISA assay will have different responses for different PST congeners based the toxin functional group(s) used to link the toxin with enzyme conjugate and immunogen [61–63]. For PSTs other than STX analyzed by the assay, the equilibrium
Figure 3.1. Saxitoxin (STX) and functional group modifications for a selection of common marine and freshwater paralytic shellfish toxins (PSTs). Not all STX derivatives are shown, with a number of other PST derivatives sharing functional groups with STX.
between the PSTs in a sample and the STX-enzyme conjugate is changed, favoring the binding of the STX-enzyme conjugate, producing the lower cross-reactivity and detection limits for these PSTs. Heterologous toxin conjugates have been produced to increase the cross-reactivity of these kits with multiple PSTs. This approach has suffered from inconsistency between batches of antibody and therefore inconsistency between assays [64]. The Abraxis STX-ELISA kit in this study used a proprietary STX-conjugate, with cross-reactivity for other PSTs ranging between 0.2-29% [65].

HPLC with fluorescence detection detects PSTs through a chemical approach that converts the PST backbone into a fluorescent derivative (Figure 3.2). This can be performed with either post-column (PCOX) [66] or pre-column derivatization [67]. Both the pre- and post-column methods are AOAC certified for the analysis of PSTs in shellfish [67,68]. The reaction converting the PST backbone to fluorescent derivatives is not specific to individual variants, but occurs for the majority of known PSTs. Oxidation can detect multiple PSTs including LWTXs 1-6 and other unelucidated PSTs containing the STX tricyclic ring system [24,28]. The fluorescent conversion of the STX ring system cleaves the bond between carbons 4 and 12, followed by aromatization to produce a fluorescent derivative [69,70]. Hydrolysis of the carbamoyl functional group from C-13 can occur, but was not necessary to form a fluorescent product [71].
**Figure 3.2.** Scheme showing the conversion of saxitoxin to a fluorescent compound (a).

Compound a converts to a second non-fluorescent compound b during isolation [14,71].
Different functional groups impact how well the reaction forms fluorescent products. A sulfate at C-11 increases the response 3 to 5-fold over STX due to electron withdrawal from C-12, while hydroxylation at N-1 reduces the reaction efficiency 2-fold as the hydroxyl group is eliminated prior to hybridization of the ring [28]. Reduced saxitoxin derivatives containing an alcohol at the C-12 position have a conversion efficiency lower than the oxidized ketone/diol counterparts, with the abundance of fluorescent products formed by LWTX 1 and dc-saxitoxinol 10-fold lower than STX [28,55]. A ketone/diol at the C-12 position is required for oxidation to fluorescent derivatives. Synthetic saxitoxinol derivatives were unable to undergo the Baeyer-Villiger oxidation using hydrogen peroxide as the oxidant [14]. Periodic acid is able to oxidize the alcohol to a ketone, which makes it possible for the saxitoxinol derivatives to be detected by PCOX [72]. In saxitoxinol derivatives, changes in other functional groups do not increase or decrease fluorescent conversion, with the C-11 sulfate on LWTX 1 producing no increase in conversion relative to dc-saxitoxinol [55]. The efficiency of the fluorescent conversion is limited by the oxidation of the C-12 alcohol back to a ketone, which is not affected by the electron withdrawing nature of the 11-sulfate.

Most assays are reported in STX equivalents, however different assays rely on different properties of the PST tricyclic system for detection. The STX-ELISA is a structure-based assay, where cross-reactivity depends on the portion PST backbone, as determined by the STX-enzyme conjugate epitope that can bind to an anti-toxin
antibody. Conversely, the receptor-binding assay is an activity-based assay, quantifying different PSTs variants based on their ability to bind to sodium channels, their biological target. Unlike the two assays, PCOX analysis detects the STX ring system after separation of the individual PSTs by HPLC. Individual PSTs are then added together to give a cumulative concentration of PSTs in STX eq., using toxin equivalency factors determined by the mouse bioassay [73]. Targeted LC-MS/MS analysis of PSTs performs similarly to PCOX where individual PSTs quantified prior to an estimation of toxicity using toxin equivalency factors. This stands in contrast to untargeted methods for PSTs, including high resolution mass spectrometry, which detect a property of the PST backbone rather than individual PST congeners.

There are few comparisons of the different methods in their application to analysis of freshwater cyanobacterial PSTs [28,74]. Here we report on the analysis of selected samples from New York State lakes collected through the Citizens Statewide Lake Assessment Program (CSLAP) [75] using three untargeted methods for PSTs including the Abraxis STX-ELISA, the receptor-binding assay, and PCOX. Additionally, PSTs were evaluated by three independent laboratories using mass spectrometry for the detection of PSTs; two laboratories used targeted LC-MS/MS with and without a solid-phase extraction (SPE) sample clean-up prior to analysis. The third laboratory used untargeted high-resolution mass spectrometry (HRMS) to detect PSTs. A comparison of
these methods and their application to PST analysis in New York cyanobacteria blooms is described.

3.3 Methods

3.3.1 Sample Collection, Sample Selection, Extraction of PSTs, and Sample Transport

Samples were collected between 2016-2018 from lakes in the Citizens Statewide Lake Assessment Program (CSLAP) by volunteers following the methods prescribed by the New York State CSLAP program [76]. Samples consisted of an 100-250 mL mixture of water and algal material associated with pelagic blooms near the shoreline. Samples were shipped overnight on ice to SUNY ESF where 100 mL of the sample was immediately lyophilized. PSTs were extracted from lyophilized material using 10 mL of 50% methanol containing 1% acetic acid (v/v) followed by sonication (3 × 20 s at 32 watts). The resulting slurry was centrifuged at 15,000× g for 10 min, clarified through a 0.45 µm nylon syringe filter, and kept at -20 °C until analysis. In total, 1,882 samples from 245 lakes were analyzed by PCOX. From these, 46 were selected for further analysis by ELISA, and a subset of 21 and 12 of these samples were analyzed by the receptor-binding assay, tandem mass spectrometry, and high resolution mass spectrometry.

Samples analyzed by the receptor binding assay were shipped on ice and dry ice to the NOAA Charleston, North Carolina. Samples analyzed by LC-MS/MS method 1
and LC-HRMS method 3 were transported on dry ice to Virginia Beach, Virginia and Halifax, Nova Scotia. For LC-MS/MS method 2, 500 μL of extract was evaporated to dryness, placed under N₂, and transported to Dorset, United Kingdom on dry ice.

3.3.2 PCOX

PSTs were analyzed using AOAC 2011.02 post-column chemical oxidation modified for water samples and algal powders [68]. Separation was performed using a Waters Alliance 2695 solvent delivery system (Waters, Milford, MA, USA), a Chromenta KB 3µ 150 × 4.6 mm column with an ACE (ACE Ltd., Aberdeen, Scotland) 3µ guard cartridge assembly and a flow rate of 0.8 mL/min. Guard cartridges were swapped every 400 injections. The solvent system was: (A), 2 mM heptanesulfonate (Regis Technologies Inc., Morton Grove, IL, USA) in 10 mM ammonium phosphate adjusted to pH 7.1; (B), 500 mL 2 mM heptane sulfonate in 30 mM ammonium phosphate adjusted to pH 7.1 plus an additional 150 mL of acetonitrile [77]. The separation gradient was: 0% B for 0–3 min, 40% B for 3–5 min, 100% B for 5–13 min, and 100% B until 20 min, followed by equilibration of the column back to 0% B for 10.5 min. The injection volume was 25 μL. Post-column oxidation of the PST ring used 9 mM periodic acid (Macron Fine Chemicals, Radnor, PA, USA) in 50 mM potassium phosphate at pH 9 at 0.45 mL/min added before a 25m 0.25 mm i.d. reaction coil (total volume 1 mL) maintained at 65 °C. Following the coil, 0.5 M acetic acid was added at a
flow of 0.45 mL/min. PSTs were detected at 330 nm and 390 nm excitation and emission wavelengths. PSTs were differentiated from interfering naturally fluorescent compounds by re-injection of the sample with water in place of the oxidant in the post-column system.

PST standards were obtained from the National Research Council Canada (Institute for Marine Biosciences, Halifax, Canada) and United States Food and Drug Administration (FDA) (Silver Spring, MD, USA). The FDA STX standard was diluted 1:50 to a concentration of 4 µM prior to use. An STX standard was injected every 10 samples to ensure stability of the response, with PSTs quantified using a linear regression. Saxitoxin (STX) instrument LODs were 0.9 pg, while method LODs ranged between 1-2 µg STX/L. STX was linear between 9.9 to 198 ng on column which corresponded to 9.3 and 729.5 µg/L in lake water based on a 100 mL sample (slope, 4.23×10^{-4}; y-int, -5.8×10^{-4}; R^2, 0.99). An STX standard was injected every 10 samples to ensure stability of the response, with PSTs quantified using a linear regression. Saxitoxin (STX) instrument LODs were 0.9 pg, while method LODs ranged between 1-2 µg STX/L. STX was linear between 9.9 to 198 ng on column which corresponded to 9.3 and 729.5 µg/L in lake water based on a 100 mL sample (slope, 4.23×10^{-4}; y-int, -5.8×10^{-4}; R^2, 0.99). An STX standard was injected every 10 samples to ensure stability of the response, with PSTs quantified using a linear regression. Saxitoxin (STX) instrument LODs were 0.9 pg, while method LODs ranged between 1-2 µg STX/L. STX was linear
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between 1-2 µg STX/L. STX was linear between 9.9 to 198 ng on column which
corresponded to 9.3 and 729.5 µg/L in lake water based on a 100 mL sample (slope,
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3.3.3 Receptor-Binding Assay

Toxin extracts were analyzed for STX-like activity by the receptor-binding assay for PSTs in a microplate format as detailed in Van Dolah et al. [59]. Total potency of a sample was estimated by competition between radiolabeled STX [11^3H] (American Radiolabeled Chemicals, Inc., Saint Louis, MO, USA) and any STX-like activity present
in samples for binding to voltage-gated sodium channels in a crude rat brain membrane preparation. Total PSTs were expressed as STX eq. using a calibration curve (Figure S3.1) prepared from a STX dihydrochloride reference standard acquired from NIST (NIST reference material 8642, National Institutes of Standards and Technology, Gaithersburg, MD). To evaluate and avoid interference from methanol in the extraction solvent due to denaturation of protein and to evaluate matrix effects, samples were diluted 1/5 (10% methanol) and 1/50 (1% methanol) prior to analysis. The method detection limit was 2 µg STX eq./L.

### 3.3.4 STX-ELISA

All bloom extracts were diluted to a maximum concentration of 5% methanol prior to analysis. Further dilutions were made when sample concentrations fell outside the linear range of the STX-ELISA calibration curve (0.02-0.4 µg STX eq./L). STX concentrations were determined using Abraxis ELISA (Abraxis LLC, Warminster, PA, USA) according to the manufacturer’s instructions. The STX-ELISA method LOD was 0.75 µg, corresponding to 0.015 µg STX eq./L in a sample, while the LOQ was 0.02 µg STX eq./L.
3.3.5 LC-MS/MS Method 1

Analysis was performed using an Acquity Binary UPLC system coupled to a Xevo TQD triple quadrupole mass spectrometer (Waters, Milford, MA, USA). Separation was achieved with a Waters (Waters, Milford, MA, USA) BEH Amide 1.7-μm column (2.1 × 100 mm) coupled to a BEH Amide 1.7-μm guard column maintained at 60 °C. Injections volumes were 1.3 μL. The mobile phase A consisted of water/formic acid (Fisher Scientific, Hampton, NH, USA)/NH₄OH (Fluka, Charlotte, NC, USA) (500:0.075:0.3 v/v/v), and mobile phase B was acetonitrile/water/formic acid (700:300:0.1 v/v/v). Flow rates were operated based on those described by Boundy et al. [51]. Tune parameters were 150 °C source temperature, 600 °C desolvation temperature, and 500 L/h desolvation gas flow. The capillary voltage was held at 4 kV. The qualification and confirmation transitions and LODs used for the different PSTs are shown in Table S3.2 and Table S3.3.

3.3.6 LC-MS/MS Method 2

Solid-phase extraction (SPE) was performed both manually and on a GilsonAspec XL4 SPE liquid handling robot with amorphous graphitized polymer carbon Supelco ENVI-Carb 250 mg/3 mL SPE cartridges (Sigma–Aldrich, St. Louis, MO, USA). The cartridges were conditioned with 3 mL of acetonitrile/water/acetic acid (20:80:1 v/v/v) with 200 μL air push, followed by 3 mL of water/NH₄OH (1000:1 v/v)
with a 200 μL air push. A 400 μL sample extract was loaded onto the conditioned cartridge with a 200 μL air push, and were subsequently washed with 700 μL of deionized water with a 400 μL air push, eluting salts to waste. PST were then eluted with 2 mL of acetonitrile/water/acetic acid (20:80:1 v/v/v) with a 400 μL air push into a 5 mL polypropylene test tube. The eluent was diluted by 1/4 with acetonitrile.

An Acquity I-Class UPLC system coupled to a Xevo TQ-S triple quadrupole mass spectrometer (Waters, Milford, MA, USA) was used for analysis. Separation was achieved with a BEH Amide 1.7 μm column (2.1 × 100 mm) (Waters, Milford, MA, USA) coupled to a BEH Amide 1.7 μm guard column maintained at 60 °C. The injection volume was 2 μL. The mobile phase A consisted of 0.015% formic acid (Fisher Scientific, Loughborough, UK) and 0.06% NH₄OH (Fisher Scientific, Loughborough, UK) in water (v/v), and B was 70% acetonitrile with and 0.01% formic acid (v/v). Flow rates and UPLC gradients were operated based on those described by Boundy et al. [51]. Tune parameters were 150 °C source temperature, 600 °C desolvation temperature, 1000 L/h desolvation gas flow, 7.0 bar nebulizer gas flow, 150 L/h cone gas flow, and 0.15 mL/min collision gas flow. The capillary voltage was held at 0.5 kV and 2.5 kV for positive and negative ionization modes, respectively. LWTXs 1-6 were only measured for sample 12 using this method. The qualification and confirmation transitions and LODs used for the different PSTs are shown in Table S3.2 and Table S3.3.
3.3.7 LC-HRMS Method 3

LC-HRMS analyses were performed on an Agilent model 1200 LC system that consisted of a binary pump, auto-sampler and column oven (Agilent, Santa Clara, CA, USA) coupled to a Q Exactive HF Orbitrap mass spectrometer with a HESI-II heated electrospray ionization interface (ThermoFisher Scientific, Waltham, MA, USA). All LC separations were performed, as previously reported [46,78], with an Amide-80 TSK-gel (TosoHaas, Grove City, OH) column (5 μm, 2 × 250 mm) held at 40 °C with mobile phases of aqueous 50 mM formic acid and 2 mM ammonium formate (A) and acetonitrile (B). The elution gradient (0.2 mL/min) included a linear gradient from 10 to 45% A over 25 min, then to 70 % A by 27 min, followed by an isocratic period at 70% for 13 min and re-equilibration at 10% for 20 min. Injection volume was 2 μL. The MS conditions, included a sheath and auxiliary gas flow rates of 35 and 10 (arbitrary units), S-lens RF level of 50 (arbitrary units), capillary temperature of 300 ºC and auxiliary gas heater temperature of 300 ºC.

Data were acquired using separate positive and negative methods in full scan/data dependent acquisition (FS/DDA) mode. Full scan data were collected from m/z 200–500 using the 30, 000 resolution setting, an AGC target of 1×10⁶ and a maximum inject time of 100 ms. MS/MS data were collected using the 15,000 resolution setting and an AGC target of 5×10⁵. An isolation window of 0.7 m/z and a loop count of 10 was used to select the top 10 ions detected in each FS for subsequent isolation and CID. A
minimum AGC target of $1 \times 10^3$ was used in MS/MS selection that resulted in an intensity threshold in of $1 \times 10^4$ for precursor ions. The peptide match function was set to preferred and isotope exclusion was used. Dynamic exclusion was set to 5 sec and a static exclusion list was populated at the beginning of each sequence from ions detected from a blank injection. An inclusion list was populated using the unique masses of reported PSTs and LWTXs [29,32,78,79]. In positive ion mode the mass spectrometer was calibrated from $m/z$ 74–1622 using a Pierce LTQ Velos EIS positive calibrations solution (Thermo, Waltham, MA, USA). The spray voltage was 2.5 kV and a stepped collision of 20 and 35 and 40 V was used. In negative mode, the mass spectrometer was calibrated from $m/z$ 69–1780 using Pierce ESI negative calibration solution (Thermo, Waltham, MA, USA). The spray voltage was −4.5 kV and a stepped CE of 20 and 35 V was used.

Paralytic shellfish toxins were identified through critical evaluation of retention time and exact mass of precursor ([M+H]+ or [M-H]-) ions as well as MS/MS dissociation. All reported PSTs had observed accurate masses < 5 ppm of the formulae for the precursor ions. For PSTs detected in samples for which chemical standards are available (i.e. dcSTX, dcGTX 2, dcGTX 3 and LWTX 1), MS/MS spectra were compared between samples and standards, including considerations of fragment ion accurate mass and relative abundance. For putative PSTs detected in samples for which no authentic standard was available (i.e. LWTX 2, LWTX 3, LWTX 5, LWTX 6), exact mass and previously reported fragmentation in FS/DDA were used to support identification.
[32,79]. MS/MS spectra of PSTs from samples and standards used to support identification of putative PSTs are shown in supporting information (Figures S4-13). Transitions and LODs for the different PSTs are shown in Table S3.2 and Table S3.3.

3.3.8 Matrix Susceptibility of the Abraxis ELISA

To determine the susceptibility of the Abraxis STX-ELISA to matrix interferences, a larger subset of 118 samples was randomly selected from each lake sampled in 2016, 2017, and 2018, that contained no PSTs as measured by PCOX. If there were not enough samples from one year to fill a plate (40 lakes), the rest of the PST-negative samples were randomly selected from Chautauqua Lake. All samples were diluted 10-fold to 5% methanol and analyzed as described previously.

3.4 Results

3.4.1 Quantification of PSTs using PCOX, STX-ELISA and the Receptor Binding Assay

Total PST concentrations as determined by PCOX and STX-ELISA ranged between 31.2-923 µg STX eq./L and 0-772.6 µg STX eq./L, while the concentrations ranges of PSTs as measured by the receptor-binding assay were smaller, with concentrations ranging between 3.8-69.2 µg STX eq./L. There was little agreement between the three methods for both water samples or benthic Microseira that contained
PSTs (Figure 3.3) [28]. PST concentrations measured by PCOX were generally higher than STX-ELISA (Figure 3.3A), or the receptor-binding assay (Figure 3.3B) with one exception (Cayuga Lake). Increasing concentrations of PSTs as measured by PCOX did not produce increases in ELISA or receptor-binding assay response. Additionally, 35% (16/46) of samples that were positive for PSTs by PCOX were negative for PSTs by STX-ELISA and 62% (13/21) by the receptor binding assay.

Paralytic shellfish toxin (PST) concentrations measured STX-ELISA and receptor-binding assay were similarly variable (Figure 3.3C). While in PST concentrations measured by STX-ELISA varied by five orders of magnitude, PST concentrations measured by the receptor-binding assay showed much less variation. STX-ELISA concentrations were higher than the receptor binding assay for only six samples, while three had PST concentrations in the receptor binding assay higher than the STX-ELISA. Twelve samples positive by STX-ELISA showed no PST activity in the receptor binding assay.

3.4.2 Evaluation of STX-ELISA Susceptibility to Matrix Interferences

To determine the effect of the lake matrix on the STX-ELISA analyses, we evaluated false-positive rate of STX-ELISA in multiple matrices. All lakes with samples that tested positive for PSTs also had samples that tested negative for PSTs, therefore all lakes containing PSTs were included in this subset.
Of the 118 samples evaluated, 18 samples that were PST-negative by PCOX contained detectable PSTs (> 0.015 µg STX eq./L) using the STX-ELISA. Three of these samples were strongly positive (>0.70 µg STX eq./L), four were weakly positive (0.25-0.035 µg STX eq./L), while eleven samples were just above the ELISA LOD but below the LOQ with concentrations between 0.15-0.20 µg STX eq./L. This corresponded to a 15% false positive rate if all samples above the LOD were included. Eliminating the samples between the LOD (0.015 µg/L) and LOQ (0.02 µg/L) reduced the false positive rate to 6%. These false positives were not evenly distributed by year, with 12 false positives in 2018 versus 3 in each of 2016 and 2017. Conversely, any false positives in the STX-ELISA could also be considered false negatives for PCOX.
Figure 3.3. Comparison of PSTs concentrations determined by HPLC with post column chemical oxidation, STX-ELISA, and the receptor binding assay. Black diamonds (◆) represent samples positive by both methods, while gray diamonds (◆) represent samples positive by only one method. Samples labeled with squares (■) were Microseira extracts described in Smith et al. [28]. Lines represent the regression for a 1:1 correlation in concentration for the three methods. Six samples were negative by both the STX-ELISA and the receptor binding assay (shown as overlapping points at (0.01,0.01) in panel C), while two other points had similar receptor binding assay and STX-ELISA concentrations and also overlap.
Figure 3.4. PST Concentrations as measured by STX-ELISA (LOD and LOQ 0.015 and 0.02 µg STX eq./L respectively) in 118 samples that contained no PSTs as measured by PCOX. Dashed line represents the LOD. Samples were collected in 2016, 2017, and 2018, and were from 36, 38, and 36 water bodies, respectively, with bloom(s) during that year. Raw concentrations for samples that tested negative for PSTs by ELISA, that were not corrected for the blank, are shown as open symbols below the LOD.
3.4.3 Identification and Quantification of PSTs by Mass Spectrometry

There was significant variability in the total PSTs quantified by each of the LC-MS/MS methods. LC-MS/MS identified only one or two PST congeners in most samples, while PCOX identified more than five in most samples (Figure 3.6, Figure S3.14). A summary of the compounds identified by mass spectrometry is shown in Table S3.15.

Ion suppression was observed in LC-MS/MS method 1 for the later eluting PSTs (e.g. STX, NEO) in extracts combined with a mixture of authentic PSTs. This led to the differences in PSTs measured by LC-MS/MS methods 1 and 2. Following the SPE cleanup used in LC-MS/MS method 2, a loss of dcGTX 3, an increase in dcGTX 2, loss of doSTX, and loss of all LWTXs was observed.

LC-MS/MS methods 1 and 2 suggested the presence of GTX 5 in 2 samples (Technical Note S3.16) based on a transition from 380.1 → 300.1. LC-HRMS could not confirm the presence of GTX 5, and the retention times did not match that of a GTX 5 standard.
Table 3.1. PSTs measured in 12 samples by six methods for PSTs. Units for STX-ELISA, PCOX, and the receptor-binding assay are reported in µg STX eq./L, while the three LC-MS/MS methods are in µg PSTs/L. Specific toxins variants detected by each mass spectrometry method are discussed in the text.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Lake</th>
<th>STX-ELISA</th>
<th>PCOX</th>
<th>Receptor-binding Assay</th>
<th>LC-MS/MS Method 1</th>
<th>LC-MS/MS Method 2</th>
<th>LC-HRMS Method 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cayuga Lake</td>
<td>128.0</td>
<td>48.7</td>
<td>84.1</td>
<td>16.7</td>
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<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>Cayuga Lake</td>
<td>237.5</td>
<td>27.4</td>
<td>3.8</td>
<td>ND</td>
<td>0.14</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>Cayuga Lake</td>
<td>232.4</td>
<td>31.2</td>
<td>3.7</td>
<td>ND</td>
<td>0.16</td>
<td>ND</td>
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<tr>
<td>4</td>
<td>Cayuga Lake</td>
<td>343.0</td>
<td>51.4</td>
<td>4.9</td>
<td>ND</td>
<td>1.00</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>Chautauqua Lake</td>
<td>ND</td>
<td>138.9</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>Chautauqua Lake</td>
<td>0.32</td>
<td>552.4</td>
<td>ND</td>
<td>ND</td>
<td>0.032</td>
<td>3.6</td>
</tr>
<tr>
<td>7</td>
<td>Chautauqua Lake</td>
<td>ND</td>
<td>54.4</td>
<td>not determined*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>8</td>
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<td>ND</td>
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<td>12</td>
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<tr>
<td>9</td>
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<td>250.8</td>
<td>2.6</td>
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<td>ND</td>
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<tr>
<td>10</td>
<td>Indian Lake</td>
<td>0.40</td>
<td>703.6</td>
<td>3.8</td>
<td>0.59</td>
<td>ND</td>
<td>10</td>
</tr>
<tr>
<td>11</td>
<td>Indian Lake</td>
<td>772.6</td>
<td>923.2</td>
<td>69.2</td>
<td>300.4</td>
<td>22.3**</td>
<td>1,180</td>
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<tr>
<td>12</td>
<td>Lake Placid</td>
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<td>252.8</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Matrix effects led to significant variation across dilutions and therefore no result was reported for this sample.

**Any LWTXs detected were not included in the final concentration of PSTs reported for this sample.
There was significant variation in the concentrations of PSTs measured by the six methods (Table 3.1). PST concentrations and PST profiles in Cayuga Lake had similar PSTs concentrations by PCOX analysis, but there was more variation in these samples by STX-ELISA. Three samples from Cayuga Lake had similar concentrations of STX equivalents as measured by the receptor-binding assay, whereas the fourth had a higher apparent concentration of toxin, even above that measured by PCOX. Unlike most samples analyzed by STX-ELISA, which had lower concentrations of toxin relative to PCOX, the samples from Cayuga Lake as measured by STX-ELISA had concentrations of PSTs greater than those measured by PCOX. Two samples from Indian Lake, had similar PSTs concentrations as measured by PCOX, but varied by 20-times in the receptor-binding assay and by three orders of magnitude in the STX-ELISA. Notably, PCOX detected PSTs in several samples where no PSTs were detected by the other methods.

3.4.4 Structural Verification and Quantification of PSTs by LC-MS/MS and Chemical Investigation into the “GTX 3 Imposter”

Although the PSTs profiles in New York were highly variable, a compound initially identified as GTX 3, was ubiquitous. The compound coeluted with authentic GTX 3 standard added to fix samples. However, this GTX 3-like compound, although co-eluting with GTX 3, could not be converted to its epimer (GTX 2) after heating at 50
°C for 50 h or by acid catalysis. This epimerization normally occurs in 20 min under these conditions [80,81]. GTX 3 was not found in any of the mass spectrometric methods in these samples despite an apparent concentration of the compound being above the LOD for each of the methods.

3.4.5 Distribution of Blooms and Detection of PSTs in Cayuga Lake, New York

PSTs were detected in a number of samples collected on Cayuga Lake in the summer and fall of 2017 and 2018 (Figure 3.5). Blooms were temporally irregular and localized in 2017, but appeared at both the north and south ends of the lake, with a bloom at the end of July spanning several square miles at the southern end of the lake. The blooms occurring toward the end of July, in both the northern and southern portions of the lake, were heavily dominated by Dolichospermum, with the bloom in the northern half of the lake lasting for several weeks. Blooms in the northern portion of the lake reappeared for several weeks in September and were dominated by Microcystis although Dolichospermum was still present at a much lower abundance. Samples were taken nine times from the blooms in 2017, with 99% of fluoroprobe total chlorophyll associated with cyanobacteria, and mean concentrations of 2,469 µg cyanobacteria chlorophyll/L respectively.

Blooms were widespread throughout the entire lake in 2018 with high levels of chlorophyll (mean 1,419 µg cyanobacteria chlorophyll/L, n = 37) starting at the
beginning of July with blooms dissipating at end of September \[82\]. Blooms in the beginning of 2018 were dominated by *Dolichospermum*, while in mid-September blooms were primarily dominated by *Microcystis*.

A number of PSTs were identified in Cayuga Lake in 2017 and 2018 (Figure 3.6). The profiles between the two years were dissimilar, with more PST variants and higher concentrations of toxin as determined by PCOX in 2017 compared to those in 2018 (Figure 3.7). Five PSTs were present in both years, with the three most common being an unknown PST eluting at 13.5 minutes (compound 12 found in 15/23 samples), the GTX 3 imposter (13/23 samples), and STX (9/23 samples).

Samples collected from both ends of the lake at similar times in 2017 had matching PST profiles, with the profiles remaining relatively unchanged when blooms were resampled in September. The GTX 3 imposter was identified in all samples in this year, along with STX and C1/C2 and a number of unknown PSTs. Samples collected in the July 2018 blooms contained 1 or 2 of three late eluting compounds, including two unknown PSTs at 13 and 13.5 minutes, and STX. Samples collected in September contained a number of earlier eluting compounds eluting between 6.5-12.5 minutes in addition to STX (Figure 3.7).
Figure 3.5. Map of samples collected from Cayuga Lake for PSTs in 2017 (▲) and 2018 (●) with samples containing PSTs and non-detect samples represented by filled and empty shapes.
**Figure 3.6.** Chromatogram showing PSTs analyzed by PCOX with post-column chemical oxidation in a sample from a bloom collected from Cayuga Lake on 7/26/17. Chromatogram conditions are described in section 3.3.2. The black line was the chromatogram of the sample analyzed in the presence of the periodic acid oxidant, while the dashed red line is the same sample analyzed with water in place of the oxidant. Integrated peaks and their labels represent PSTs. The compound at 9.1 min was not included in the total PSTs due to its co-elution with other interfering compounds. Compound 7 was detected in samples with and without the co-eluting interference. For consistency between samples Compound 7 was included in quantification of this sample by subtracting the peak areas of the oxidized and non-oxidized peaks. One PST co-eluted with an interference at approximately 9.2 min and was therefore the contribution of this PST was not included.
Figure 3.7. Concentration and distribution of PSTs as determined by PCOX in samples collected from Cayuga Lake in 2017 and 2018. Compounds are listed in order of PCOX elution (C1/C2) to late eluting (STX). Compound 7, identified in 2017 and eluting at 10.1 min, was present in several samples especially in July 2018, but was found co-eluting with a matrix interferent so the contribution of this toxin was not included in these profiles.
3.5 Discussion

STX was a minor component of the PSTs congeners identified in New York. The relative abundance of STX was low (<1-15%) relative to total PSTs, and was exceptionally rare in more than 1,800 blooms collected from 245 lakes (Chapter 4). Paralytic shellfish poisoning toxin (PST) concentrations have been historically low in most surveys of PSTs [9,31,35,83–86]. These surveys most often used the STX-ELISA as STX is often a component of PST mixtures in marine environments [87–89]. STX was not a dominant variant in New York lakes, which may explain the low PST detection rate in freshwater systems and inconsistency between results (Figure 3.3, Table 3.1).

The three methods, STX-ELISA, receptor-binding assay, and PCOX detect PSTs by different mechanisms and should not necessarily agree with each other. This is emphasized by varying cross-reactivities of different PST variants relative to STX (Table 3.2). HPLC with chemical oxidation utilizes chemistry inherent to the PST ring system for fluorescence. The reaction is affected by changes in the functional groups on PST variants, but there are no direct links between toxicity and the efficiency of the fluorescent conversion. The PCOX method can be challenging to operate [58,90], but is a robust analytical method for PSTs in shellfish [66]. Application to freshwater systems required modification of the AOAC certified method (Technical Note S3.22), with a major limitation for freshwater PST analysis being the lack of analytical standards for many of the less common PSTs and with only one LWTX of six available.
Table 3.2. Relative Response to STX for Different Analytical Methods and Assays for PSTs.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>PCOX Response Relative to STX</th>
<th>Mouse Bioassay Relative Toxicity [73]</th>
<th>Abraxis STX-ELISA Cross-reactivity* [65]</th>
<th>Receptor Binding Assay Cross-reactivity [91]</th>
</tr>
</thead>
<tbody>
<tr>
<td>STX</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>NEO</td>
<td>0.41</td>
<td>0.5-1.2</td>
<td>0.013</td>
<td>0.73</td>
</tr>
<tr>
<td>GTX1</td>
<td>0.10</td>
<td>0.8-1</td>
<td>&lt;0.02</td>
<td>1.04**</td>
</tr>
<tr>
<td>GTX2</td>
<td>4.66</td>
<td>0.4</td>
<td>0.23</td>
<td>0.34**</td>
</tr>
<tr>
<td>GTX3</td>
<td>3.52</td>
<td>0.6-1.1</td>
<td>0.23</td>
<td>0.34**</td>
</tr>
<tr>
<td>GTX4</td>
<td>0.08</td>
<td>0.3-0.7</td>
<td>&lt;0.02</td>
<td>1.04**</td>
</tr>
<tr>
<td>GTX5</td>
<td>0.71</td>
<td>0.1-0.2</td>
<td>0.23</td>
<td>0.033</td>
</tr>
<tr>
<td>GTX6</td>
<td>0.44</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>dcSTX</td>
<td>1.13</td>
<td>0.4-1.02</td>
<td>0.29</td>
<td>0.10</td>
</tr>
<tr>
<td>dcNEO</td>
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<td>0.02-0.4</td>
<td>0.06</td>
<td>-</td>
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<tr>
<td>dcGTX1</td>
<td>-</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>dcGTX2</td>
<td>2.71</td>
<td>0.2-0.3</td>
<td>0.014</td>
<td>-</td>
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<tr>
<td>dcGTX3</td>
<td>2.46</td>
<td>0.2-0.5</td>
<td>0.014</td>
<td>-</td>
</tr>
<tr>
<td>dcGTX4</td>
<td>-</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LWTX1</td>
<td>0.09</td>
<td>0 [29]</td>
<td>0.13*</td>
<td>-</td>
</tr>
<tr>
<td>LWTX2</td>
<td>-</td>
<td>0.11 [29]</td>
<td>0.13*</td>
<td>-</td>
</tr>
<tr>
<td>LWTX3</td>
<td>-</td>
<td>0.06 [29]</td>
<td>0.13*</td>
<td>-</td>
</tr>
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<td>LWTX4</td>
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<td>0 [29]</td>
<td>0.13*</td>
<td>-</td>
</tr>
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<td>-</td>
<td>0 [29]</td>
<td>0.13*</td>
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<tr>
<td>C1+C2</td>
<td>1.20</td>
<td>0-0.2</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

*a* Abraxis cross reactivities for GTX 2,3, GTX 1,4, dcGTX 2,3 reported as epimeric mixtures for each pair of compounds.

*Cross reactivity for all LWTXs reported as “Lyngbyatoxin”. Individual LWTXs are unlikely to have the same cross reactivity.

**Receptor binding activity for GTX 2,3 and GTX 1,4 determined using epimeric mixtures.
LC-MS/MS methods for PSTs can offer similar or better separation for many of the PST congeners and lower LODs than PCOX [41,51,52]. However, LC-MS/MS method 1 was heavily impacted by ion suppression for the late eluting PSTs, where no response for the late eluting PSTs (e.g. STX) was observed after authentic toxins were added to natural samples. This was unexpected as the same extracts were used for quantification and detection of microcystins and anatoxin-a without issue. The charge states of PSTs may contribute to their ionization being more sensitive to matrix interferences. The addition of an SPE cleanup in LC-MS/MS method 2 resolved ion suppression somewhat; STX was detected in several samples by this method that were not detected by LC-MS/MS method 1. However, the SPE introduced additional losses including the loss of all LWTXs when evaluated with sample 11.

The STX-ELISA is highly sensitive to structural modifications of STX [65] and to the process used to link toxin to the immunogen for production of toxin antibodies or enzyme conjugates. As congener patterns in freshwater PST producers were variable between strains [27–29,32], the STX-ELISA was expected to have more variability when compared to other analyses. The STX-ELISA estimated lower concentrations of PSTs than PCOX, and provided no information about the PST congeners present in a sample.

PSTs response in the receptor-binding assay also varies for the individual PST congeners. As the LWTXs had low toxicities as measured by intraperitoneal injection in mice [29], the receptor binding assay should have a limited response to these PSTs. The
receptor-binding assay should not detect non-toxic congeners [57], including congeners that can be converted to more toxic derivatives (e.g. C1-C4) [12]. Low cross-reactivity with LWTXs and the reduced toxicity for some PST congeners [12,73], may lead to an a non-detect for PSTs even when PSTs are present.

Concentrations of PSTs in New York were high enough to be an environmental concern. However, there was little consistency in the absolute PST concentrations determined by the different methods leading to confusion as to what results should be used for regulatory purposes. Each of the three untargeted methods could be the primary analytical method, however the receptor-binding assay and the STX-ELISA do not identify PST congeners in a sample. Therefore, these methods should be paired with PCOX or LC-MS/MS when the PST congener information is important. A combination of analytical techniques may be the best approach the use of multiple methods would eliminate false negatives and help explain the variability in estimates of toxin concentrations.

Conversion of toxin concentrations to actual toxicity was also problematic, as larger PST concentrations measured by PCOX or STX-ELISA did not produce corresponding increases in the PST concentrations measured by the receptor-binding assay. We conclude that neither PCOX nor STX-ELISA provided good estimates of toxicity. However, PCOX and STX-ELISA would over-estimate PST concentrations
relative to the receptor-binding assay, and both methods should be protective with regards to human health effects.

Neither targeted LC-MS/MS method included the LWTXs in their analyses. However, the LWTXs were found in four samples using the untargeted LC-HRMS method. The presence of the LWTXs in open water samples suggests these toxins may not be limited to the benthos. The producers of these LWTXs is unclear, as these toxins are generally associated within benthic Microseira [27,29,92]. LWTXs were not detected in whole water samples collected above Microseira mats in Butterfield Lake [28]. However, they may have been released by benthic organisms in the lakes studied here, or may have been produced by planktonic cyanobacteria. Lyngbyatoxins may more common in planktonic environments than previously believed (Table 3.1).

PCOX was effective at detecting some of the LWTXs (Figure S3.14). LWTXs 1, 4 and 6 are reduced saxitoxinol derivatives, and due to the poor reactivity of saxitoxinol derivatives to chemical oxidation, these compounds were only detectable at higher concentrations (Table 3.2, [55]). The cross reactivity for the LWTXs in the STX-ELISA was ~10-fold less than STX, while these LWTXs were found to be non-toxic by the mouse bioassay and therefore may not be detectable by the receptor binding assay. LWTX 1 was widespread in the St. Lawrence river [32]. The LWTXs 1, 4 and 6 are best detected using mass spectrometry; however, reference standards are currently unavailable to provide accurate quantification.
There were also unknown PSTs in New York lakes. LC-HRMS detected a number of PST-like compounds with similar nitrogen and ring-double bond equivalent properties that did not share molecular weights with known PSTs. A GTX 3-like compound was detected by PCOX in Cayuga Lake with a STX-like fluorescence response that disappeared upon removal of the oxidant. This compound co-occurred with a number of other PSTs. However, this unknown PST co-eluting with GTX 3 did not epimerize to form GTX 2, nor was GTX 3 detected by any of the three mass spectrometric methods. The STX-ELISA response for samples from Cayuga Lake was greater than the other analytical methods, where known PSTs generally responded less efficiently than STX in the Abraxis ELISA [65]. This suggested that there were other compounds being detected by the STX-ELISA method that were not included in the targeted LC-MS/MS methods. Although the PST congeners identified in Cayuga Lake in 2017 were similar by PCOX analysis, the receptor binding assay was 20-fold higher for one sample, suggesting these unknown PSTs may be toxic.

Both known and unknown PSTs were not limited to small localized water bodies, but were found to be distributed throughout the state in critical water bodies. Cayuga Lake is home to 120,000 people and serves as drinking water for several cities [93]. Samples from Cayuga Lake measured by the receptor-binding assay all had PST concentrations above the 3 µg STX eq./L “do not use” limit set by the state of Ohio [94], and the other untargeted methods also detected PSTs in concentrations well above 3 µg
Low concentrations of PSTs have been found in past lake surveys in the United States [9,83], Ohio [31], Germany [35,84], and Russia [36]. PST concentrations may actually be much higher than those estimates depending on the method used for their analysis of PSTs. For the protection of human and environmental health, PST analysis should involve multiple methods to better estimate PST concentrations for risk assessments.

3.6 References


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3.7 Supplementary Information

**Figure S3.1.** Standard curve used for the analysis of PSTs by the receptor binding assay for this study. Saxitoxin receptor binding assay calibration curve prepared with STXdiHCl, EC50= 2.58 nM, slope= -0.802.
Table S3.2. LC-MS/MS MRM transitions used in methods 1-3. Transitions that are not in bold are run in positive mode, while bold transitions are in negative mode. Injection volumes were 1.3, 2, and 2 µL for methods 1, 2, and 3, respectively.
<table>
<thead>
<tr>
<th>Toxin</th>
<th>LC-MS/MS Method 1</th>
<th>LC-MS/MS Method 2</th>
<th>Method 3 HRMS Screen</th>
</tr>
</thead>
<tbody>
<tr>
<td>STX</td>
<td><strong>316.1 &gt; 298.1</strong></td>
<td><strong>316.1 &gt; 220.1</strong></td>
<td><strong>316.1 &gt; 126.1</strong></td>
</tr>
<tr>
<td>NEO</td>
<td><strong>410.1 &gt; 367.1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GTX1</td>
<td><strong>394.1 &gt; 351.1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GTX2</td>
<td>396.1 &gt; 298.1</td>
<td>394.1 &gt; 351.1</td>
<td></td>
</tr>
<tr>
<td>GTX3</td>
<td><strong>412.1 &gt; 314.1</strong></td>
<td><strong>394.1 &gt; 333.1</strong></td>
<td><strong>394.1 &gt; 298.1</strong></td>
</tr>
<tr>
<td>GTX4</td>
<td>380.1 &gt; 300.1</td>
<td>412.1 &gt; 314.1</td>
<td></td>
</tr>
<tr>
<td>GTX5</td>
<td>380.1 &gt; 300.1</td>
<td>380.1 &gt; 300.1</td>
<td><strong>378.1 &gt; 122.0</strong></td>
</tr>
<tr>
<td>GTX6</td>
<td>257.1 &gt; 126.1</td>
<td>396.1 &gt; 316.1</td>
<td><strong>394.1 &gt; 122.0</strong></td>
</tr>
<tr>
<td>dcSTX</td>
<td><strong>273.1 &gt; 255.1</strong></td>
<td><strong>273.1 &gt; 126.1</strong></td>
<td><strong>257.1 &gt; 222.0</strong></td>
</tr>
<tr>
<td>dcNEO</td>
<td></td>
<td>273.1 &gt; 126.1</td>
<td>273.1 &gt; 225.1</td>
</tr>
<tr>
<td>dcGTX1</td>
<td>353.1 &gt; 273.1</td>
<td><strong>367.1 &gt; 274.1</strong></td>
<td><strong>367.1 &gt; 349.1</strong></td>
</tr>
<tr>
<td>dcGTX2</td>
<td>353.1 &gt; 255.1</td>
<td>351.1 &gt; 164.0</td>
<td><strong>351.1 &gt; 333.1</strong></td>
</tr>
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<td>353.1 &gt; 255.1</td>
<td>351.1 &gt; 333.1</td>
<td><strong>351.07285</strong></td>
</tr>
<tr>
<td>dcGTX4</td>
<td>396.1 &gt; 316.1</td>
<td>369.1 &gt; 271.1</td>
<td><strong>367.1 &gt; 349.1</strong></td>
</tr>
<tr>
<td>C1</td>
<td>396.1 &gt; 298.1</td>
<td><strong>474.1 &gt; 351.0</strong></td>
<td><strong>474.03548</strong></td>
</tr>
<tr>
<td>C2</td>
<td><strong>316.1 &gt; 298.1</strong></td>
<td><strong>316.1 &gt; 220.1</strong></td>
<td><strong>316.1 &gt; 126.1</strong></td>
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<tr>
<td>C3</td>
<td><strong>490.1 &gt; 410.1</strong></td>
<td><strong>412.1 &gt; 332.1</strong></td>
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<td><strong>490.1 &gt; 392.1</strong></td>
<td><strong>490.03039</strong></td>
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<td>412.1 &gt; 314.1</td>
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<td>396.1 &gt; 178.1</td>
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<td>241.1 &gt; 206.1</td>
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<td>LWTX2</td>
<td>395 &gt; 315*</td>
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<td><strong>393.08342</strong></td>
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<td>LWTX4</td>
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<td><strong>241.14077</strong></td>
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<tr>
<td>LWTX5</td>
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<td>299.14625</td>
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<td>LWTX6</td>
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<td><strong>283.15134</strong></td>
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<tr>
<td>11-OH-dcSTX</td>
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<td></td>
<td>273.13059</td>
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*LWTX transitions were based on those reported by Onodera et al. used when analyzing sample 12 only [29].
Table S3.3. LODs for each of the LC-MS/MS methods reported in fmol of PSTs on column based on the injection volumes for the respective analytical method.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>LC-MS/MS Method 1 LODs</th>
<th>LC-MS/MS Method 2 LODs</th>
<th>LC-HRMS Method 3 LODs</th>
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<td>NEO</td>
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<td>80</td>
<td>170</td>
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<td>88.9</td>
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<td>41</td>
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<td>150.5</td>
<td>100</td>
<td>42</td>
</tr>
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<td>GTX3</td>
<td>41.1</td>
<td>97.5</td>
<td>15</td>
</tr>
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<td>18.5</td>
<td>150</td>
<td>99</td>
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<td>GTX5</td>
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<td>110</td>
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<td>GTX6</td>
<td></td>
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<td>466.4</td>
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**Figure S3.4.** Extracted LC-HRMS chromatogram showing full-scan mass range of m/z 351.0729 ± 0.5 ppm extracted from NRC certified reference materials for dcGTX 2 and 3 (A) and *Microseira wollei* from Butterfield Lake (B).
Figure S3.5. Full scan (A and C) and MS/MS (B and D) HRMS spectra of dcGTX2 from NRC certified reference materials for dcGTX2 and 3 (A and B) and *Microseira wollei* from Butterfield Lake (C and D).
Figure S3.6. Full scan A and C) and MS/MS (B and D) HRMS spectra of dcGTX3 from NRC certified reference materials for dcGTX2 and 3 (A and B) and *Microseira wolle* from Butterfield Lake (C and D).
**Figure S3.7.** Extracted LC-HRMS chromatogram showing full scan mass range of $m/z$ 377.0885 ± 0.5 ppm extracted from NRC certified reference materials for LWTX 1 (A) and sample 11 (B).
Figure S3.8. Full scan (A and C) and MS/MS (B and D) HRMS spectra of LWTX 1 from NRC certified reference materials for LWTX 1 (A and B) and sample 11 (C and D).
Figure S3.9. Extracted LC-HRMS chromatogram showing full scan mass range of m/z 257.1359 ± 0.5 ppm extracted from NRC certified reference materials for dcSTX (A) and sample 11 (B).
Figure S3.10. Full scan (A and C) and MS/MS (B and D) HRMS spectra of dcSTX from NRC certified reference materials for dcSTX (A and B) and Microseira wolleii from Butterfield Lake (C and D).
Figure S3.11. Extracted LC-HRMS chromatograms of LWTX 1 eluting at 23.91 min (A) and putative LWTX 5 eluting at 30.40 min (B) from and *Microseira wolleii* from Butterfield Lake. Positive ionization full scan (C) and MS/MS (D) of LWTX 5. Product ions detected are consistent with those previously reported by Foss et al [79].
Figure S3.12. Extracted LC-HRMS chromatograms of putative LWTX6 (A) at m/z 283.1513 ± 5 ppm from Microseira wollei from Butterfield Lake. Positive ionization full scan (B and D) and MS/MS (C and E) of putative LWTX 6 eluting at 29.27 min (B and C) and an unknown isomer eluting at 28.65 min (D and E). Product ions detected are consistent with those previously reported by Lajeunesse et al [32].
Figure S3.13. Extracted LC-HRMS chromatograms of putative LWTX 2 and LWTX3 (A) at \( m/z \) 393.0834 ± 5 ppm from sample 11. Negative ionization full scan (B and D) and MS/MS (C and E) of putative LWTX 2 eluting at 22.80 min (B and C) and LWTX 3 eluting at 24.29 min (D and E).
**Figure S3.14.** PCOX chromatogram showing the PSTs identified in Sample 12 with labels based on the authentic standards of marine PSTs, and the less common PST transitions, including the LWTXs, detected in this sample by LC-MS/MS method 2. Y-axis was normalized for clarity as the baselines were different between the analyses with and without oxidant. Peaks with retention times but no assigned PST variant were PSTs, but were not identified as there were no authentic standards to confirm their retention time.
Table S3.15. A summary of PSTs identified in samples by mass spectrometry.

<table>
<thead>
<tr>
<th>Sample</th>
<th>LC-MS/MS Method 1</th>
<th>LC-MS/MS Method 2</th>
<th>Method 3 HRMS Screen</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>GTX 5?</td>
<td></td>
<td>LWTX 1, LWTX 3, LWTX 5, LWTX 6</td>
</tr>
<tr>
<td>6</td>
<td>C1, C2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>GTX 2</td>
<td></td>
<td>LWTX 5</td>
</tr>
<tr>
<td>8</td>
<td>STX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>STX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>STX</td>
<td></td>
<td>LWTX 3, LWTX 5</td>
</tr>
<tr>
<td>11</td>
<td>STX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>dcGTX 2, dcGTX 3, GTX 2, GTX 3, GTX 5?</td>
<td>dcGTX 2, dcGTX 3, doSTX, dcSTX, LWTXs</td>
<td>dcGTX 2 dcGTX 3, LWTX 1, LWTX 2, LWTX 3, LWTX 5, LWTX 6, dcSTX*, 11OH-dcSTX (dcM2)*</td>
</tr>
</tbody>
</table>

*Not included in the total concentrations of PSTs for the sample.
Technical Note S3.16. Ionic compounds as part of the environmental matrix led to excessive peak tailing for certain PSTs, such as GTX 3 (but not GTX 2), after 300-500 injections. This could be mitigated with the use of guard cartridges that were swapped after 400 injections, however only the Ace 3µ guard cartridges were able to stop breakthrough of these matrix interferents into the column. Other cartridge types, including the Ace 5µ, would not retain the interferents, which would eventually contaminate the column, with this damage being irreversible.
**Table S3.17.** Sample information for PSTs measured in 12 samples by six analytical methods for PSTs.

<table>
<thead>
<tr>
<th>ESF Code</th>
<th>Sample</th>
<th>Lake</th>
<th>Date Collected</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-901</td>
<td>1</td>
<td>Cayuga Lake</td>
<td>7/18/2017</td>
<td>42.82897, -76.73415</td>
</tr>
<tr>
<td>17-993</td>
<td>2</td>
<td>Cayuga Lake</td>
<td>7/26/2017</td>
<td>42.538402, -76.552005</td>
</tr>
<tr>
<td>17-1024</td>
<td>3</td>
<td>Cayuga Lake</td>
<td>7/26/2017</td>
<td>Unknown</td>
</tr>
<tr>
<td>17-1395</td>
<td>4</td>
<td>Cayuga Lake</td>
<td>7/31/2017</td>
<td>42.50204, -76.52185</td>
</tr>
<tr>
<td>17-808</td>
<td>5</td>
<td>Chautauqua Lake</td>
<td>7/14/2017</td>
<td>42.152373, -79.387163</td>
</tr>
<tr>
<td>17-1070</td>
<td>6</td>
<td>Chautauqua Lake</td>
<td>7/31/2017</td>
<td>42.194033, -79.421216</td>
</tr>
<tr>
<td>17-773</td>
<td>7</td>
<td>Chautauqua Lake</td>
<td>7/13/2017</td>
<td>42.111575, -79.283107</td>
</tr>
<tr>
<td>17-911</td>
<td>8</td>
<td>Hadlock Lake</td>
<td>7/19/2017</td>
<td>43.432642, -73.568316</td>
</tr>
<tr>
<td>17-774</td>
<td>9</td>
<td>Hemlock Lake</td>
<td>7/13/2017</td>
<td>42.774013, -77.608845</td>
</tr>
<tr>
<td>17-608</td>
<td>10</td>
<td>Indian Lake</td>
<td>7/4/2017</td>
<td>41.368227, -73.891316</td>
</tr>
<tr>
<td>17-1448</td>
<td>11</td>
<td>Indian Lake</td>
<td>7/31/2017</td>
<td>41.368227, -73.891316</td>
</tr>
<tr>
<td>16-665</td>
<td>12</td>
<td>Lake Placid</td>
<td>7/11/2016</td>
<td>44.306636, -73.971484</td>
</tr>
</tbody>
</table>
Figure S3.18. Chromatogram showing retention time and separation of standards of PSTs analyzed by LC-MS/MS method 1. Molecular weights and transitions are showed in Table S3.1
Figure S3.19. Chromatogram showing the PSTs identified in Sample 11 as detected by LC-MS/MS method 1. Molecular weights and transitions are showed in Table S3.1
Figure S3.20. Chromatograms showing retention time and separation of standards of PSTs analyzed by LC-MS/MS method 2. Molecular weights and transitions are showed in Table S3.1
Occurrence and Distribution of Neurotoxins in New York State

4.1 Abstract

The neurotoxic anatoxins (ATXs), including anatoxin-a, homo-anatoxin, dihydro-anatoxin, paralytic shellfish poisoning toxins (PSTs), and hepatotoxic microcystins were surveyed in shoreline blooms in 245 New York lakes. All toxins were detected at high concentrations and in the case of the PSTs at higher frequencies than have previously been reported. The PSTs and ATXs were found in ~15% and ~10% of blooms (N = 1,882 and 3035), while in a number of lakes these toxins were far more prevalent and may have posed health risks comparable to those of the microcystins. The neurotoxins were detected in lakes much earlier in the year than the microcystins, and each of the toxin classes showed significant inter- and intra-year variation. The health risks from these toxins for recreational users and their potential to contaminate drinking water is discussed. The neurotoxins were widely distributed in New York lakes, and national regulatory guidelines will be important for the protection of lake users from exposure to these toxins.

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2To be submitted to Toxins as Smith, Z.J.; Boyer, G.L., Occurrence and Distribution of Neurotoxins in New York State.
4.2 Introduction:

Cyanobacterial blooms are a global phenomenon identified in more than 40 countries worldwide [1]. Cyanobacterial blooms occur in every state in the United States [2], and have garnered attention due to the recurring nature of blooms in priority waterbodies such as the Great Lakes. Blooms have occurred in Lake Erie [3], Green Bay, Lake Michigan [4], Saginaw and Sturgeon Bays, Lake Huron [5] and in the nearshore areas of Lake Ontario [6] and Lake Superior [7]. Blooms also occur in lakes in the surrounding states including but not limited to New York [8], Ohio [9], Pennsylvania [10], and Wisconsin [11]. Many of these cyanobacterial blooms produce cyanobacterial toxins, including microcystins (MCs), anatoxins (ATXs), and paralytic shellfish toxins (PSTs).

MCs are hepatotoxic peptides and are the primary focus of most cyanotoxin monitoring programs. There are over 250 known congeners of MC [12], most are identified by variable amino acids in the MC ring. Although congener profiles vary spatially and temporally, MC-LR, with leucine (L) and arginine (R) in the variable amino acid positions, are the most abundant congener in the United States [8,13,14], with other common congeners including MC-LA, -RR, -YR, and -LY. MCs are produced by a number of cyanobacterial genera, including Dolichospermum (basionym Anabaena), Anabaenopsis, Aphanocapsa, Arthrospira, Hapalosiphon, Microcystis, Nostoc, Oscillatoria, Planktothrix, Snowella, Synechocystis, and Woronichinia [5].
While the hepatotoxic MCs are the focus of most monitoring programs, cyanobacteria produce a number of other toxic compounds. Anatoxins are lethal neurotoxins known for their acute toxicity [15]. The first ATX derivative, anatoxin-a, was described in 1977 by Devlin et al. [16]. Since that time, a number of other ATX congeners have also been elucidated including homo-anatoxin [17], dihydro-anatoxin [18], dihydro-homoanatoxin and the anatoxin epoxides. ATX binds to nicotinic acetylcholine receptors (nAChR) [19] in both muscle and neuronal receptors [20] producing its toxic effects. ATXs are produced by members of the cyanobacterial genera Dolichospermum (basionym Anabaena), Cuspidothrix (basionym Aphanizomenon), Arthrospira, Cylindrospermum, Microcystis, Oscillatoria, Phormidium, Planktothrix, and Raphidiopsis (basionym Cylindrospermopsis) [5].

Paralytic shellfish poisoning toxins (PSTs) are a second class of algal neurotoxins which bind to the sodium channel of higher organisms [21–23]. Marine PSTs are produced by dinoflagellates, where the toxins can be accumulated by shellfish leading to significant human health problems. Saxitoxin (STX), the parent compound in the PST group, is one of the most toxic non-protein natural products [24], and because of its toxicity, shellfish are heavily monitored in the regions where PSTs-producing dinoflagellates are common. There are more than 60 known analogs of STX [25]. These STX analogs are often more common than STX in both freshwater and marine systems, but are generally believed to be less toxic than STX when measured by the mouse.
bioassay [26,27]. PSTs have been detected in the freshwaters of Australia [28,29], Brazil [30], United States (USA) [9,13,27,31–34], Canada [35], Germany [36], France [37], Portugal [38], Russia [39], and New Zealand [40]. Freshwater PSTs in these countries are produced by members of the genera *Cuspidothrix* (basionym *Aphanizomenon*), *Dolichospermum* (basionym *Anabaena*), *Microseira* (basionym *Lyngbya*), *Planktothrix*, *Raphidiopsis* (basionym *Cylindrospermopsis*), and *Scytonema*.

While MCs are universally included freshwater cyanobacterial surveys due to their widespread distribution and known toxicity, other toxins including ATXs and/or PSTs may or may not be included in these monitoring programs. The 2007 EPA National Lake Assessment Program evaluated the distribution of PSTs in 1161 lakes and reservoirs across the US, identifying only six lakes with saxitoxin concentrations greater than 0.2 µg STX eq./L. PSTs were identified in 4 lakes at concentrations between 0.02-0.2 µg STX eq./L in a more detailed assessment of toxin mixtures in 23 eutrophic midwestern lakes [14]. PSTs are widespread in Ohio, but their extent and the congeners detected remains poorly described [9]. These three prior US surveys used the STX-ELISA to detect PSTs. For the Ohio survey, samples containing PSTs greater than 0.15 µg STX eq./L as measured by STX-ELISA were also analyzed by LC-MS/MS [41].

In Germany, PSTs were identified in 34% of 29 lakes [42] and two of five lakes had PST producing strains of *Aphanizomenon* (*Cuspidothrix*) [36]. PSTs were identified in 14% of the samples from ten French lakes, but at concentrations less than 0.05 µg/L [43].
PSTs were found in New Zealand lakes examined between 2001-2004 using ELISA in 90% (38/42) water bodies, but again in low ng per liter concentrations [44]. This study selected only samples from lakes containing cyanobacterial genera known to produce PSTs.

Historical evaluations have shown ATXs to be more abundant than the PSTs. Anatoxin-a was detected in 25% of samples and in 29 and 80 German lakes in two separate surveys [42,45]. The highest concentration was 13.1 µg/L, although most concentrations were under 1.0 µg/L. Within the US, anatoxin-a was found in 7 of 23 (30%) of midwestern lakes, with mean and median concentrations of 1.6 and 0.16 µg/L [14]. A separate study of lakes across 11 states found 7% of samples contained anatoxin-a with median concentrations of 0.75 µg/L [46]. Recently, anatoxin-a, homo-anatoxin and dihydro-anatoxin were detected in streams in California, USA [47] and in New Zealand [48,49]. These toxins were produced by benthic cyanobacteria, with ATXs produced by benthic cyanobacteria having been implicated in the deaths of dogs in New Zealand [50], France [51] and the United States [52]. There was concern that the constant release of ATXs from the benthos into streams used for drinking water may pose a chronic risk to consumers [53].

The occurrence and distribution of ATX derivatives other than parent compound anatoxin-a has not been widely assessed. Anatoxins are commonly quantified using LC-MS/MS techniques, such as EPA method 545 [54], which targets a single congener.
Similarly, most PST analysis has used an STX-ELISA, which has poor cross-reactivity to congeners other than STX [55], or LC-MS/MS which targets the common PSTs found in marine systems. Freshwater PSTs have a very different congener profile as exemplified by the discovery of the lyngbyatoxins (LWTXs) [27] from *Microseira wollei* (basionym *Lyngbya wollei*) [56].

There have been only a few surveys evaluating the distribution of cyanobacteria neurotoxins in lakes containing chronic cyanobacterial blooms in New York state. Here we evaluated the statewide occurrence and temporal distributions of anatoxin-a and its two derivatives, dihydro-anatoxin and homo-anatoxin. Paralytic shellfish poisoning toxins (PSTs) were analyzed using a non-targeted screening method for PSTs that relied on the conversion of tricyclic PST ring system into a fluorescent derivative [57]. This oxidation approach was applicable to a wide range of PSTs, and allowed for the detection of PST congeners where standards were not readily available [33]. A comparison of this oxidation approach versus the STX-ELISA, receptor binding assay, and mass spectrometry for the detection of freshwater PSTs was presented in Chapter 3. Additionally, we evaluated the association of ATXs and PSTs with the occurrence of shoreline blooms and selected cyanobacterial genera.
4.3 Methods

4.3.1 Sample Collection, Chlorophyll and Visual Analysis, and Toxin Extraction

Samples were collected from 245 lakes and water-bodies in New York State between 2016-2018 through the Citizen State-wide Lake Assessment Program (CSLAP). Scums and other algal material from shoreline blooms was collected by volunteers following the instructions prescribed by the CSLAP program [58]. Samples were shipped overnight to the lab where 100 mL aliquots were immediately lyophilized to dryness. Total chlorophyll and cyanobacterial chlorophyll were measured using a FluoroProbe (bbe Moldaenke, Schwentinental, Germany). Major cyanobacteria species were qualitatively identified in a 500 µL aliquot using an inverted microscope at 50-200x. Separate whole water samples from Sodus Bay and Lake Neatahwanta were collected in 1 L bottles, stored on ice during transport to the lab, and immediately lyophilized. Samples from Sodus Bay were collected weekly from Oak Park Marina, Katlynn Harbor, and the Public Beach as detailed in Perri et al. [59]. Samples were collected weekly from a pier extended 60 m into Lake Neatahwanta (43°18'48.9”N 76°25'46.7”W).

4.3.2 Toxin Extraction and Analysis

Microcystins, ATXs, and PSTs were extracted from freeze dried material using 10 mL of 50% methanol containing 1% acetic acid (v/v) followed by sonication (3 × 20 s at
32 watts). The resulting slurry was centrifuged at 15,000× g for 10 min, passed through a 0.45 µm nylon syringe filter, and kept at -20 °C until analysis.

MCs were analyzed by LC-MS as described in Tang et al. [62]. The microcystin-LR instrument LOD was 4 ng, while method LODs ranged between 0.10-1 µg/L, and the response linear between 1.2 and 250 ng oc which corresponded to 7.2-2065 µg MC-LR/L in a lake based on a 100 mL sample (slope, 6×10^6; y int, 4.0×10^6; R², 0.99).

Anatoxin-a was analyzed by LC-MS/MS using a modified version of EPA method 545 that included one quantification and two confirmation ions for each toxin [54], while homo-anatoxin and dihydro-anatoxin were also analyzed in the same analysis using one quantification and two confirmation ions for each compound (Table S4.1). Anatoxin-a was purchased from BioMol (Biomol GmbH, Hamburg, Germany), homo-anatoxin-a was purchased from Abraxis (Abraxis LLC, Warminster, PA, USA), and dihydro-anatoxin was obtained from BioMol and also prepared by sodium borohydride reduction of anatoxin-a as described in Yang [60]. The structures of the three compounds were confirmed by NMR spectrometry prior to use. Anatoxin-a standards were calibrated gravimetrically. Dihydro-anatoxin and homo-anatoxin were in limited supply, so quantification of these compounds was performed using response factors relative to the response of anatoxin-a using the transitions in Table S4.1 [61]. Homo-anatoxin and dihydro-anatoxin were calibrated in MS1 mode scanning molecular weights between 100-450 Da, using extracted ions at 180.1 and 168.2 Da for
homo-anatoxin and dihydro-anatoxin, respectively, using anatoxin-a as the calibrant. This calibration assumed that the ionization of the derivatives was similar to anatoxin-a. The response factors for the homo-anatoxin and dihydro-anatoxin were determined for the 180.0 → 163.1 and 168.0 → 43.1 transitions for the two anatoxin derivatives, with relative response factors calculated relative to anatoxin-a. The relative response factors were determined to be 2.74, 4.14, and 0.55 for the quantification of α-dihydro-anatoxin, β-dihydro-anatoxin, and homo-anatoxin relative to anatoxin-a by LC-MS/MS. Dihydro-anatoxin concentrations were reported as a sum of the α and β congeners.

Quantification was performed using a linear regression of anatoxin-a with an anatoxin-a standard every 15 samples to verify the stability of the response. Instrument LODs for anatoxin-a, homo-anatoxin, α-dihydro-anatoxin, and β-dihydro-anatoxin were 2, 4, 0.6, and 0.2 pg on column. Corresponding method LODs ranged between 4-10 ng/L, 7-18 ng/L, 1-3 ng/L and 1-2 ng/L, respectively. Anatoxin-a response was linear between 5 ng and 48 ng oc, which corresponded to 0.96-73.0 µg anatoxin-a/L in a lake based on a 100 mL sample (slope, 8.6×10^4, y int, 4.0×10^4, R^2, 1.0).

PSTs were analyzed using the AOAC 2011.02 post-column chemical oxidation method modified for water samples and algal powders [62]. Separations were performed using a Waters Alliance 2695 solvent delivery system (Waters, Milford, MA, USA), and a Chromenta KB 3µ 150 x 4.6 mm column with an ACE (ACE Ltd., Aberdeen, Scotland, UK) 3µ guard cartridge assembly at a flow rate of 0.8 mL/min.
The solvent system was: (A), 2mM heptanesulfonate (Regis Technologies Inc., Morton Grove, IL, USA) in 10 mM ammonium phosphate adjusted to pH 7.1; (B), 500 mL 2 mM heptane sulfonate in 30 mM ammonium phosphate adjusted to pH 7.1 plus an additional 150 mL of acetonitrile [57]. The separation gradient was: 0% B for 0–3 min, 40% B for 3–5 min, 100% B for 5–13 min, and 100% B for 20 min, followed by equilibration of the column back to 0% B for 10.5 min. The injection volume was 25 µL.

Post-column oxidation of the PST ring used 9 mM periodic acid (Alfa Aesar, Ward Hill, MA, USA) in 50 mM potassium phosphate at pH 9 at a flow rate of 0.45 mL/min entering a 25m 0.25 mm i.d. reaction coil (1 mL total volume) maintained at 65 °C. Following the coil, 0.5 M acetic acid was added at a flow of 0.45 mL/min. PSTs were detected at 330 and 390 nm excitation and emission.

Because the detector response varied between days, we determined individual method limits of detection for each toxin and each analysis from their average daily response factors. To quantitate the PST toxins, primary PST standards were obtained from NRC Canada (Institute for Marine Biosciences, Halifax, Canada) and the United States Food and Drug Administration (FDA) (Silver Spring, MD, USA). The FDA STX was diluted 1:50 to a concentration of 4 µM prior to use. Other standards of saxitoxin, decarbamoylsaxitoxin, neosaxitoxin, decarbamoylneosaxitoxin, gonyautoxin 1, gonyautoxin 2, gonyautoxin 3, gonyautoxin 4, gonyautoxin 5, decarbamoylgonyautoxin 2, decarbamoylgonyautoxin 3, lyngbyatoxin 1, and C1+C2 were used to calculate
relative response factors for the different PSTs relative to STX. An STX standard was injected every 10 samples to ensure stability of the response, with PSTs quantified using a linear regression. Saxitoxin (STX) instrument LODs were 0.9 pg, while method LODs ranged between 1-2 µg STX/L. STX was linear between 9.9 to 198 ng on column which corresponded to 9.3 and 729.5 µg/L in lake water based on a 100 mL sample (slope, 4.23×10^4; y-int, -5.8×10^4; R^2, 0.99). To differentiate PSTs from naturally occurring fluorescent compounds, samples that contained any putative PST peak in the chromatogram were reanalyzed using water in place of the periodic acid oxidant in the post-column system. PST-related compounds were only fluorescent in the presence of the oxidant. Retention times for standards of GTX 3 and STX varied by less than 6% and 2% respectively over >1 year of analysis.

4.3.3 Statistical Analysis

All analyses were performed using R version 3.5.1. Statistical differences between cyanobacterial chlorophyll and toxins were determined using a one-way ANOVA and Tukey’s honestly significant difference (HSD) tests on natural log transformed cyanobacterial chlorophyll, MC, anatoxin-a, and PST concentrations. Transformed cyanobacterial chlorophyll concentrations had 1 added to each value to include samples containing 0 µg/L of cyanobacterial chlorophyll. Following transformation, the distribution of cyanobacterial chlorophyll concentrations was zero-
inflated (Figure S4.2), but the number of low and high outliers in each of the years was similar, while the sample sizes were sufficiently large for the test to remain robust. The use of the non-parametric Kruskal-Wallis test did not lead to any change in the post-hoc comparison conclusions. For the toxins, non-detect values were not included in statistical analyses, and following natural log transformation the residual distributions were normal or near-normal. Kruskal-Wallis analysis that included the samples containing no detectable toxin were not performed due to the number of zeroes for each toxin [63]. For anatoxin-a, 2014 was not included in an ANOVA as there was a change in the analytical method in that year. Regression statistics were determined from ordinary least squares linear regression models.

4.4 Results

4.4.1 Occurrence of Cyanobacteria Toxins in New York State

A summary of the concentrations of MCs, ATX, and PSTs detected in 3,035 bloom samples retrieved from 245 lakes between the years 2014-2018 are shown in Table 4.1. Concentrations of all toxins ranged by three orders of magnitude, from trace concentrations near the LOD for the respective methods for toxin quantification, up to thousands of µg toxin per liter (Figure 4.2). Mean and median concentration for each toxin by year are provided in Table 4.1. Each toxin had multiple extreme outliers where
toxin concentrations were orders of magnitude higher than the mean and median concentrations of toxin.

Concentrations of MCs were strongly correlated to the concentration of cyanobacterial chlorophyll in all of the years (Figure S4.3, Table 4.1, mean of all years, $R^2 = 0.36$). A similar correlation was not observed with the neurotoxins, with only anatoxin-a strongly correlated with cyanobacterial chlorophyll ($R^2 = 0.29$) in 2018 (Figures S4.4-S4.7). There was a weak relationship between anatoxin-a and cyanobacterial chlorophyll in 2015 ($R^2 = 0.11$), and for PSTs and cyanobacterial chlorophyll in 2017 ($R^2 = 0.04$) (Table 4.1). Unlike MCs, many samples containing high concentrations of ATXs or PSTs did not contain high concentrations of cyanobacterial chlorophyll, and conversely, dense cyanobacteria blooms were not consistently associated with high concentrations of neurotoxins.

MCs were the most common toxins in New York water bodies in all years, appearing in 20-40% of the collected samples depending on the year, with only 3 of the 30 most frequently sampled lakes (Figure 4.1) having no samples with detectable concentrations of MCs. Within individual lakes, up to 86% of the samples tested positive for MCs. Mean toxin concentrations were higher than median concentrations, driven by numerous samples with extreme concentrations of MCs (Table 4.1). Additionally, both mean and median concentrations of MCs across the state were above the 0.3 µg/L concentrations for drinking water [64] and 4 µg/L recreational
concentration [65] thresholds recommended by the New York State Department of Health. The number of bloom samples did not change greatly between years, nor were there more lakes containing toxin, but the number of toxic samples trended upwards from 2016. There was significantly less MC in 2017 than in other years, but the net difference in this year versus the others was small (Figure 4.2). Common MC congeners identified in New York included MC-LR, -RR, -YR, and -LA. Other congeners present were -H4YR, -LL, -mLR, -dLR, -LY, -mLA, -dLA, -WR, and -LF, but with few exceptions the contribution of these compounds to the total MC concentrations quantified in blooms was small.

Anatoxin-a concentrations were lower than MC concentrations, with fewer outliers resulting in mean and median anatoxin-a concentrations being closer to each other. The occurrence of anatoxin was consistent between years with the exception of 2014, where anatoxin-a was detected at low frequency. There were significant differences in the concentrations of anatoxin-a between years, although the differences were small with the exception of 2018 (Table 4.1, Figure S4.2).

Concentrations of dihydro-anatoxin, measured in 2016-2018, and homo-anatoxin, measured in 2017 and 2018, concentrations were more variable than anatoxin-a both within and between years. Dihydro-anatoxin occurred less often than anatoxin-a both number of samples and in number of lakes containing toxin. However, the concentrations of dihydro-anatoxin were often much higher than anatoxin-a. Homo-
anatoxin was detected more slightly more often (~5% of samples) than dihydro-anatoxin (~3-4% of samples), but still less frequently than anatoxin-a (~11% of samples) (Table 4.1). Unlike anatoxin-a, where detections were relatively consistent between years with the exception of 2014, the prevalence of homo-anatoxin was highly variable, with double the number of positive samples observed in 2017 as compared to 2018. When present, toxin concentrations for homo-anatoxin were often greater than anatoxin-a, with >50% of the samples containing greater than 1 µg/L of toxin (Figure 4.2).

The occurrence of PSTs was similar to anatoxin-a, and PSTs were found in a similar number of lakes. The concentrations of total PSTs were generally higher than anatoxin-a or its two congeners. Median PST concentrations were similar to median MC concentrations, but PSTs had lower mean concentrations as there were fewer blooms containing extreme levels of PSTs. Concentrations of PSTs in 2017 were significantly higher than those in 2016 and 2018 (Figure 4.2). Samples often contained many PST congeners, including the *Lyngbya wolfei* PSTs [27,33], that were not a part of the suite common marine PSTs (Chapter 3). One compound, an unknown PST co-eluting with GTX 3 (Chapter 3), was ubiquitous in many New York lakes.
4.4.2 Bloom Intensity and the Associated Cyanobacterial Genera in 2014-2018

Cyanobacterial chlorophyll concentrations varied widely between blooms within a year (Figure 4.3). Average cyanobacterial chlorophyll concentrations between years were significantly different between 2014 and the other years, including 2016 ($p < 0.0001$), 2017 ($p < 0.0001$), and 2018 ($p < 0.01$), while there was no difference in 2015 ($p = 0.11$). Post-hoc analysis identified cyanobacterial chlorophyll was similar between 2014 and 2015, 2016 and 2017, and 2017 and 2018. Average cyanobacterial chlorophyll concentrations were 5 to 10-fold lower in 2014 and 2015 than in other years, where concentrations of cyanobacterial chlorophyll increased in the years 2016-2018.

Mean and median cyanobacterial chlorophyll concentrations between 2014-2018 were 4580 and 31.5 µg/L respectively, with extreme blooms containing high chlorophyll concentrations responsible for the differences between the mean and median. Most blooms collected by the CSLAP program were dominated by cyanobacteria. The use of total chlorophyll rather than cyanobacterial chlorophyll did not change any of the qualitative or quantitative assessments of cyanobacteria abundance between years.

Of twelve common cyanobacteria genera known to produce MCs, ATX, and/or PSTs that were evaluated here [5], only the genera *Microcystis* and *Dolichospermum* were consistently found at high frequency (>50%) in New York blooms. *Cuspidothrix* (basionym *Aphanizomenon*) and *Woronichinia* were identified at higher frequencies for some years and locations, but still less than *Microcystis* and *Dolichospermum* (Figure 4.4).
Table 4.1. Total microcystins (MC), anatoxins, and total paralytic shellfish toxin (PST) concentrations in New York lakes between 2014-2018. MC concentrations (µg MC-LR eq./L) and PST concentrations (µg STX eq./L) are sum of all congeners. Anatoxin-a, dihydro-anatoxin, and homo-anatoxin concentrations are reported for the individual congeners in µg per L. Zero values where toxins were below the limit of detection were excluded in mean or median values. Variation in yearly toxin concentrations are shown as ±1 standard deviation of the mean. Symbols (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) on years represent the significance of linear correlations between natural log transformed toxin and natural log transformed cyanobacterial chlorophyll + 1.
<table>
<thead>
<tr>
<th>Toxin</th>
<th>Year</th>
<th>Lakes Containing Toxin</th>
<th>Samples Toxic/Samples Tested (%)</th>
<th>Range (µg/L)</th>
<th>Median (µg/L)</th>
<th>Mean (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Microcystin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>2014***</td>
<td>36</td>
<td>111/555 (20)</td>
<td>0.32 - 7,032</td>
<td>11.0</td>
<td>188 ± 762</td>
<td></td>
</tr>
<tr>
<td>2015***</td>
<td>43</td>
<td>143/598 (24)</td>
<td>0.15 - 18,720</td>
<td>24.8</td>
<td>335 ± 1,622</td>
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</tr>
<tr>
<td>2016***</td>
<td>54</td>
<td>200/523 (38)</td>
<td>0.02 - 4,299</td>
<td>12.0</td>
<td>186 ± 518</td>
<td></td>
</tr>
<tr>
<td>2017***</td>
<td>47</td>
<td>281/708 (40)</td>
<td>0.02 - 23,351</td>
<td>4.9</td>
<td>236 ± 1,495</td>
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<tr>
<td>2018***</td>
<td>52</td>
<td>261/651 (40)</td>
<td>0.21 - 3,167</td>
<td>13.3</td>
<td>133 ± 317</td>
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<tr>
<td><strong>Anatoxin-a</strong></td>
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<tr>
<td>2014</td>
<td>5</td>
<td>6/555 (1)</td>
<td>0.010 - 11.2</td>
<td>0.38</td>
<td>2.4 ± 4.4</td>
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<tr>
<td>2015*</td>
<td>22</td>
<td>58/598 (10)</td>
<td>0.002 - 79.2</td>
<td>0.30</td>
<td>2.3 ± 10.5</td>
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<tr>
<td>2016</td>
<td>31</td>
<td>103/523 (20)</td>
<td>0.016 - 32.7</td>
<td>0.64</td>
<td>2.1 ± 4.7</td>
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<tr>
<td>2017</td>
<td>28</td>
<td>87/708 (12)</td>
<td>0.001 - 5.5</td>
<td>0.092</td>
<td>0.41 ± 0.93</td>
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<tr>
<td>2018***</td>
<td>23</td>
<td>72/651 (11)</td>
<td>0.001 - 17.4</td>
<td>0.046</td>
<td>0.53 ± 2.3</td>
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<tr>
<td><strong>Dihydro-anatoxin</strong></td>
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<tr>
<td>2016</td>
<td>20</td>
<td>30/523 (6)</td>
<td>0.017 – 2213</td>
<td>0.89</td>
<td>148.9 ± 458</td>
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<tr>
<td>2017</td>
<td>8</td>
<td>13/708 (2)</td>
<td>0.009 - 9.6</td>
<td>0.19</td>
<td>1.2 ± 2.6</td>
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<tr>
<td>2018</td>
<td>12</td>
<td>17/651 (3)</td>
<td>0.004 - 388</td>
<td>0.25</td>
<td>27.3 ± 93.8</td>
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<tr>
<td><strong>Homo-anatoxin</strong></td>
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<tr>
<td>2017</td>
<td>21</td>
<td>52/708 (7)</td>
<td>0.001 - 575</td>
<td>0.51</td>
<td>14.0 ± 79.6</td>
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<tr>
<td>2018</td>
<td>14</td>
<td>23/651 (4)</td>
<td>0.004 - 24.0</td>
<td>0.40</td>
<td>2.5 ± 5.6</td>
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<tr>
<td><strong>Total Paralytic Shellfish Poisoning Toxins</strong></td>
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<tr>
<td>2016</td>
<td>36</td>
<td>101/523 (19)</td>
<td>0.38 – 396</td>
<td>8.1</td>
<td>22.1 ± 47.4</td>
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<tr>
<td>2017*</td>
<td>34</td>
<td>108/708 (15)</td>
<td>0.65 – 923</td>
<td>14.0</td>
<td>43.8 ± 123.4</td>
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<tr>
<td>2018</td>
<td>29</td>
<td>73/651 (11)</td>
<td>0.48 – 142</td>
<td>6.1</td>
<td>12.8 ± 21.3</td>
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<tr>
<td>Location</td>
<td>Percent Total Samples Containing Toxin</td>
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<td>Chautauqua Lake</td>
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<td>Sodus Bay</td>
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<td>Lake Neatiahwanta</td>
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<td>Honeoye Lake</td>
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<td>Skaneateles Lake</td>
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<td>Cayuga Lake</td>
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<td>Lake Mohegan</td>
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<td>Java Lake</td>
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<td>Otisco Lake</td>
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<td>Wallkill River</td>
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<td>Lawson Lake</td>
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</table>
Figure 4.1. Occurrence of microcystins, anatoxin-a, dihydro-anatoxin, homo-anatoxin, and paralytic shellfish toxins from 245 New York Lakes. Of the 245 lakes sampled, only the 30 lakes with the largest number of samples collected over the five-year period are shown for clarity. The number of samples for each of the lakes shown are in Table S4.8.
Figure 4.2. Box-and-whisker plots of anatoxin-a, dihydro-anatoxin (DHATX), homo-anatoxin (HTX), and paralytic shellfish poisoning toxins (PSTs) across New York lakes over the years 2014-2018. Dihydro-anatoxin (DHATX) and PSTs were not analyzed in samples collected in 2014 and 2015, while HTX was not analyzed in samples collected in 2014, 2015, and 2016. Letters refer to the groupings evaluated by Tukey’s honestly significant differences test on log transformed toxin concentrations for anatoxin-a, MCs, and PSTs. Upper and lower bounds of the boxes are the 25th and 75th percentiles, while the bar represents the mean. Whisker lengths are 1.5× the distance between the 25th and 75th percentiles, with any samples containing toxins outside this range are shown as points.
Microcystis and Dolichospermum frequently co-occurred in the same samples, with 50-70% of blooms containing both genera (Table 4.2). Microcystis abundance relative to Dolichospermum increased in 2017 and 2018. Microcystis was also found more frequently without Dolichospermum in 2017 and 2018, while blooms containing Dolichospermum with co-occurring Microcystis increased at the same time.

Other potential toxin-producing genera in New York included Oscillatoria, Microseira (basionym Lyngbya), Phormidium, Nostoc, Raphidiopsis (basionym Cylindrospermopsis), and Anabaena. These were infrequently identified in blooms during this 5-year period (data not shown). When these genera were detected, they likely originated from filaments or material that may have detached from benthic substrate. Raphidiopsis, a potential cylindrospermopsin producer, was rarely detected in New York (<10 samples), as was the toxin.

4.4.3 Association of Cyanobacterial Genera with Cyanobacterial Toxins

To examine which genera were associated with toxin production, we examined the association of cyanobacteria genera with the occurrence of toxins (Figure 4.5). MCs were detected in samples with Microcystis and Dolichospermum in 76 and 49% of samples, respectively. The simple presence of either of the genera Microcystis or Dolichospermum
Figure 4.3. Box and whisker showing concentrations of cyanobacterial chlorophyll measured in blooms collected during each year plotted on a log scale. Letters refer to the groupings evaluated by Tukey’s honestly significant differences test on natural log transformed cyanobacterial chlorophyll (not shown). Upper and lower bounds of the boxes are the 25th and 75th percentiles, while the bar represents the mean. Whisker lengths are 1.5× the distance between the 25th and 75th percentiles, with any samples containing toxins outside this range are shown as points.
**Figure 4.4.** Numerical occurrence of five different cyanobacterial genera in all bloom samples collected in the years 2014-2018. The lower black bars represent the number of samples containing the cyanobacterium in the specific year, while the upper grey bars represent the number of samples without the cyanobacterium. Other cyanobacteria genera evaluated but not included in the plot were *Oscillatoria, Microseira* (basionym *Lyngbya*), *Phormidium, Nostoc, Raphidiopsis* (basionym *Cylindrospermopsis*), and *Anabaena*. 
did not predict toxicity, with 50 and 61% of samples containing one or both of these, genera, respectively containing no detectable MCs (data not shown). MCs were found co-occurring with Microcystis at rates well above the overall occurrence of Microcystis in

Table 4.2. Co-occurrence of Microcystis and Dolichospermum genera in New York Blooms.

<table>
<thead>
<tr>
<th>Year</th>
<th># of Samples with co-occurring Dolichospermum and Microcystis</th>
<th>Total Microcystis Samples (Percent Co-Occurrence with Dolichospermum)</th>
<th>Total Dolichospermum Samples (Percent Co-Occurrence with Microcystis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2014</td>
<td>144</td>
<td>262 (55%)</td>
<td>252 (57%)</td>
</tr>
<tr>
<td>2015</td>
<td>182</td>
<td>310 (59%)</td>
<td>287 (63%)</td>
</tr>
<tr>
<td>2016</td>
<td>158</td>
<td>256 (62%)</td>
<td>223 (71%)</td>
</tr>
<tr>
<td>2017</td>
<td>170</td>
<td>340 (50%)</td>
<td>249 (68%)</td>
</tr>
<tr>
<td>2018</td>
<td>174</td>
<td>348 (50%)</td>
<td>257 (68%)</td>
</tr>
</tbody>
</table>

blooms, with Microcystis likely to be a major producer of MCs in New York. However, Dolichospermum co-occurred with Microcystis 57-71% of the time, and therefore Dolichospermum may also be an important MC producer in New York (Table 4.2). PSTs co-occurred in the presence of Microcystis or Dolichospermum 69% and 62% of the time.
This was similar to the occurrence of MCs with these two genera. The presence of these genera did not predict the presence of PSTs.

The presence of anatoxin-a was not related to any genera, with the highest occurrence in blooms with *Microcystis* and *Dolichospermum* (43% and 39% respectively), similar to the overall occurrence for these two genera in all samples. Nor was the presence of dihydro-anatoxin and homo-anatoxin associated with any particular genera.

*Microcystis* and *Dolichospermum* occurred without any detectable toxins other than MCs >70% of the time. Ten other cyanobacteria genera were evaluated for their co-occurrence with cyanobacteria toxins, none appeared to be closely associated with any of the toxins.

### 4.4.4 Temporal Evaluation of Cyanotoxins in Five Lake

Five lakes or embayments were selected for detailed examination of the occurrences of MCs, ATXs, and PSTs over the growing season and in-between years (Figures 4.6-7, Figures S4.9-11). There were important differences between the lakes, with different toxins, months when these toxins were identified, and
**Figure 4.5** Numerical occurrence of five different cyanobacterial toxins with different cyanobacterial genera. Y-axis shows the sum of samples collected over 2014-2018 containing the specific genera. Lower black bars represent a sample containing the toxin and the cyanobacterium, while the upper grey bars are the proportion of samples containing the cyanobacterium but not the toxin. Other cyanobacteria genera evaluated but not included in the plot were *Oscillatoria*, *Microseira* (basionym *Lyngbya*), *Phormidium*, *Nostoc*, *Raphidiopsis* (basionym *Cylindrospermopsis*), and *Anabaena*. 
in the abundance of these toxins. As the different toxins have different recreational and drinking water thresholds, the hazards from exposure to toxin(s) varied between the five lakes.

Blooms in Sodus Bay had concentrations of MCs exceeding the recommended children’s drinking water threshold of 0.3 µg/L [66] only in 2016. The other four lakes, had higher concentrations of MCs exceeding the 0.3 µg/L drinking water threshold and, in many cases, exceeding the 4 µg/L shoreline bloom recreational threshold used in New York [65] and the 8 µg/L recreational guidance for recreational contact set by the EPA [67].

The neurotoxic PSTs were detected frequently in four of the five lakes, where Findley Lake had only one bloom where PSTs were detected. Many blooms contained PSTs above the proposed 3 µg STX eq./L drinking water threshold for PSTs used several countries [68,69], although few exceeded the 75 µg STX eq./L recreational threshold guideline for PSTs set by Washington State [70].

Anatoxin (ATX) concentrations were much lower than PST concentrations in the five lakes. The highest concentrations of anatoxins were above or near the common drinking water thresholds (6-20 µg/L), while far more blooms exceeded the lower drinking water guidelines of 1-3 µg/L [68]. Only Owasco Lake had appreciable concentrations of the ATX derivative, homo-anatoxin. In 2017, Owasco Lake blooms regularly exceeded the 2 µg/L guideline set by New Zealand for this toxin [71].
Temporal variation of the MCs and PSTs within years tended to be similar in these five lakes. MCs frequently occurred in the late-summer and fall, whereas PSTs often appeared earlier in the summer and remained throughout the growing season. Anatoxins (ATXs) were detected in four of the five lakes, where ATX detections in three of the lakes started in July and August, while ATXs in Owasco Lake were observed starting in September.

Inter-year variation of toxins within these five lakes was high. In three of the lakes, MCs were only detected in some of the five years, while in the other two MCs were quite common but the concentrations could vary by several orders of magnitude between years. PSTs were similar to MCs in these two latter lakes, where they were detected in all years in Owasco Lake, Cayuga Lake, and Chautauqua Lake, but with a wide range of toxin concentrations. In Sodus Bay and Findley Lake, the opposite was true, where PSTs were only detected in 2018 and 2017 respectively. In Sodus Bay, most ATX detected was associated with 2016, while in Owasco Lake, there were high concentrations of homo-anatoxin in 2017, but little homo-anatoxin was detected in 2018. Similarly, 70% of all samples positive for anatoxin-a in Chautauqua Lake were collected in 2016.
Figure 4.6. Yearly and temporal concentrations of microcystins, anatoxins, and paralytic shellfish toxins in Sodus Bay, New York. Homo-anatoxin and dihydro-anatoxin are shown grey-filled, while anatoxin-a is shown as open symbols.
**Figure 4.7.** Yearly and temporal concentrations of microcystins, anatoxins, and paralytic shellfish toxins in Owasco Lake, New York. Homo-anatoxin and dihydro-anatoxin are shown grey-filled, while anatoxin-a is shown as open symbols.
4.5 Discussion

Microcystins (MCs) are the focus of most state-wide monitoring efforts. This is due in part the clear guidelines for drinking water supplies and recreation issued by the US-EPA [66]. Cyanobacterial blooms in New York contained a number of cyanotoxins other than the MCs, often at concentrations of concern. The neurotoxic anatoxins, including anatoxin-a, dihydro-anatoxin, and homo-anatoxin, and paralytic shellfish poisoning toxins (PSTs), including saxitoxin (STX) and related derivatives, were all detected in multiple lakes located throughout New York.

There are significant gaps in our understanding of the human health risks posed by both ATXs and freshwater PSTs. The US-EPA, in determining the toxicological information needed to issue the guideline values for MCs and cylindrospermopsin also evaluated the toxicological information for anatoxin-a. They determined the available toxicological data were insufficient to issue a guidance value for anatoxin-a at that time [72]. Similar conclusions were reached in Canada and by the Cawthron Institute of New Zealand [73,74]. Missing information needed for the establishing a short-term oral reference dose included long-term chronic studies, especially those investigating neurotoxicity and late stage neuropathy. Limiting toxicological studies for the ATXs was the lack of readily available toxin in quantities and/or purity for large scale exposure studies. While synthetic anatoxin-a could be used for these studies rather than
purified toxin, the expense associated with the synthesis of the compound in the amounts required was prohibitive.

While toxicological information for ATXs is limited, drinking water and recreational guidelines for ATXs have been established (Table 4.3). Many of the guidelines for ATX in drinking water are based upon a limited set of toxicological studies [75]. These same set of toxicology studies has produced a range of drinking water and recreational thresholds, which has led to a state of regulatory confusion (Table 4.1). New York anatoxin-a concentrations frequently exceeded the 0.1 µg/L guideline established in Minnesota, while anatoxin-a exceedances above 1 µg/L were less common. It is unclear which guideline is most appropriate, and by extension what level of risk is posed by ATXs in New York.

Compounding the issuing of an appropriate guidance value is the lack of information about the chronic toxicity of anatoxin-a [73]. Anatoxins (ATXs) have been implicated in the deaths of dogs and other livestock globally [76]. Anatoxins (ATXs) may also be constantly released in New Zealand streams [53], which could chronically pollute drinking water. This has spurred recognition that assessment of health effects from chronic exposure to ATXs are needed. Anatoxin-a induces conformational changes in nAChR receptors, changing the binding affinity of other ligands to the channel, although there is no evidence that
Table 4.3. Drinking water and recreational exposure guidelines for anatoxins and paralytic shellfish poisoning toxins (PSTs). All values are reported in µg/L, with PST limits reported in µg STX eq./L.

<table>
<thead>
<tr>
<th>Source</th>
<th>Drinking Water Exposure</th>
<th>Recreational (Short Term) Exposure</th>
<th>Recreational (Subchronic) Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>California (anatoxin-a) [77]</td>
<td>-</td>
<td>90</td>
<td>-</td>
</tr>
<tr>
<td>Minnesota (anatoxin-a) [78]</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>New Zealand (anatoxin-a) [71]</td>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>New Zealand (homo-anatoxin) [71]</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ohio (anatoxin-a) [41]</td>
<td>20</td>
<td>300</td>
<td>-</td>
</tr>
<tr>
<td>Oregon (anatoxin-a) [79]</td>
<td>3</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>Quebec (anatoxin-a) [80]</td>
<td>3.7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Washington State (anatoxin-a) [81]</td>
<td>1</td>
<td>450</td>
<td>75</td>
</tr>
<tr>
<td>Australia (PSTs) [82]</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Brazil (PSTs) [83]</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>New Zealand (PSTs) [71]</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Oregon (PSTs) [79]</td>
<td>1</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Ohio (PSTs) [41]</td>
<td>0.3</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Washington State (PSTs) [70]</td>
<td>3</td>
<td>75</td>
<td>-</td>
</tr>
</tbody>
</table>
these changes are retained and would lead to chronic neurological damage [19]. In a similar vein, it remains unclear whether exposure to chronic or sub-lethal anatoxin-a concentrations would lead to behavioral and/or chemical changes in higher organisms [76].

While anatoxin-a was the most common ATX congener in New York waters, the ATX derivatives homo-anatoxin and dihydro-anatoxin were detected less frequently than anatoxin-a but at higher concentrations. Homo-anatoxin activity and toxicity has been compared to anatoxin-a in biological and biochemical assays. Homo-anatoxin bound neuronal nAChR sites with the same affinity as anatoxin-a in potency assays as a nicotinic agonist, however, homo-anatoxin was ~10-fold less effective nicotinic agonist than anatoxin-a [84]. Anatoxin-a and homo-anatoxin had similar effects on phrenic nerve-diaphragm preparations [17] and similar LD-50’s in mice as measured by intraperitoneal injection [85]. The combinatorial evidence from these studies suggests that homo-anatoxin has a toxicity similar to anatoxin-a. The New Zealand Ministry of Health established a permissible limit for homo-anatoxin in drinking water 2 µg/L [71]. This homo-anatoxin regulatory limit was lower than anatoxin-a due to an additional 5-fold safety factor relative to anatoxin-a due to database uncertainties [86].

The toxicological evidence regarding dihydro-anatoxin toxicity is less clear. Dihydro-anatoxin was non-lethal in cyanobacterial extracts evaluated by bioassay [18,87]. In contrast, synthetic dihydro-anatoxin was highly potent as measured by the
mouse bioassay by Bates and Rapoport, although it was 12.5 fold less toxic than
anatoxin-a [85]. An in vitro assay of dihydro-anatoxins’ contracture potency by Swanson
et al. found 10-fold reduced effect compared to anatoxin-a [88]. These studies suggest
dihydro-anatoxin is less toxic than anatoxin-a. However, there is significant uncertainty
regarding the toxicity of this derivative, and whether dihydro-anatoxin is a risk to
environmental health. No exposure guideline values have been established for dihydro-
anatoxin.

Saxitoxins (STXs) have been intensively studied in marine systems since the mid-
1960s due to their association with paralytic shellfish poisoning. However, the route of
exposure from contaminated shellfish (consumption following cooking) is different
from PST exposures from contaminated drinking water. The different toxin congener
distributions in marine versus freshwater systems further complicates the comparison.

The acute health risks from marine PSTs by consumption of contaminated
shellfish is established [89–92]. Regulatory limits for total PSTs in shellfish are 80 µg
saxitoxin eq./100g of shellfish tissue. These limits are widely adopted in the European
Union, Australia, Canada, and the USA [93] and have been critical in reducing the
incidence of illness caused by the consumption of contaminated shellfish.

There are no universal exposure limits for PSTs in freshwater for drinking water
or recreational contact. The shellfish regulatory guideline and the guidelines established
in Table 4.3 are derived from the European Food Safety Authority (EFSA) reference
dose for STX of 0.5 µg STX eq./kg body weight. This reference dose is for acute exposure, based off of human exposure PSTs and bioassays, although this dose has been re-evaluated, as it may be under-protective of human health. The minimum dose for 10% of consumers to experience symptoms of toxin exposure was determined to be 0.37 µg STX eq./kg body weight [93], far lower than the original measured lowest observed effect level (LOEL) of 1.5 µg STX eq./kg body weight dose recommended by the EFSA [94]. The no observed effect level (NOEL) reference dose (0.5 µg STX eq./kg) was estimated by dividing the LOEL of by 3. Most safety regulations use this NOEL reference dose. A reevaluation of the LOEL finding it to be five times lower than its original estimate suggests that drinking water and recreational guidelines for PSTs may require revision.

Unlike acute poisonings of PSTs, effects from long-term exposure to PSTs are unclear. Toxicological studies of PSTs investigating chronic exposure are limited, with current chronic drinking water guidelines based on acute effects of PST intoxication. In cases of acute exposure, symptoms resolve within a short time frame [95] and the majority of nerve function returns within weeks [96,97]. However, long-term low-level exposure to PSTs may produce different physiological effects. Further evaluations of the chronic effects of exposure to PSTs is warranted.

Compounding the issuing of an acceptable guidance value — most toxicological studies have used the parent compound STX. There is less information for the toxicity
of other PST congeners. There is a wide range of in toxicity between the different toxin congeners as measured by the mouse bioassay [25], and molar toxicity of different PST congeners is expressed in STX equivalents [26]. Three freshwater LWTX congeners showed no toxicity after intraperitoneal injection in the mouse bioassay [27]. The high concentrations and abundance of these congeners complicates the evaluation of the health effects from PSTs in freshwater systems [27].

PCOX reports not toxicity but the sum of all of the fluorescent PST derivatives identified by the analysis. Analysis of a subset of samples by the receptor-binding assay suggested that for most samples, PCOX overestimated the actual toxicity (Chapter 3). We have limited understanding on the metabolism of these toxins, and there is potential for interconversion of congeners in the environment and after consumption, therefore an overestimate of toxicity better protects human health.

The neurotoxins showed different temporal variation than the MCs. This poses additional concerns for management. PSTs occurred in New York lakes in June and July, several weeks earlier than the MCs, which are detected in late summer and fall (Figure 4.6-7, Figures S4.9-11). Microcystin (MC) producers such as *Microcystis* proliferate in late summer and fall. PST producers such as *Microseira wollei* produce PSTs throughout the year [32,33,98]. *Dolichospermum circinalis* blooms produced PSTs early in the spring growing season [28,99], and the PST producing *Aphanizomenon* strain NH-5 from New Hampshire was collected early in the season in July [31]. PSTs in New
York were continuously detected from early to late into the season, although concentrations were often lower in samples collected earlier in the season.

Inter-year heterogeneity makes it challenging to determine whether ATXs are a public health risk. Ohio has limited monitoring for anatoxin-a due to historically low anatoxin-a occurrence [41,100]; however inter-year variation could lead to limited anatoxin-a detections or reduced concentrations in some years. Additionally, anatoxin-a did not necessarily co-occur with homo-anatoxin or dihydro-anatoxin (Figure 4.7).

Anatoxin (ATX) congeners such as epoxy-anatoxin [101] and epoxy-homoanatoxin [87], dihydro-homoanatoxin [53], 4-hydroxyhomo-anatoxin [102] may also be present, but their distributions have only been assessed in lotic systems [53,103,104]. These other ATX congeners should be incorporated into freshwater lake monitoring programs as they may also pose an unknown public health risk [101].

*Microcystis* and *Dolichospermum* were observed to dominate many of the blooms in New York. *Microcystis* can contain the genes for MC production within the state [105], but *Dolichospermum* currently has not been identified as containing the *mcy* gene cluster (unpublished). *Microcystis* likely produces microcystins in New York, as this genera frequently co-occurred with *Microcystis*. However, the occurrence of toxins with specific genera does not mean that a genera was producing toxins. Although PSTs co-occurred frequently with *Dolichospermum* or *Microcystis*, there is no evidence that these two genera were also producing PSTs, only that PSTs co-occurred frequently with
the two genera. Importantly, the presence of one or both genera did not indicate toxicity, with 50% or more blooms containing either *Dolichospermum* or *Microcystis* containing no toxins, while the presence or absence of the cyanobacteria genera alone did not provide useful information about the risks from toxin exposure.

Benthic cyanobacteria also produce cyanobacterial toxins, and these toxins may contribute to those detected in shoreline composite samples. Production and release of ATXs by benthic cyanobacteria may contribute to some of the ATXs observed in New York waters. PSTs were produced by benthic cyanobacteria in Butterfield Lake [33], the St. Lawrence River [35], Indian Lake (Chapter 3), Lake Erie (unpublished), and in other New York lakes [106]. The distribution of benthic cyanobacteria and their toxins should be assessed on a wider scale in New York.

The choice of sampling site has a major impact on detection of cyanobacterial toxins. Lake monitoring programs such as the New York CSLAP [8,107,108] and the EPA National Lakes Assessment monitoring programs [13] traditionally used a limited number of open water sites sampled on regular intervals to assess water quality. Several states now combine this traditional spot sampling [109] with remote sensing to detect blooms [100]. Sample designs focused on mid-lake sites heavily underestimated toxin prevalence in New York, as shoreline samples contained far more toxin and may give a better indication of the recreational exposure risk. Limited temporal sampling designs may miss the heterogeneity in bloom density, and toxin presence and abundance.
Extrapolation of toxin concentrations from particular time points to other locations, or to estimate future toxin concentrations, is not recommended due to significant intra- and inter- year variation [110].

This natural variability presents a problem for assessing water quality and the risks posed from cyanobacteria. Toxin concentrations changed quickly, and determining when and where to sample presented a problem. Assessment criteria often uses indirect measurements such as cyanobacterial chlorophyll concentrations [79,111] or biovolume [100,112] to assess the potential for toxin occurrence. These approaches protect from exposure to microcystin toxins where bloom density was correlated with toxin concentrations (Table 4.1). However, neither the ATXs or PSTs correlated with cyanobacterial chlorophyll, and the majority of blooms in New York had no detectable levels of cyanotoxin. Biovolume and/or chlorophyll concentrations heavily overestimated the potential of exposure to cyanotoxins. Even when toxins were present, cyanobacterial chlorophyll was only weakly related to toxin concentrations (Figures S4.3-S4.7). The historical presence of a toxin in a given waterbody may give an indication of the toxins which may be present. However, only direct measurements of toxin can accurately assess their concentrations.

Improvements in the analytical methodologies are critical in the assessment of ATXs and PSTs. Currently, most freshwater monitoring programs only include anatoxin-a in their analytical methods. Homo-anatoxin likely poses an equal or greater
threat to human health and should be included into analytical methods such as EPA method 545 as well as further incorporation into cyanotoxin monitoring programs, [54]. Similarly, LC-MS/MS methods have gained popularity as a method for marine PSTs. These methods include only a limited number of freshwater congeners. These improvements are needed to implement effective monitoring programs for the neurotoxic PSTs and ATXs in freshwater ecosystems.

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Supplementary Information

Table S4.1. Multiple reaction monitoring (MRM) transitions used for the detection of anatoxin, homo-anatoxin, and dihydro-anatoxin.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Quantitation Transition</th>
<th>Confirmation Transitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anatoxin-a</td>
<td>166.0 → 131.0</td>
<td>166.0 → 148.0; 166.0 → 90.90</td>
</tr>
<tr>
<td>Homo-anatoxin</td>
<td>180.0 → 163.1</td>
<td>180.0 → 145.1; 180.0 → 105.0</td>
</tr>
<tr>
<td>Dihydro-anatoxin</td>
<td>168.0 → 43.1</td>
<td>168.0 → 55.9, 168.0 → 67.0</td>
</tr>
</tbody>
</table>
Figure S4.2. Histogram of natural log transformed cyanobacterial chlorophyll concentrations.
Figure S4.3. Relationship between natural log cyanobacterial chlorophyll and natural log total microcystins. Summary statistics of regressions from each year 2014-2018: $R^2$, 0.38, 0.49, 0.18, -1.15, 0.37; slope, 0.48, 0.65, 0.44, 0.62, 0.61; y-int, -0.63, -0.71, 0.33, 0.39, -0.54.
Figure S4.4. Relationship between natural log cyanobacterial chlorophyll and natural log anatoxin-a. Summary statistics of regressions from each year 2014-2018: $R^2$, 0.0, 0.11, 0.0, 0.0, 0.29; slope, -0.01, 0.35, 0.06, 0.0, 0.35; y-int, -1.23, -2.48, -0.70, -1.87, -4.0.
Figure S4.5. Relationship between natural log cyanobacterial chlorophyll and natural log dihydro-anatoxin. Summary statistics of regressions from each year 2016-2018: $R^2$, 0.0, 0.0, 0.19; slope, -0.03, -0.02, 0.38; y-int, 0.89, -0.98, -1.88.
Figure S4.6. Relationship between natural log cyanobacterial chlorophyll and natural log homo-anatoxin. Summary statistics of regressions from each year 2017-2018: $R^2$, 0.0, 0.11; slope, 0.04, 0.19; y-int, -0.33, -2.1.
Figure S4.7. Relationship between natural log cyanobacterial chlorophyll and natural log total paralytic shellfish toxins. Summary statistics of regressions from each year 2016-2018: $R^2$, 0.0, 0.04, 0.0; slope, 0.03, 0.13, 0.03; y-int, 1.92, 1.68, 1.58.
Table S4.8. Total number of samples (N) collected for each lake shown in Figure 4.1.

Samples from 2014-2018 were used to determine the percent occurrence for microcystins and anatoxin-a; samples from 2016-2018 were used for paralytic shellfish poisoning toxins and dihydro-anatoxin; samples from 2017-2018 were used for homo-anatoxin.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Chautauqua Lake</td>
<td>803</td>
<td>524</td>
<td>396</td>
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<td>Sodus Bay</td>
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<td>176</td>
<td>135</td>
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<tr>
<td>Lake Neatahwanta</td>
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<td>Lawson Lake</td>
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Figure S4.9. Yearly and temporal concentrations of microcystins, anatoxins, and paralytic shellfish toxins in Cayuga Lake, New York. Homo-anatoxin and dihydro-anatoxin are shown grey-filled, while anatoxin-a is shown as open symbols.
Figure S4.10. Yearly and temporal concentrations of microcystins, anatoxins, and paralytic shellfish toxins in Chautauqua Lake, New York. Homo-anatoxin and dihydro-anatoxin are shown grey-filled, while anatoxin-a is shown as open symbols.
Figure S4.11. Yearly and temporal concentrations of microcystins, anatoxins, and paralytic shellfish toxins in Findley Lake, New York. Homo-anatoxin and dihydro-anatoxin are shown grey-filled, while anatoxin-a is shown as open symbols.
**Figure S4.12.** Histogram of log transformed anatoxin-a concentrations showing near normal distribution for analysis by one-way ANOVA with year as the independent variable.
**Figure S4.13.** Histogram of log transformed microcystin concentrations showing near normal distribution for analysis by one-way ANOVA with year as the independent variable.
Figure S4.14. Histogram of log transformed paralytic shellfish poisoning toxin concentrations showing near normal distribution for analysis by one-way ANOVA with year as the independent variable.
Spatial and Temporal Variation in Paralytic Shellfish Toxin Production by Benthic Microseira (Lyngbya) wollei in a Freshwater New York Lake

5.1 Abstract

Butterfield Lake is a mesotrophic lake in New York State where residents and pets have experienced unexplained health issues. Microseira wollei (basionym Lyngbya wollei) was found at two of 15 sites in Butterfield Lake and analyzed for microcystins, anatoxins, cylindrospermopsins, and paralytic shellfish poisoning toxins (PSTs). Only PSTs and trace levels of anatoxin-a were detected in these samples. This is the first published report of PSTs in a New York State lake. To evaluate the environmental and temporal drivers leading to the observed toxicity, PST content at the two sites was examined in detail. There were distinct differences in the total PST content, filament nutrient, filament chlorophyll, and relationship to environmental drivers between the sites, as well as distinct differences in the total PST content measured using different analytical techniques. A multivariate model containing site, temperature, and filament chlorophyll explained 85% of the variation in PSTs observed over the growing season. This work emphasizes the importance of proper site selection and choice of analytical technique in the development of monitoring programs to protect lake users from the occurrence of benthic cyanobacteria toxins.

5.2 Introduction

Paralytic shellfish poisoning toxins (PSTs) are a class of algal toxins which inhibit the sodium channel of higher organisms, including humans and marine mammals [1–3]. Produced by a variety of algae and cyanobacteria, these toxins create significant human health and economic problems in regions where they occur. Saxitoxin (STX), the parent compound of the PSTs family, is extremely toxic and is closely monitored where there is a high potential for exposure. There are more than 50 known analogs of STX [4]; many are common, but most are less toxic than STX as measured by the mouse bioassay [5–7]. Marine PSTs are produced by many dinoflagellate species and lead to illnesses every year through consumption of contaminated fish and shellfish [8–10]. PST contamination of shellfish can result in significant economic losses through the closure of shellfish beds and the added costs of monitoring for PSTs [11]. Oral and written records suggest that PST-producing blooms are not new and that poisonings from consumption of contaminated shellfish (known as paralytic shellfish poisoning) have been occurring for centuries [12,13].

PSTs were originally thought to predominately occur in marine environments. This assumption has been challenged as reports of PSTs produced by freshwater cyanobacteria grow in number. Freshwater PST-producing cyanobacteria occur in Australia [14], Brazil [15], United States [5,16,17], Canada [18], Germany [19], Russia [20], and New Zealand [21,22], suggesting a world-wide distribution. PST-producing
genera in these countries included *Dolichospermum* (*basionym Anabaena*) (Australia), *Raphidiopsis* (*basionym Cylindrospermopsis*) (Brazil), *Microseira* (*basionym Lyngbya*) (USA and Canada), *Scytonema* (New Zealand), and *Cuspidothrix* (*basionym Aphanizomenon*) (USA). PST production is highly variable within each clade as even closely related species do not always produce toxins [23].

Benthic cyanobacteria produce a number of cyanotoxins including microcystins, anatoxins, cylindrospermopsins, and PSTs [24]. Benthic cyanobacteria and their toxins have been associated with the deaths of dogs in New Zealand [22], France [25], California (USA) [26], and of cows in Switzerland [27]. Benthic PSTs have not been linked with these major exposure events, but PST-producing *Microseira wollei* (*basionym Lyngbya wollei*) [28] have been reported in several freshwater North American water bodies. Within the United States, six new analogs of PSTs referred to as the lyngbyatoxins (LWTXs; not to be confused with the dermatoxins with the same name) were purified in 1997 from *M. wollei* collected from the Guntersville Reservoir, Alabama, USA [5]. These new freshwater PSTs were found in the St. Lawrence River near Montreal and in Florida [16,18], but they were not detected in cultures of PST-toxic cyanobacteria from New Zealand and Brazil [29]. Freshwater PST production, as measured by the ELISA assay, is widespread in Ohio, though the extent of the problem and the PST variants involved remains poorly characterized [30]. Benthic cyanobacteria
have been observed in a number of New York lakes (personal observation), but little is
known about potential toxin production by benthic cyanobacteria in these water bodies.

Butterfield Lake (44°19′10.4″N 75°46′29.0″W) is a mesotrophic lake in the foothills
of the New York State Adirondack Mountains (Figure 1). The region surrounding the
lake is rural and sparsely populated, with small stretches of farmland and a limited
number of small towns and scattered individual homes. Butterfield Lake is at the lower
dermal. The region surrounding the end of the Indian River chain of lakes and the watershed area (4419 ha) is mostly
forests, shrubs and grasses. For the region, the lake is relatively large (407 ha), with an
average depth of 4 m, and is 14 m deep at its deepest point. It has one public access boat
launch and two small private marinas on the southwestern shoreline. Butterfield Lake
has been part of New York State’s Citizen Statewide Lake Assessment Program
(CSLAP) for water quality since 1986 and has been routinely sampled for nutrients,
chlorophyll (Chl), and other physical parameters [31]. In the years 2015–2017, the
average surface water total phosphorus was 0.014 ± 0.003 mg P/L, with a hypolimnetic
total phosphorus average of 0.27 ± 0.08 mg P/L. Surface water total nitrogen and total
dissolved nitrogen averaged 0.44 ± 0.02 mg N/L and 0.008 ± 0.001 mg N/L, respectively,
over the same time period. Planktonic algae in the lake were likely to be phosphorus
limited as the planktonic N:P ratio was near 40. As a result, the average summer surface
chlorophyll (Chl) has exceeded 20 µg Chl/L only twice since 1986 [31].
In 2015–2018, samples from Butterfield Lake were tested for microcystins, cylindrospermopsins, and anatoxin-a through the CSLAP program [21]. One planktonic water sample tested positive for anatoxin-a in 2017, but this report was associated with a dense bloom of the chlorophyte Spirogyra leaving the source of this toxin uncertain. In 2016–2018, large plumes of the green alga, Mougeotia, were observed in the water column, likely associated with infusions of phosphorus from the hypolimnetic waters into surface waters during spring and fall turnover. Despite few reports of planktonic cyanobacteria toxins, there is a history of health issues associated with Butterfield Lake. In 2015, a CSLAP volunteer reported rashes on their arms after working on the lake [32]. In 2018, a dog became lethargic from an unknown element after swimming in a bay on the southeastern shore and eventually expired [33]. In 2017 and 2018, a resident living just south of the current Channel sample site reported health problems leading to hospitalization [33]. The causative factor of these health events, including whether cyanobacteria toxins were involved, remains unclear.

We had previously observed M. wollet in Butterfield Lake; however, the spatial extent of its distribution and its production of toxins was unknown. As planktonic cyanobacterial toxins did not appear to be associated with the reported health concerns, we investigated whether benthic cyanobacteria could be a contributing factor. Here, we describe the spatial, temporal, and between-site variation of M. wollet and its cyanotoxin
production in Butterfield Lake, as well as a preliminary investigation into the environmental drivers associated with PSTs occurrence in \textit{M. wollei}.

5.3 Methods

5.3.1 Sample Collection and Processing

Ten locations along the southwestern shoreline and five locations on the eastern shoreline of the lake were investigated for the presence of \textit{M. wollei} (Figure 5.1). The collections sites were 50–300 m apart on the western shore, and 300–1000 m apart on the eastern shore. At each of these 15 locations, 1–5 rakes were tossed in different directions from the boat, dragged across the bottom and hand-sorted back in the boat to find \textit{M. wollei}. For temporal analysis, the rake was tossed three times into different portions of the \textit{M. wollei} mat and a ~10g wet weight portion of cyanobacteria from each toss was combined to form one sample. Samples were collected every two weeks between July and October. Plant material and detritus were handpicked out from the samples of \textit{M. wollei}, and the sample was washed in the field to remove sediments. For spatial coverage, a petite ponar (Wildco, Yulee, FL, USA) was dropped vertically into the \textit{M. wollei} mat and the resulting mud mixture/biomass mixture was placed into a bucket. Variation due to sampling technique was evaluated by collecting three replicates in close proximity. Cyanobacteria mat material was picked out by hand, washed using lake water and returned to the lab. In the lab, the samples were cleaned again to further
remove sediment. Both rake and ponar samples were placed on ice until their return to the lab. Once in the lab, the samples were weighed, immediately frozen at −80 °C, and freeze-dried. The freeze-dried samples were homogenized in a mortar and pestle using liquid nitrogen, and the powder stored at −20 °C until further analysis. Measurements of water column chlorophyll, phycocyanin, temperature, pH, and conductivity were collected with a Hydrolab DS 5X Sonde, (HACH Environmental, Loveland, CO, USA) at each collection site.

5.3.2 Nutrient Analyses

Total nitrogen and phosphorus content in the cyanobacteria filaments were measured in duplicate on ~30 mg of portions of lyophilized M. wollei powder. Material was fragmented by sonification (3 × 20 s at 32 watts), and a ~20 mL aliquot sonicated further (12 × 20 s at 32 watts) to lyse the filament prior to analysis. Lake water was collected and maintained in the field at 4 °C for analysis of total phosphorus and total nitrogen, or filtered (0.22 µm) in the field and maintained at −20 °C for later analysis of total dissolved nitrogen. Total phosphorus (EPA method 365.1) and total nitrogen (EPA method 353.2) were measured using these samples using a SEAL autoanalyzer model AA3 (SEAL Analytical, Mequon, WI, USA).
Figure 5.1. Map of Butterfield Lake (44°19′10.4″N 75°46′29.0″W) showing the location of the Channel, Dock and Citizens Statewide Lake Assessment Program (CSLAP) sampling sites. Other sampling sites where Microseira or other benthic cyanobacteria were not found are indicated by the blue points. The insert shows the location of Butterfield Lake within New York State.
5.3.3 Chlorophyll, Metal, and Total Carbon Analyses

Chlorophyll-a was determined by extraction of duplicate lyophilized powders using a modification of EPA method 445.0 and analyzed using a Turner Design TD-700 fluorimeter (Turner Design, San Jose, CA, USA). Total carbon was determined on ~2 mg of sample with a Thermo Scientific FlashEA 1112 elemental analyzer (Thermo Scientific, Waltham, MA) interfaced with a thermal conductivity detector. Filament-associated metal content was determined by ICP-OES (Perkin Elmer Optima 3300DV, Waltham, MA, USA). Approximately 50 mg of *M. wolles* powder was heated to 75 °C overnight in 10 mL concentrated nitric acid. Samples were then centrifuged at 10,000× *g* for 5 min, diluted to a final concentration of 7.5% nitric acid, and infused into the ICP-OES (Perkin Elmer Optima 3300DV) for measurement of the common soil elements: iron, aluminum, calcium, magnesium, and sulfur.

5.3.4 DNA Extraction and Analysis

Genomic DNA was extracted from freeze-dried environmental samples using a method modified from Kurmayer et al. [34]. Ten milligrams of freeze-dried cells were incubated in 750 µL of hydration/osmotic shock buffer containing 100 mM EDTA, 50 mM Tris-HCl (pH 8.0), and 25% (w/v) sucrose for 2 h on ice. Lysozyme (25 µL of 100 mg mL⁻¹) was added and incubated at 37 °C for 20 min. Proteinase K (50 µL of 1 mg mL⁻¹) and 50 µL of 10% SDS (w/v) were added and incubated at 50 °C for 2 h. DNA was
collected with three extractions using phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) followed by two extractions of chloroform/isoamyl alcohol (24:1, v/v). DNA was precipitated with sodium acetate (0.3 M) and 100% ethanol, and then washed with 70% ethanol using standard methods [35]. DNA was quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and stored at −20 °C until used as a template in PCR. Partial sequence of the 16S rRNA gene was amplified using the cyanobacteria-specific primer set 27F and 809R [36]. PCR reaction mixtures were formulated with EconoTaq Plus Green 2× Master Mix (Lucigen, Middleton, WI, USA) using ~30–50 ng of genomic DNA as a template. Thermal cycling conditions were 94 °C for 2 min, followed by 37 cycles of 94 °C for 30 s, 64 °C for 30 s, and 72 °C for 90 s, followed by a final extension cycle of 72 °C for 5 min. The PCR product size was verified in agarose gels. PCR products were cleaned using the QIAquick PCR purification kit (Qiagen, Hilden, North Rhine-Westphalia, Germany). PCR products were sequenced on the Applied Biosystems 3730 Genetic Analyzer (Thermo Scientific, Waltham, MA, USA) at the Genomics Core Facility at the University of Tennessee, Knoxville. Phylogenetic analysis and tree construction were conducted in MEGA7 [37]. Sequences were aligned using MUSCLE and default settings. Alignment gaps were deleted. Trees were constructed using the maximum likelihood method based on the general time reversible model and a discrete Gamma distribution with five categories. Support for tree topology was assessed with 100 bootstrap iterations.
5.3.5 Toxin Extraction and Analysis

Microcystins, cylindrospermopsins, anatoxins, and PSTs were extracted 50% methanol containing 1% acetic acid (v/v). Ten milliliters was added to ~700 mg of cyanobacterial powder prior to probe sonication (3 × 20 s at 32 watts) on ice. The slurry was centrifuged at 15,000× g for 10 min, passed through a 0.45 µm nylon syringe filter, and was the same sample used for HPLC-FL, ELISA, LC-MS, and LC-MS/MS analyses. SPATT bags were constructed as described in Lane et al. [38], using 5 g of DIAION HP20 resin. SPATT bags were deployed 1 m under the surface of the water at the Channel and Dock sites for two weeks. Toxins were extracted from the resin using 100% MeOH. The solvent was removed in vacuum and the sample reconstituted into 2 mL of distilled water. Microcystins were analyzed by LC-MS as described in Tang et al. [39]. Anatoxin-a and homo-anatoxin-a were analyzed by LC-MS/MS. Cylindrospermopsin, epi-cylindrospermopsin and deoxycylindrospermopsin were determined by LC-MS/MS in the same run again using one quantification ion and two confirmation ions [45]. PSTs were analyzed using AOAC 2011.02 post-column chemical oxidation modified for water samples and algal powders [40]. Separation used a Waters Alliance 2695 solvent delivery system (Waters, Milford, MA, USA), and a Chromenta KB 3µ 150 × 4.6 mm column with an ACE (ACE Ltd., Aberdeen, Scotland) 3µ guard cartridge assembly at 0.8 mL/min. The solvent system was: A, 2 mM heptanesulfonate (Regis Technologies Inc., Morton Grove, IL, USA) in 10 mM ammonium phosphate adjusted to
pH 7.1; B, 500 mL 2 mM heptane sulfonate in 30 mM ammonium phosphate adjusted to pH 7.1 + 150 mL of acetonitrile [41]. The separation gradient was: 0% B for 0–3 min, 40% B for 3–5 min, 100% B for 5–13 min, and 100% B for 20 min, followed by equilibration of the column back to 0% B for 10.5 min. Oxidation of the PST ring used 9 mM periodic acid (Alfa Aesar, Ward Hill, MA, USA) in 50 mM potassium phosphate at pH 9 in a reaction coil temperature maintained at 65 °C. The acid modifier was 0.5 M acetic acid with a flow of 0.45 mL/min. PSTs were differentiated from interfering fluorescent compounds by re-injection of the sample with water in place of the oxidant. Individual method LODs were determined for each toxin from their average daily response factors for powders and for water samples. Microcystin LODs averaged 0.20 µg/g and 0.50 µg/L for *M. wollei* samples and whole water samples, respectively. Method LODs for anatoxin-a and homo-anatoxin were 0.005 µg/g and 0.02 µg/L for *M. wollei* and whole water samples, respectively. Cylindrospermopsin and deoxycylindrespermopsin LODs were 0.05 µg/g and 0.1 µg/L, respectively. PST LODs were 0.15 µg/g and 0.10 µg/L, respectively. Primary PST standards were purchased from the NRC Canada (Institute for Marine Biosciences, Halifax, Canada) and United States Food and Drug Administration (FDA) (Silver Spring, MD, USA). FDA STX was diluted 1:50 to a concentration of 4 µM prior to use. STX standard response converted into an equivalent *M. wollei* concentration was linear between 1.3 and 184.5 µg/g dry weight of *M. wollei*. NRC standards of STX, NEO, dcNEO, dcSTX, GTX-1, GTX-2, GTX-3, GTX-4, GTX-5,
dcGTX-2, dcGTX-3, LWTX-1, and C1+C2 were used to calculate relative response factors. Response factors for other variants of PSTs relative to STX ranged from 0.1 to 4.6 with an average of 1.44. All PSTs, both known and unknown, were quantified using an STX standard curve with single injections of STX standards to verify the stability of the response factor. For comparison of analytical methods, three samples were chosen from the Dock site and three samples were chosen from the Channel site, with three samples from the beginning, middle, and end of the sampling period each. PSTs were analyzed by LC-MS/MS per the method described in Armstrong et al. [42] using a Waters Xevo TQD mass spectrometer (Waters, Milford, MA, USA) in the laboratory of Professor Juliette Smith at the Virginia Institute of Marine Science, and by Pearse McCarron at the National Research Council of Canada. STX was determined using Abraxis ELISA (part number 52255B, Abraxis LLC, Warminster, PA, USA) according to the manufacturer’s instructions. Samples for ELISA were diluted to achieve a maximum methanol concentration of <5% prior to analysis.

5.3.6 Statistical Analysis and Model Selection

Statistical analyses were performed in R version 3.5.1 (R Core Team, Vienna, Austria) with base package tools. Multiple comparisons for paired t-test p-values were corrected with Holm’s stepdown procedure (n = 6 parameters). Durbin–Watson tests assessed autocorrelation from temporal pseudoreplication in parameters used in the
ANCOVA and multiple regression models, with significant autocorrelation found only in total PSTs collected from the Channel ($p < 0.05$). Small deviations from normality were observed in the residuals for some tests, but were not deemed sufficient enough to warrant transformation. Transformation did not change the significance or interpretation of the tests or results, with the loss of information in the magnitude of differences deemed excessively detrimental. ANCOVA maximal models started with predictor, site, and predictor site interaction, and were reduced to a minimum explanatory model using $F$ tests ($p < 0.05$), testing first for the removal of the interaction term, followed by site if $p$ was greater than 0.05. The retention of site in the model reflected the lack of explanatory power from the continuous predictor by itself in explaining the variation in the response variable.

The explanation of the variation in PSTs from both sites was performed by a multiple linear regression containing biological and environmental variables collected from the *M. wollei* and the surrounding water. The full model started with the following predictors: filament chlorophyll, filament nitrogen and phosphorus, temperature, site, temperature/site interaction, and CSLAP water column total phosphorus and total nitrogen, and was simplified to be minimally adequate using manual and automated forwards and backwards stepwise addition or removal of parameters. Model simplification was done by a combination of $F$ tests and AIC ($p < 0.05$, $\Delta$AIC = 2).

Coefficients for the final model were intercept (25.71), temperature (−1.53), site (149.92),
chlorophyll (0.015), and interaction (−5.66). Final model statistics ($R^2 = 0.858$, $F_{4,13} = 19.56$). Filament chlorophyll and filament nitrogen were covariate so only one term was kept in the reduced model. Non-nested models containing either filament chlorophyll or filament nitrogen were compared using Akaike information criterion (AIC) and predicted residual error sum of squares (PRESS) [43]. Relative importance of the terms in the final model was evaluated by comparison of each nested model to the final model by AIC and PRESS. Site, temperature, and filament chlorophyll explained a majority of the variation in total PSTs over 2017.

5.4 Results

5.4.1 Site Selection

Benthic rake collections made at 15 locations in shallow waters along the western and southern portions of the lake identified two sites with large *M. wolleii* mats (Figure 5.1). No *M. wolleii* was found at the other 13 sites. One site (Channel) was located at a man-made channel inside of a wetland that extended approximately 300 m long and was 5 m wide. This channel was used to connect the lake to several homes and a private boat launch and marina. A second site (Dock) was located at the southern end of the lake by the public boat launch and fishing dock. *M. wolleii* at the Dock site was patchier and growing amongst more aquatic vegetation when compared to the Channel site. *M. wolleii* at the Channel site was found growing on or in the muddy substrate, whereas *M.
*wollei* at the Dock site was found on the muddy substrate, on aquatic vegetation, and in detached clumps floating on or near the shoreline.

### 5.4.2 Comparison of Analytical Methods

Six samples, three from each site, were analyzed for PSTs by LC-MS/MS, ELISA, and HPLC with fluorescence detection (HPLC-FL). Higher concentrations of PSTs were found in Dock site samples relative to the Channel site samples using all three methods; however, the absolute concentrations as measured by the three analytical methods were markedly different (Table 5.1). The relative abundance of PST variants, as determined by HPLC-FL, was as follows: GTX-3 (0–12%), GTX-5 (25–50%), dcGTX-3 (<1%), dcGTX-2 (<1%), STX (0–3%), dcSTX (0–3%), LWTX-2/3 (30–80%) and LWTX-5 (3–30%). The presence of these PSTs was confirmed by LC-MS/MS [44]. The LWTXs, along with GTX-3 and GTX-5, accounted for most of the observed PSTs. The other PSTs, including STX, fluctuated, but represented a small portion of the total PST concentrations. The toxin profiles were similar between the sites. HPLC-FL measured 1.5-10× higher concentrations of toxin than ELISA or LC-MS/MS and thus HPLC-FL was used as the primary analytical method for these studies.
5.4.3 Species Identification

To determine if differences in PST occurrence at the two sites were associated with a difference species composition, 16S rRNA gene sequence analysis was conducted on *M. wollei* from the Dock and Channel sites. Samples from both sites yielded partial 16S rRNA gene sequences (682 and 678 at the Channel and Dock, respectively) that were identical over the 678 bp alignment. These sequences showed greater than 99% identity to 16S rRNA gene of *M. wollei* originating from the Guntersville Reservoir, a known PST producer [5]. In a maximum likelihood tree comprising 16S sequences from PST-producing strains of *M. wollei*, the Butterfield Lake sequences formed a highly supported cluster with the strain from Guntersville Reservoir (Figure 5.2).
### Table 5.1. Paralytic shellfish poisoning toxin (PST) concentrations measured in six samples by three different analytical methods.

<table>
<thead>
<tr>
<th>Sample Date</th>
<th>Dock HPLC-FL</th>
<th>ELISA 1</th>
<th>LC-MS/MS 2</th>
<th>Channel HPLC-FL 1</th>
<th>ELISA 1</th>
<th>LC-MS/MS 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>7/4/2017</td>
<td>33.77</td>
<td>6.94</td>
<td>22.33</td>
<td>10.23</td>
<td>5.75</td>
<td>3.63</td>
</tr>
<tr>
<td>9/10/2017</td>
<td>58.98</td>
<td>4.18</td>
<td>20.12</td>
<td>10.81</td>
<td>3.27</td>
<td>4.85</td>
</tr>
<tr>
<td>10/22/2017</td>
<td>101.25</td>
<td>36.24</td>
<td>37.95</td>
<td>16.00</td>
<td>2.58</td>
<td>9.56</td>
</tr>
</tbody>
</table>

1 Total PSTs calculated in µg saxitoxin (STX) eq./g dry wt. 2 LC-MS/MS PSTs were quantified using 12 common marine PST standards. Standards were not available for the lyngbyatoxins (LWTXs) so the contributions of these toxins to the total PST pool as measured by LC-MS/MS were not included.
Figure 5.2. Maximum likelihood tree of 16S rRNA gene sequences from strains of *Microseira wollei*. Support values of 100 bootstrap iterations are shown at the nodes. Known paralytic shellfish poisoning toxin (PST) producers (✳) and cylindrospermopsin producers (×) are labeled; strains without labels were not tested for toxins. *M. wollei EU603708* was the PST producer collected from Guntersville Reservoir, Alabama [5].
5.4.4 Spatial Variation within the Channel

Transects along the length of the Channel site demonstrated that *M. wollei* coated the bottom in a thick mat (Figure 5.3). *M. wollei* coverage per square centimeter varied along the Channel (Figure 5.3A) with an average of 90.8 ± 38.9 mg dry weight/cm². Three replicates collected in close proximity varied by less than 10% in their dry weight. The *M. wollei* at transect locations 54 m and 72 m were associated with lower filament chlorophyll per g dry wt. (Figure 5.3B; 159 ± 43.0 µg Chl/g dry wt.) and contained no detectable PSTs (Figure 5.3C). These two sites had much less filament chlorophyll than the other five sites (average 505 ± 115 µg Chl/g dry wt.). There was little difference in the filament nitrogen (11.4 ± 1.1 mg N/g dry wt.) and filament phosphorus (1.23 ± 0.24 mg P/g dry wt.) of the *M. wollei* across all seven transect locations (Figure 5.3C,D). Five of the seven samples tested positive for PSTs (average 8.45 ± 2.30 µg STX eq./g dry wt.). PSTs/cm² and Chl/cm² were negatively correlated to biomass/cm² ($R^2 = 0.9; p < 0.02$) if the sites without detectable PSTs were excluded from the regression. The relationship between total PSTs per area or filament chlorophyll per area to biomass/cm² was less clear when the 54 m and 72 m samples were included in the regression ($R^2$: 0.14–0.16). None of the samples tested positive for microcystins, anatoxins or cylindrospermopsins.
**Figure 5.3.** Spatial variation in different parameters related to *Microseira wollei* collected at 18 m intervals along a 108 m transect at the Channel site: (A) *M. wollei* density in mg dry wt., (B) filament chlorophyll content in µg Chl per g dry wt., (C) total nitrogen content of the filaments in mg N per g dry wt., (D) total phosphorus in the filament in mg P per gram dry wt., (E) total paralytic shellfish poisoning toxins (PSTs) in saxitoxin (STX) equivalent per g dry weight as determined by HPLC-FL, and (F) µg STX equivalents per square centimeter coverage of the benthos. Error bars for filament chlorophyll represent one standard deviation from duplicate measurements. Error bars for measurements of filament nitrogen, filament phosphorus, and total PSTs were <5% and are not shown.
5.4.5 Temporal Variation Between Sites

A comparison of the basic water quality parameters for the Dock and Channel sites are shown in Table 5.2. Both nutrient and physical parameters at the Channel and Dock were markedly different, with the Channel being 1.6 °C colder, a higher conductance, and a lower pH than at the Dock (Table 5.2). The calculated 1% light level for both sites reached the sediment surface of 1.5 m and 1.5–1.75 m for the Channel and Dock, respectively. Total phosphorus and total dissolved nitrogen in the Channel were elevated over the Dock by about 50%, and total nitrogen was elevated in the Channel over the Dock by about 30%. Physical parameters and surface water nutrient concentrations at the Dock were representative of the conditions of the southern basin of the lake and CSLAP site (Table S5.1).

*M. wollei* measurements included average filament chlorophyll, filament nitrogen, filament phosphorus, N:P ratio, total PSTs per g dry wt., and PSTs per chlorophyll. All concentrations were higher at the Dock site than at the Channel site for most of 2017 (Figure 5.4). Trace levels of anatoxin-a (8–70 ng anatoxin-a/g dry wt.) were occasionally detected in benthic mats at both sites, but no toxins were detected in water samples from either site. Anatoxin-a and PSTs were detected in the water column using Solid Phase Adsorption Toxin Tracking (SPATT) bags at both sites (21–513 µg STX eq./g resin and 0.04–1.2 µg anatoxin-a/kg resin), but again at trace levels. *M. wollei* in the Channel and Dock sites had weight-based N:P ratios of 5.92 ± 0.82 and 6.65 ± 0.92,
respectively (Figure 5.4). The N:P ratios at both sites were lower than the weight-based Redfield ratio of 7.2:1 [45]. There were no differences in total carbon per g dry wt. *M. wolleii* between the Channel and Dock sites (38.7 ± 1.95% and 38.7 ± 0.86%, respectively) [46,47], nor were there consistent differences in the summed concentrations of Al, Fe, Mg, and Ca in the *M. wolleii* filaments, indicating variation in the degree of sediment contamination of the samples collected from the two sites was unlikely.

5.4.6 Environmental Factors as a Predictor of Total PSTs and Chlorophyll

Analysis of covariance (ANCOVA) regression models with either filament phosphorus, filament nitrogen or temperature as independent variables had complex relationships with site, filament chlorophyll and total PSTs (Figure 5.5). Filament nitrogen affected total PSTs and filament chlorophyll similarly at both sites (Figure 5.5A,B). Filament phosphorus (Figure 5.5C,D) had little or no impact on filament chlorophyll concentrations, but *M. wolleii* at the Dock site had approximately 30% more filament chlorophyll per g dry wt. at the same concentration of filament phosphorus. There was a negative relationship between PSTs and filament phosphorus, and for the same concentrations of filament phosphorus, there were approximately 2.5 times more PSTs in *M. wolleii* collected at the Dock site than at the Channel site. Temperature (Figure 5.5E) had a strong negative relationship with PSTs, and there was no relationship between temperature and filament chlorophyll (data not shown). The relationships between
Table 5.2. Average water quality parameters for the Dock and Channel sites measured between 4th July and 22nd October, 2017 (n = 9). Temperature, conductivity, and pH were measured every two weeks. Water column nutrients were measured three times over the 4 month period. Light attenuation and depth were measured once in the middle and once at the end of the July-to-October sampling period.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dock</th>
<th>Channel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depth (m)</td>
<td>1.5–1.75</td>
<td>1.5</td>
</tr>
<tr>
<td>Average Temperature (°C)</td>
<td>22.22 ± 3.38</td>
<td>20.66 ± 3.94</td>
</tr>
<tr>
<td>Conductivity (μS)</td>
<td>197.92 ± 19.25</td>
<td>225.61 ± 17.51</td>
</tr>
<tr>
<td>pH</td>
<td>8.27 ± 0.69</td>
<td>7.14 ± 0.28</td>
</tr>
<tr>
<td>TP (planktonic) (mg P/L)</td>
<td>0.0112 ± 0.0035</td>
<td>Below detection *</td>
</tr>
<tr>
<td>TDP (planktonic) (mg P/L)</td>
<td>Below detection *</td>
<td>Below detection *</td>
</tr>
<tr>
<td>TN (planktonic) (mg N/L)</td>
<td>0.320 ± 0.009</td>
<td>0.482 ± 0.077</td>
</tr>
<tr>
<td>TDN (planktonic) (mg N/L)</td>
<td>0.254 ± 0.009</td>
<td>0.419 ± 0.028</td>
</tr>
<tr>
<td>Light Attenuation Coefficient (k)</td>
<td>−1.87 ± 1.16</td>
<td>−2.12 ± 0.40</td>
</tr>
<tr>
<td>Calculated 1% light level (m)</td>
<td>1.51–6.48</td>
<td>1.83–2.68</td>
</tr>
</tbody>
</table>

* LOD < 0.009 mg P/L. TP, total phosphorus. TDP, total dissolved phosphorus. TN, total nitrogen. TDN, total dissolved nitrogen.
Figure 5.4. Box-and-whisker plots for six parameters measured in *Microseira wollei* filaments collected biweekly at both sites in 2017. Paired two-tailed $t$-tests were significantly different for all parameters. The $p$-values are adjusted for multiple comparisons using Holm’s correction with 8 degrees of freedom; tested parameter, difference in means, Holm’s adjusted $p$-value: Total PST: 33.19, and 0.012; filament chlorophyll: 778.78, and 0.026; filament phosphorus: 0.50, and 0.005; filament nitrogen: 5.25, and 0.0007; toxin/chlorophyll: 0.014, and 0.026; N:P ratio: 0.73, and 0.026. Upper and lower bounds of the box-and-whisker plots are the 25th and 75th percentiles, while the bar represents the mean. Whisker lengths are $1.5 \times$ the distance between the 25th and 75th percentiles, with any samples outside of this range shown as points.
temperature and total PSTs were different for the two sites, with decreases in temperature at the Dock leading to a 5 fold greater increase in toxin than at the Channel site. Filament chlorophyll did not relate to total PSTs (Figure 5.5F), with the Channel having a weakly negative slope and the Dock having a weakly positive slope; neither slope was significantly different from a slope of 0.

The minimally adequate model needed to predict total PST concentrations consisted of three parameters listed in their order of importance: site, temperature, and filament chlorophyll (Figure 5.6). The model contained these three terms with an interaction between temperature and site. By itself, filament nitrogen was a better predictor of PSTs when compared to filament chlorophyll, but these two prediction terms were covariate and the inclusion of filament chlorophyll over filament nitrogen produced a marginally better model (ΔAIC, 2.32; ΔPRESS, 405.98; ΔR², 0.02).
**Figure 5.5.** The correlations between filament nitrogen and total paralytic shellfish poisoning toxins (PSTs) (A) and filament chlorophyll (B), between filament phosphorus and total PSTs (C) and filament chlorophyll (D), between total PST and temperature (E) and between total PST and filament chlorophyll (F) using the best analysis of covariance (ANCOVA) model at the Dock (◆) and Channel (▲) sites. The maximal ANCOVA model of continuous predictor, site, and the interaction of site, and predictor were reduced to be minimally explanatory. $R^2$ represents the variation explained by the entire model, not the variation explained by individual regression models. Each point represents a sample collected every two weeks over the summer and fall.
Figure 5.6. Total paralytic shellfish poisoning toxins (PSTs) and predicted PSTs from the final model of site, temperature, and filament chlorophyll ($R^2 = 0.858$, $F_{4,13} = 19.56$).

Dashed black and grey lines are the 95% confidence intervals for predicted total PST concentrations at each site. The full model of 8 terms was reduced to minimally adequate model using AIC and PRESS. Total PSTs on 07/17/2017, 09/25/2017, and 10/09/2017 fell outside the 95% CI for the model.
5.5 Discussion

The choice of analytical method for PST analysis is non-trivial. Four analytical methods for PST have replaced the original mouse bioassay (AOAC 959.08): HPLC with fluorescence detection after pre-column chemical oxidation (AOAC 2005.06) [48] or post-column chemical oxidation (AOAC 2011.02) [49], LC-MS/MS (interlaboratory certification currently in process) [50–52], or an enzyme-linked immunosorbent assay (ELISA) [53]. All four methods have advantages and disadvantages as a primary analytical tool. The two oxidation methods convert the tricyclic PST ring system into a fluorescent derivative. Functional groups change the conversion efficiency, but most of the ~60 known PST congeners form a fluorescent product. LC-MS/MS is slowly being adopted for regulatory use with shellfish, but is limited by the availability of certified standards. Currently, standards are commercially available for 17 PSTs; sixteen of these standards correspond to marine PSTs, which may or may not be the predominant variants in freshwater systems. Only one freshwater PST, LWTX-1, is commercially available as a certified standard. ELISA is a useful screening tool but is not recommended for quantification [54], as it provides no structural information about the PST variants in a sample, and most ELISA methods for PSTs were developed to monitor for shellfish toxins such as STX. The cross-reactivity to PST congeners present in cyanobacteria, including the LWTXs, is highly variable if known at all.
Six *M. wollei* samples from Butterfield Lake were measured by post-column HPLC-FL, LC-MS/MS, and ELISA. As expected, HPLC-FL, with its ability to respond to a broader range of PSTs, reported a higher concentration of total PSTs than the ELISA assay. Concentrations of PSTs measured by LC-MS/MS analysis were also lower than total PSTs as measured by HPLC-FL. This was expected, as additional toxins such as LWTX-2 through LWTX-6 were present in the HPLC-FL analysis that were not included in the LC-MS/MS analysis. Despite these quantitative differences, the methods were internally consistent, with ~4-fold higher concentrations of PSTs observed at the Dock site relative to the Channel site with all three techniques.

Because of the limited availability of freshwater PST standards and the abundance of the LWTX analogs in our samples, we chose HPLC-FL with post-column oxidation as our primary analytical tool. One complication is that different congeners have different conversion efficiencies in forming fluorescent product(s) as calculated using NRC certified reference materials. We adopted a single response factor using STX, fully recognizing that some congeners (GTX-2 and GTX-3) had a higher response factor, whereas other congeners (GTX-1, GTX-4, and LWTX-1) had a lower response factor relative to STX. In this approach, the HPLC-FL method was operated as an assay (as opposed to an analysis), mimicking the ELISA which quantified total PSTs in µg STX eq. using a single congener (STX) for the standard curve. This allowed us to estimate PST concentrations where standards were not available, such as the LWTXs.
Butterfield Lake represents the second documented case of PST production by *M. wollei* in Northeastern USA and the first documented case of benthic production of PSTs within New York [18]. *M. wollei* dominated in mats in Butterfield Lake and PSTs were present in most samples. *M. wollei* was the most likely source of the PSTs identified in Butterfield Lake based on the similarity of the 16S rRNA gene sequence of *M. wollei* from Butterfield Lake to the PST-producing *M. wollei* strain from Alabama, the similarity of the PST congener profiles between the Alabama *M. wollei* and the profiles observed in the two Butterfield Lake sites, and the dominance of *M. wollei* in the benthic mat from both sites. Genetic work to confirm the presence of the *stx* biosynthetic operon in the *M. wollei* from Butterfield Lake is currently in progress. The concentrations of PSTs reported here were comparable to the total amounts of PSTs found by Hudon et al. in the St. Lawrence River [55].

We tested for several other types of cyanobacteria toxins in Butterfield Lake. Only trace levels of anatoxin-a were detected in addition to the PSTs. There were no detectable levels of microcystins, homo-anatoxin, cylindrospermopsins or deoxycylindrospermopsin in these samples. The source of anatoxin-a was uncertain. While *Anabaena* and *Phormidium* were associated with anatoxin-a production in wadeable streams in California and New Zealand [56–58], these genera were not observed during microscopic examination of the cyanobacterial mats from Butterfield Lake. These and/or other anatoxin-a producing benthic cyanobacteria may have been
present in the mat in small quantities and could account for the low levels of anatoxin-a.

Regardless of the source, the low measured concentrations of anatoxin-a suggest there was little or no risk to lake residents and other users of the lake by toxins other than PSTs. Total PSTs concentrations ranged over the season but they were always the major cyanobacterial toxin found in Butterfield Lake.

We considered both intra- and extracellular toxin concentrations when evaluating the risk to humans from exposure to these toxins. Routes of exposure to PSTs in Butterfield Lake are comparable to their planktonic counterparts [24,59], where recreational exposure through bathing and/or drinking water dominate the discussion of planktonic blooms [60]. In Butterfield Lake, recreational or other direct contact with M. wolfei was more likely to occur at the Dock site than at the Channel site due to its proximity to people. However, even at the Dock site, most M. wolfei was located far enough from the shoreline to limit potential human contact. Unlike planktonic cyanobacteria blooms, swimmers were unlikely to encounter the toxic benthic mats unless they detached from the bottom substrate. Additionally, the highest toxin concentrations occurred later in the season, coinciding with decreasing water temperatures. Lower temperatures would correspond with less recreation at the lake, further decreasing the risk of direct exposure to M. wolfei containing high levels of toxin.

A second route of exposure is through consumption of drinking water contaminated by PSTs. Many lake residents pump water directly from the lake into
their homes. The residents use different approaches to treat their water, with filtration being the most common but some using UV-disinfection or chlorination [33,61]. PSTs are quite polar and residential charcoal filtration units, generally designed for non-polar taste and odor compounds, are not very effective at removing PSTs from the water. Dissolved toxins were never detected in water samples collected from either the Channel or the Dock sites; anatoxin-a and PSTs were only detected in SPATT bags that were deployed in the water column for two weeks. The concentrations of anatoxin-a and PSTs in the water were always lower than the recommended regulatory limit for these toxins, suggesting risk from direct exposure in drinking water was low [24,62,63].

The presence of benthic cyanobacteria mats in Butterfield Lake was very consistent at the two sites, but offers a different challenge for monitoring when compared to a traditional planktonic bloom. The New York CSLAP program is well suited to monitoring planktonic algal blooms and their corresponding toxins; using a combination of temporal sampling over the growing season and samples from visible blooms to track the temporal and spatial variation of the bloom. The original purpose of CSLAP was as to tracking long-term changes in water quality, and CSLAP samples are usually collected from a limited number of open water sites in the center of the lake. A similar approach was used in the EPA National Lakes Assessment to evaluate cyanobacterial toxins [64]. These procedures are not well suited to monitoring for cyanotoxins from benthic mats where the choice of sampling site becomes increasingly
important. If collections were made in Butterfield Lake at only the Channel or Dock sites, or any of the 13 other sites from around the lake, it would have resulted in an incomplete assessment of toxin content in Butterfield Lake. Extrapolating toxin content to an entire waterbody from a limited number of samples would therefore potentially misrepresent toxin concentrations and their exposure risk.

The choice of site was the most important parameter in our multivariate models when evaluating what factors influenced the presence of PSTs. These parameters were different from those derived for planktonic cyanobacterial blooms, which often contain nutrient parameters, while sampling site is often not included as a covariate [65]. While Butterfield Lake was phosphorus-limited in the surface water with a weight-based N:P ratio around 40, M. wollei filaments were generally nitrogen-limited, with N:P ratios below 7.2 for the majority of samples. Only three samples, all at the Dock, had N:P ratios above the Redfield ratio. Changes in filament nitrogen content were closely linked to changes in total PSTs and filament chlorophyll, while filament phosphorus content had no relationship with filament chlorophyll at either site. Benthic cyanobacteria grow in close proximity to the sediments and phosphorus content in Butterfield Lake sediments can be quite high (420 mg P/kg sediment [33]). Thus M. wollei could acquire the phosphorus needed for growth directly from the sediments, even when surface water phosphorus concentrations were low [66]. Unlike benthic cyanobacteria found in the Eel River [67], M. wollei in Butterfield Lake was never
phosphorus-limited. In contrast, sediment N was low (<110 mg N/kg sediment [33]) and filament nitrogen was closely related to both total PSTs and filament chlorophyll. These results are very different from those results obtained in culture using *M. wollei* isolated from Alabama where both high nitrate and high phosphate resulted in a marked decrease in PSP toxicity [68].

Despite this lack of phosphorus limitation, there was still a weak negative relationship between total PSTs and filament phosphorus (Figure 5.5). Boyer et al. [69] observed a similar trend in dinoflagellates grown under phosphorus-limited conditions, where decreasing phosphorus increased the toxicity per cell. A similar trend was observed here with *M. wollei*. Boyer et al. attributed this increase to a decreased growth rate that in turn led to an increase in toxin content per cell. A similar relationship may occur with *M. wollei* in Butterfield Lake.

The relationships between PSTs, environmental parameters, and site were different depending on the predictor and the response variables chosen. Still the choice of site remained the most important variable in explaining the PST concentrations in Butterfield Lake. The similarity in 16S rRNA segments between the Dock site and Channel site indicate that changes in toxin production were not due to changes in species, but that environmental and/or biological differences between the sites may be important. Although there were important relationships between nutrients and total PSTs, models to predict total PST content did not include filament nutrient
concentrations as predictors, only environmental variables in addition to filament chlorophyll. Filament chlorophyll, which may reflect filament nitrogen content, was a better predictor of total PSTs than the filament nitrogen content itself. Toxin concentration was primarily driven by site, temperature, and filament chlorophyll, with significant loss in explanatory power when each of these terms was removed. Calcium content, an important predictor of biomass and toxicity in culture [68], was not different between the two sites and could not explain the observed differences. Temperature effects were highly dependent on the site, where temperature may have been covariate with some other parameter, such as photosynthetically active radiation. As temperatures dropped later in the season, total PSTs, both measured and predicted by the model, diverged at the two sites; decreases in temperature had a larger impact on PST concentrations at Dock site relative to the Channel site. Filament PST content at the two sites were similar early in the season, but the total PST content increased much more at the Dock site than at the Channel site as the season extended into September and October. The cause of the differences between the sites are unknown, but might be linked to the close association of *M. wolfei* with submerged aquatic vegetation at the Dock site, where aquatic vegetation was largely absent in the Channel.

The environmental drivers of *M. wolfei* PSTs were interesting in the context of freshwater planktonic blooms, where planktonic bloom size is closely related to phosphorus, while microcystins and anatoxin-a content is more closely related to
nitrogen availability [39,70–73] and genetic factors [74]. The drivers of benthic toxin production may not be the same as those for planktonic cyanobacteria, with genetic factors, physical parameters, and nutrient parameters all playing important roles with respect to toxin production [57,75]. This neglects the important role sediments play as a source of nutrients to the cyanobacteria. Hudon et al. [55] collected *Lyngbya* (potentially *Microseira*) samples from two large fluvial lakes in the St. Lawrence River and examined the correlation of several environmental parameters to LWTX-1. They identified depth, dissolved organic carbon, and the 1% light level as important contributors for toxin occurrence, while water flow was important in mat proliferation. Compared to those found in fluvial environments, water flow did not change in Butterfield Lake, while the 1% light level was at or below the sediment water interface at both sites. Further studies would be needed to better define the role of light in this lacustrine system. Hudon et al. reported no significant differences in LWTX-1 concentrations between their two fluvial lakes. This was very different from what we observed in Butterfield Lake where LWTX-1 was absent and there was a large variation in the other lyngbyatoxins. This emphasizes the importance of a representative and balanced sample design that employs a broad-spectrum analytical technique and multiple sites when trying to assess the overall risk from benthic PSTs.
5.6 References


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**Supplementary Information**

**Table S5.1.** Sample locations where lake-wide nutrient measurements were collected.

<table>
<thead>
<tr>
<th>Sample Location</th>
<th>Total Phosphorus (mg/L)</th>
<th>Total Nitrogen (mg/L)</th>
<th>Total Dissolved Nitrogen (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>44°18′30.3″N 75°47′14.4″W</td>
<td>0.0079</td>
<td>0.314</td>
<td>0.269</td>
</tr>
<tr>
<td>44°18′42.9″N 75°47′07.3″W</td>
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<td>0.348</td>
<td>0.279</td>
</tr>
<tr>
<td>44°19′09.3″N 75°47′13.3″W (Channel)</td>
<td>0.0249</td>
<td>0.567</td>
<td>0.443</td>
</tr>
<tr>
<td>44°19′57.8″N 75°45′57.7″W</td>
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<td>0.404</td>
<td>0.293</td>
</tr>
<tr>
<td>44°20′19.9″N 75°45′21.0″W</td>
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<td>0.434</td>
<td>0.327</td>
</tr>
<tr>
<td>44°18′56.2″N 75°46′33.4″W</td>
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<td>0.323</td>
<td>0.289</td>
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<tr>
<td>44°18′06.5″N 75°46′56.4″W (CSLP)</td>
<td>0.0112</td>
<td>0.298</td>
<td>0.238</td>
</tr>
<tr>
<td>44°17′58.8″N 75°47′39.5″W (Dock)</td>
<td>0.0080</td>
<td>0.309</td>
<td>0.243</td>
</tr>
</tbody>
</table>
Table S5.2. Concentrations of PSTs measured with HPLC-FL and the receptor binding assay in *Microseira wollei* collected from the Dock site.

<table>
<thead>
<tr>
<th>Sample date</th>
<th>HPLC-FL</th>
<th>Receptor Binding Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>9/10/2017</td>
<td>58.98</td>
<td>7.00</td>
</tr>
<tr>
<td>10/22/2017</td>
<td>101.25</td>
<td>6.88</td>
</tr>
</tbody>
</table>
Figure S3.3: Extracted LC-HRMS chromatogram at $m/z$ 351.0729 ± 0.5 ppm extracted from a full scan of NRC certified reference materials for dcGTX 2 and 3 (A) and *Microseira wolleii* from Butterfield Lake (B).
Figure S3.4: Full scan (A and C) and MS/MS (B and D) HRMS spectra of dcGTX2 from NRC certified reference materials for dcGTX2 and 3 (A and B) and Microseira wollei from Butterfield Lake (C and D).
Figure S3.5: Full scan A and C) and MS/MS (B and D) HRMS spectra of dcGTX3 from NRC certified reference materials for dcGTX2 and 3 (A and B) and Microseira wollei from Butterfield Lake (C and D).
Figure S3.6: Full scan (A and C) and MS/MS (B and D) HRMS spectra of dcSTX from NRC certified reference materials for dcSTX (A and B) and *Microseira wollei* from Butterfield Lake (C and D).
Figure S3.7: Extracted LC-HRMS chromatograms of LWTX 1 eluting at 23.91 min (A) and putative LWTX 5 eluting at 30.40 min (B) from and *Microseira wolleii* from Butterfield Lake. Positive ionization full scan (C) and MS/MS (D) of LWTX 5. Product ions detected are consistent with those previously reported by Foss et al [76].
Figure S3.8: Extracted LC-HRMS chromatograms of putative LWTX6 (A) at m/z 283.1513 ± 5 ppm from *Microseira wollei* from Butterfield Lake. Positive ionization full scan (B and D) and MS/MS (C and E) of putative LWTX 6 eluting at 29.27 min (B and C) and an unknown isomer eluting at 28.65 min (D and E). Product ions detected are consistent with those previously reported by Lajeunesse et al [18].
Co-Occurrence of and Spatial Heterogeneity of the Anatoxins, Paralytic Shellfish Poisoning Toxins, and Microcystin Cyanotoxins in Chautauqua Lake, New York

6.1 Abstract

Chautauqua Lake is a eutrophic lake in New York characterized by the presence of a deeper, cooler, and less nutrient rich North Basin, and a warmer, shallower, nutrient replete South Basin. The lake is home to a complex mixture of cyanobacteria, with genera that have been known to produce microcystins (MCs), anatoxins, and paralytic shellfish poisoning toxins (PSTs). Samples collected from a number of sites around the lake were analyzed for these three toxin classes over a four-year period. Concentrations of the three toxin groups varied widely, from below the detection limit to thousands of µg/L, within and between years. The North Basin was found to have larger blooms with higher concentrations of toxin relative to the South Basin, while the blooms and toxins in the two basins correlated with different sets of environmental and physical parameters. Contamination of drinking water from Chautauqua Lake was unlikely to be a significant source of exposure; however, toxin levels in many blooms exceeded recreational guidelines for toxin exposure. As the lake is well-populated, and there is a large tourist population, recreational exposure to toxins rather than from exposure to contaminated drinking water is of most concern in Chautauqua Lake.
6.2. Introduction

Cyanobacteria produce a number of toxic compounds with a variety of chemical structures and biochemical activities [1–4]. Toxin classes of primary interest due to their toxicity and widespread distribution in freshwater systems are microcystins (MCs), the neurotoxic anatoxins including anatoxin-a, homo-anatoxin, dihydro-anatoxin (ATXs), cylindrospermopsin and its derivatives, and the paralytic shellfish poisoning toxins (PSTs).

MCs are a class of hepatotoxic peptides that are the primary focus for most cyanotoxin monitoring. MCs have been well-reviewed, including the global distribution of *Microcystis* [5] a major MC producer worldwide [6], analytical methods for their detection [7], the toxicity and health effects of exposure to MCs [8], and a number of MC treatment and removal strategies [9]. MCs are produced by a number of cyanobacterial genera, including *Dolichospermum* (basionym *Anabaena*), *Anabaenopsis*, *Aphanocapsa*, *Arthrospira*, *Hapalosiphon*, *Microcystis*, *Nostoc*, *Oscillatoria*, *Planktothrix*, *Snowella*, *Synechocystis*, and *Woronichinia* [8]. There are over 250 congeners of MCs [10] with the most well-known congener being MC-LR, where two variable amino acids in the characteristic seven-membered peptide ring are leucine (L) and arginine (R). Congener profiles can vary spatially and temporally. A survey of 1161 lakes in 48 states in the United States looking for seven MCs found the most common congeners were MC-LR,
YR, -RR, LY, and -LA in decreasing abundance [11]. A smaller survey in 23 eutrophic midwestern lakes in the United State found the MC-LR, -RR and -LA congeners as the most abundant MCs, with four congeners in detectable but less significant quantities [12]. A survey in New York found the most abundant congeners to be MC-LR, -RR, and -YR with ten other detectable congeners at low abundance [13].

**Analytical methods for MCs using tandem mass spectrometry (EPA Method 544) and the ADDA ELISA (EPA Method 546) are well established [14,15]. Other analytical methods include the protein-phosphatase inhibition assay [16], and HPLC with photodiode array detection and/or mass spectrometry [7].**

Anatoxins (ATXs) are neurotoxins known for their acute toxicity [17], that bind to nicotinic acetylcholine receptors (nAChR) [18]. The structure of anatoxin-a was determined in 1977 by Devlin et al. [19], and other congeners with small structural modifications include homo-anatoxin [20] dihydro-anatoxin [21], epoxy-anatoxin [22], epoxy-homoanatoxin [23], dihydro-homoanatoxin [24], and 4-hydroxyhomo-anatoxin [25]. ATXs are produced by members of the cyanobacterial genera Dolichospermum (basionym Anabaena), Cuspidothrix (basionym Aphanizomenon), Arthrospira, Cylindrospermum, Microcystis, Oscillatoria, Phormidium, Planktothrix, and Raphidiopsis (basionym Cylindrospermopsis) [8]. Most methods for analysis are based on mass spectrometry (EPA Method 545) [26]. However, commercial ELISA assays for anatoxin-a have been produced recently [27].
Paralytic shellfish poisoning toxins (PSTs) inhibit the sodium channel of higher organisms, including humans and marine mammals [28–30]. These toxins are produced by marine dinoflagellates in addition to cyanobacteria, and can accumulate in shellfish feeding on the algae. Saxitoxin (STX), the parent compound in the PSTs group, is extremely toxic and shellfish are closely monitored for PSTs where the potential for human exposure might occur. There are more than 60 known congeners of STX [31–33]. These STX congeners can be more common than the parent STX in both freshwater and marine systems, but are generally less toxic than STX as measured by the mouse bioassay [34,35]. PST-producing cyanobacteria are found in freshwater systems around the world, including in Australia [36,37], Brazil [38], United States [11,35,39–43], Canada [44], Germany [45], France [46], Portugal [47], Russia [48], and New Zealand [49]. PST producing genera include Cuspidothrix (basionym Aphanizomenon), Dolichospermum (basionym Anabaena), Microseira (basionym Lyngbya), Planktothrix, Raphidiopsis (basionym Cylindrospermopsis), and Scytonema.

Cyanobacteria blooms containing MCs have led to contamination of drinking water supplies in the United States in Toledo, Ohio [8,50], Skaneateles Lake, New York [51,52], and Salem, Oregon [53], spurring national attention and action plans to manage and reduce contamination. The United States EPA issued a guideline value for MCs in drinking water [54] and exceedances of these limits have caused health alerts for toxins in drinking water. The United States EPA did review known toxicological information
regarding anatoxin-a [55], but they did not issue a guideline value. Anatoxins (ATXs) produced by benthic cyanobacteria in New Zealand may have contaminated drinking water supplies [24], and ATXs are widespread throughout New York lakes [13,56,57].

There are few studies evaluating cyanotoxin co-occurrence within water bodies. Exposure to multiple cyanotoxins may have synergistic effects increasing the risks of exposure [58]. MCs and anatoxin-a were detected in rivers and streams in California and Pennsylvania in the United States [59–61], in lakes in Europe [62,63], and in Argentina [64]. Cyanobacteria also produce bioactive peptides other than MCs, which can co-occur with anatoxin-a [65,66], and MCs [67]. Only a limited number of assessments of the toxic effects of cyanotoxin mixtures have been reported [68].

Chautauqua Lake is a eutrophic lake in western New York (42°10′51.4″ N 79°25′50.5″ W) (Figure 6.1). The North Basin of Chautauqua Lake is the deeper and cooler of the two basins with an average depth 9.1 m, and a maximum depth of 23 m [69,70]. The North Basin stratifies, producing low oxygen conditions for large portions of the summer [71]. Comparatively, the South Basin has a maximum depth of 5.7 m and a mean depth of 4.7 m [69], and does not stratify for extended periods of time [71].

Algal blooms in Chautauqua Lake are dominated by cyanobacteria [69], where there is potential for chronic and acute exposure to cyanotoxins. Potentially toxigenic cyanobacteria genera have been identified in the lake, including *Cuspidothrix* (basionym *Aphanizomenon*), *Dolichospermum* (basionym *Anabaena*), *Microcystis*, and *Planktothrix*. The
complex mixture of cyanobacteria in the lake could lead to the co-occurrence of the three toxins classes, MCs, ATX, and PSTs. Here we evaluated the co-occurrence of cyanotoxins and their correlation to environmental and physical variables in the two basins of Chautauqua Lake. We discuss the potential for exposure to these toxins, and the broader implications for the protection of human and animal health in lakes containing multiple cyanotoxins.

6.3 Methods

6.3.1 Sample Collection and Chlorophyll Analysis

Samples were collected biweekly from the North and South Citizen’s Statewide Lake Assessment Program (CSLAP) sites (Figure 6.1) eight times over the summer and early fall each year between 2014-2017 using the methods described by the New York Department of Environmental Conservation [72]. A number of water quality parameters, including total nitrogen (TN), total phosphorus (TP), water temperature, ammonia, nitrate plus nitrite, pH, Secchi disk depth, and conductivity, as well as cyanobacterial chlorophyll and toxin abundance were collected over a four-year period totally thirty-two samples. For mid-lake stations (CSLAP North, 42°10'51.4”N 79°25’50.5”W, and CSLAP South, 42°07'23.8”N 79°21’50.0”W), 200 mL of lake water was filtered onto glass fiber (934-AH) for toxin analysis [72].
In addition to these mid-lake sites, composite samples consisting of 250 mL of lake water was collected from below the surface, containing a mix of water and cyanobacterial material, were collected weekly from the Whiteside (42°11'38.5"N 79°25'16.4"W), Bridge (42°09'08.4"N 79°23'06.6"W), and CLA (42°06'09.5"N 79°18'05.8"W) sites beginning in May-June and ending October-November. Samples were collected from locations marked in blue (Figure 6.1) 1-5 times per year, depending whether a bloom was visually observed at each site upon weekly inspection.

Both filter samples and composite bloom samples were shipped overnight to SUNY ESF for analysis. Upon receipt, total chlorophyll and cyanobacterial chlorophyll were measured using a FluoroProbe (bbe Moldanek, Schwentinental, Germany) and 100 mL of bloom material immediately lyophilized to dryness. Major cyanobacteria species were qualitatively identified in a 500 µL aliquot using an inverted microscope at 50-200x.
Figure 6.1. Map of Chautauqua Lake, New York (42°10'51.4"N  79° 25'50.5"W). The lake is divided into two basins with differing trophic states. Two sites (CSLAP North, 42°10'51.4"N 79°25'50.5"W, and CSLAP South, 42°07'23.8"N 79°21'50.0"W) were sampled regularly for basic water quality parameters. Three shoreline sites, Whiteside (42°11'38.5"N 79°25'16.4"W), Bridge (42°09'08.4"N 79°23'06.6"W), and CLA (42°06'09.5"N 79°18'05.8"W) were sampled weekly for toxin and cyanobacterial chlorophyll. Other shoreline sites, labeled in blue, were sampled when blooms were visually identified at these sites.
6.3.2 Toxin Extraction and Analysis

Microcystins, ATXs, and PSTs were extracted from filter samples and lyophilized material using 10 mL of 50% methanol containing 1% acetic acid (v/v) and sonicated (3 x 20 s at 32 watts). The resulting slurry was centrifuged at 15,000× g for 10 min, passed through a 0.45 µm nylon syringe filter, and kept at -20 °C until analysis.

MCs were analyzed by LC-MS as described in Tang et al. [62]. The microcystin-LR instrument LODs was 3 pg, while method LODs ranged between 0.10-1 µg/L, and the response was linear between 4.9-1395 µg MC-LR/L using 8 points calculated as environmental concentration of toxin in a 100 mL sample.

Anatoxin-a was analyzed by LC-MS/MS using a modified version of EPA method 545 that included one quantification and two confirmation ions [26]. Anatoxin-a was purchased from BioMol (Biomol GmbH, Hamburg, Germany). The structure of the anatoxin-a standard was confirmed by NMR spectroscopy prior to use. Anatoxin-a standards were calibrated gravimetrically. Quantification was performed using a linear regression of anatoxin-a with an anatoxin-a standard every 15 samples to verify the stability of the response. Instrument LODs for anatoxin-a, homo-anatoxin, α-dihydro-anatoxin, and β-dihydro-anatoxin were 2, 4, 0.6, and 0.2 pg on column. Corresponding method LODs ranged between 4-10 ng/L, 7-18 ng/L, 1-3 ng/L and 1-2 ng/L, respectively. Anatoxin-a response was linear between 5 ng and 48 ng oc, which corresponded to 0.96-73.0 µg anatoxin-a/L in a lake based on a 100 mL sample (slope, 8.6×10⁴, y int, 4.0×10⁴,
Dihydro-anatoxin was chemically synthesized [57] and included into the LC-MS/MS method in 2016, while homo-anatoxin was included in 2017. Analysis of these ATX derivatives, homo-anatoxin and dihydro-anatoxin, were performed as described in Chapter 4.

PSTs were analyzed using the AOAC 2011.02 post-column chemical oxidation modified for water samples and algal powders [73]. Separation used a Waters Alliance 2695 solvent delivery system (Waters, Milford, MA, USA), and a Chromenta KB 3µ 150 x 4.6 mm column with an ACE (ACE Ltd., Aberdeen, Scotland, UK) 3µ guard cartridge assembly at 0.8 mL/min. The solvent system was: (A), 2mM heptanesulfonate (Regis Technologies Inc., Morton Grove, IL, USA) in 10 mM ammonium phosphate adjusted to pH 7.1; (B), 500 mL 2 mM heptane sulfonate in 30 mM ammonium phosphate adjusted to pH 7.1 plus an additional 150 mL of acetonitrile [74]. The separation gradient was: 0% B for 0–3 min, 40% B for 3–5 min, 100% B for 5–13 min, and 100% B for 20 min, followed by equilibration of the column back to 0% B for 10.5 min. Post-column oxidation of the PST ring used 9 mM periodic acid (Alfa Aesar, Ward Hill, MA, USA) in 50 mM potassium phosphate at pH 9 at a flow rate of 0.45 mL/min entering a 25m 0.25 mm i.d. reaction coil (1 mL total volume) maintained at 65 °C. Following the coil, 0.5 M acetic acid was added at a flow of 0.45 mL/min. PSTs were detected at 330 and 390 nm excitation and emission. PSTs were differentiated from interfering fluorescent compounds by re-injection of the sample with water in place of the oxidant.
To quantitate the PST toxins, primary PST standards were obtained from NRC Canada (Institute for Marine Biosciences, Halifax, Canada) and United States Food and Drug Administration (FDA) (Silver Spring, MD, USA). FDA STX was diluted 1:50 to a concentration of 4 µM prior to use. NRC standards of saxitoxin, decarbamoylsaxitoxin, gonyautoxin 1, gonyautoxin 2, gonyautoxin 3, gonyautoxin 4, gonyautoxin 5, decarbamoylgonyautoxin 2, decarbamoylgonyautoxin 3, lyngbyatoxin 1, and C1+C2 were used to calculate relative response factors. An STX standard was injected every 10 samples to ensure stability of the response, with PSTs quantified using a linear regression. Saxitoxin (STX) instrument LODs were 0.9 pg, while method LODs ranged between 1-2 µg STX/L. STX was linear between 9.9 to 198 ng on column which corresponded to 9.3 and 729.5 µg/L in lake water based on a 100 mL sample (slope, \(4.23 \times 10^4\); y-int, \(-5.8 \times 10^4\); \(R^2\), 0.99). To differentiate PSTs from matrix interferents and naturally fluorescent compounds, samples that contained a peak in the chromatogram were reanalyzed with water in place of the periodic acid oxidant to determine compounds that were fluorescent only after the oxidation of the PST ring system.

BMAA was analyzed by LC-MS and LC-MS/MS as described in Boyer et al. [13], and cylindrospermopsins, including cylindrospermospin, epi-cylindrospermospin and deoxy-cylindrospermospin, were analyzed by LC-MS/MS as described in Smith et al. [42]. LODs for BMAA and cylindrospermospin were 150 and 500 pg oc, respectively, with method LODs of 0.3 and 0.03 µg/L, respectively.
6.3.3 Confirmation of PSTs using the Saxitoxin Receptor Binding Assay and STX-ELISA

Toxin extracts were analyzed for STX-like activity using a receptor binding assay for PSTs, utilizing microplate format methods detailed in Van Dolah et al. [75]. The total toxic potency of a sample was estimated by measuring the competition between radiolabeled STX [11-3H] (American Radiolabeled Chemicals, Inc., Saint Louis, MO, USA) and any STX-like activity present in samples for binding to voltage-gated sodium channels in a crude rat brain membrane preparation. Total PSTs were quantified as STX eq. by a calibration curve prepared from a STX dihydrochloride reference standard acquired from NIST (NIST reference material 8642, National Institutes of Standards and Technology, Gaithersburg, MD). Sample extracts in acidified 50% methanol were analyzed using dilutions of 1/5 (10% methanol) and 1/50 (1% methanol). The method detection limit was 2 µg STX eq./L.

STX concentrations were determined using Abraxis ELISA (part number 52255B, Abraxis LLC, Warminster, PA, USA) according to the manufacturer’s instructions. Bloom extracts were diluted to a maximum concentration of 5% methanol prior to analysis. Further dilutions were made when sample concentrations fell outside the linear range of the STX-ELISA calibration curve. STX-ELISA method LOD was 0.015 µg STX eq./L.
6.3.4 Calculation of Photosynthetically Active Radiation, Wind Speed, and Wind Direction

Direct normal irradiance, diffuse horizontal irradiance, wind speed, wind direction, dew point temperature, and solar zenith angle, were acquired from the National Renewable Energy Laboratory (NREL) at 42°08’39.5"N 79°23’07.9"W [76]. Photosynthetically active radiation (PAR) was calculated from NREL irradiance values using MODEL-1 developed by Alados et al., 1996 for each 30-minute point, and averaged over two-week intervals starting from January 1, 2014 until December 31, 2017 [77]. Wind speed and wind angle data consisting of wind speed and wind direction in 30-minute intervals were projected from cardinal coordinates into Cartesian coordinates, with each point converted into East/West or North/South proportions of wind speed. The average wind direction (AWD) and average wind speed (AWS) for each two-week interval starting from January 1, 2014 were determined from the average East/West and North/South wind speeds. Daily rainfall was acquired from the Chautauqua Lake-Jamestown airport METAR weather station (42°07’48.0"N 79°13’48.0"W) and averaged over two-week intervals starting January 1, 2014.
6.3.5 Model Simplification and Selection Approach for Blue-Green Algal Chlorophyll and Cyanotoxins

Models for cyanobacteria toxin and cyanobacteria abundance were developed based on the methods described by Bunea et al. and Abram et al. [78,79]. The statistical methods used a combination of bootstrap enhanced LASSO and ridge regression, as described in Technical Note S6.1. These regression techniques use a penalization term that through bootstrap iterations estimates the likelihood of any particular variable’s inclusion into a model. This is done by separating the data into equal-sized subsets that are used to create a training set versus a separate test set that can be used to evaluate the model. The importance of each predictor is evaluated by comparing the most complex model, that includes all predictors, to the most-simple model containing only an intercept and no predictors.

Penalized regression models were used here as, following the removal any data interval that had missing values for any parameter, only a limited number of points were left for model generation (Table S6.2). Due to over parameterization, penalized regression models were used in place of standard multiple regression techniques. While low sample size did not impact some parameters through overparameterization (cyanobacterial chlorophyll) and was only a small constraint for others (MCs), it resulted in full models for some response variables becoming over-parameterized (PSTs and ATX) (Table S6.2). Because standard regression techniques could not be used with
response variables that had become heavily over-parameterized, our approach allowed us to systematically produce models for each response variable. A more detailed summary of the penalized regression approach is described in Technical Note S6.1.

6.4 Results

6.4.1 Chautauqua Lake Water Quality and Algal Bloom Monitoring

Water quality measurements and total and cyanobacterial chlorophyll measurements in the two Chautauqua Lake basins are shown in Table 6.1. The South Basin was more eutrophic than the North Basin, with a 1 m shallower average Secchi disk depth, 27.1 µg/L more total phosphorus, and higher concentrations of total and cyanobacterial chlorophyll. A summary of the top 25th quartile of blooms in both basins is shown in Table 6.2. While the median cyanobacterial chlorophyll concentration was lower in the North Basin relative to the South Basin, average cyanobacterial chlorophyll was much higher in the North Basin. Maximum cyanobacterial chlorophyll concentrations in the North Basin were ~10-fold higher than in the South Basin. Blooms began to form in July, with the South Basin blooms appearing ~2 weeks earlier than those in the North Basin [80].
6.4.2 Occurrence of Cyanotoxins in Chautauqua Lake

The occurrence of the MCs, ATX, and PSTs in Chautauqua Lake is summarized for the lake as a whole and for each basin in Table 6.3. Neither the cylindrospermopsins nor BMAA were not found in any sample collected between the years 2014-2018. Microcystins (MCs) were widespread throughout Chautauqua Lake between 2014-2018. The MC congeners most frequently detected were MC-LR, -RR, or -YR while trace congeners included -H4YR [81], -dLR, -mLR, -WR, -FR, and -LA. These latter congeners were detected at low concentrations and represented a small portion of total MC concentration. However, H4YR concentrations were exceptionally high in 2017, accounting for ~30% of the total MC concentration, and were observed only in this one year.

The neurotoxic anatoxin-a and PSTs were detected in Chautauqua Lake (Table 6.3). Anatoxin-a was the predominate anatoxin congener; dihydro-anatoxin was detected in one sample in 2016, while dihydro-anatoxin and homo-anatoxin were not detected in 2017 or 2018. In contrast, PSTs were widespread in Chautauqua Lake. The bulk of the PSTs were not common marine PSTs. A single compound potentially identified as LWTX 3 by mass spectrometry was responsible for high concentrations of PSTs [35,74]. Samples without this PST contained much lower concentrations of total PSTs.
Table 6.1. Water quality measurements collected in the North and South Basins of Chautauqua Lake at biweekly intervals over 2014-2017. Statistical differences between water quality parameters were determined with paired t-tests. Significant differences ($p < 0.05$) are bolded. Water quality parameters are an average of 64 samples, 32 at each site. Chlorophyll differences were evaluated after natural log transformation due to extreme violations of normality, see text for discussion.

<table>
<thead>
<tr>
<th>Basin</th>
<th>North Basin Mean ± SD</th>
<th>South Basin Mean ± SD</th>
<th>Difference in Means from Paired t-test ($p$ value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP (µg P/L)</td>
<td>41.7 ± 18.4</td>
<td>68.9 ± 35.1</td>
<td>27.1 (&lt;0.0001)</td>
</tr>
<tr>
<td>TN (mg N/L)</td>
<td>0.57 ± 0.68</td>
<td>0.74 ± 0.33</td>
<td>0.166 (0.15)</td>
</tr>
<tr>
<td>Ammonia (mg N/L)</td>
<td>0.046 ± 0.057</td>
<td>0.034 ± 0.032</td>
<td>0.012 (0.29)</td>
</tr>
<tr>
<td>NO$_2$ - NO$_3$ (mg N/L)</td>
<td>0.029 ± 0.028</td>
<td>0.026 ± 0.038</td>
<td>0.004 (0.66)</td>
</tr>
<tr>
<td>Water Temp (°C)</td>
<td>22.4 ± 2.3</td>
<td>23.5 ± 2.6</td>
<td>0.66 (0.039)</td>
</tr>
<tr>
<td>pH</td>
<td>7.93 ± 0.39</td>
<td>8.07 ± 0.66</td>
<td>0.13 (0.23)</td>
</tr>
<tr>
<td>Secchi (m)</td>
<td>2.29 ± 0.95</td>
<td>1.09 ± 0.70</td>
<td>1.17 (&lt;0.0001)</td>
</tr>
<tr>
<td>Conductivity (µS)</td>
<td>190.4 ± 33.3</td>
<td>193 ± 31.7</td>
<td>3.35 (0.57)</td>
</tr>
<tr>
<td>Total Chlorophyll (µg/L)</td>
<td>2426 ± 16780</td>
<td>700 ± 3370</td>
<td>NA*</td>
</tr>
<tr>
<td>Cyanobacterial chlorophyll (µg/L)</td>
<td>2408 ± 16780</td>
<td>635 ± 3080</td>
<td>NA*</td>
</tr>
<tr>
<td>Log Total Chlorophyll+1*</td>
<td>3.1 ± 2.3</td>
<td>3.8 ± 2.0</td>
<td>0.70 (&lt;0.001)*</td>
</tr>
<tr>
<td>Log Cyanobacterial chlorophyll+1*</td>
<td>2.5 ± 2.6</td>
<td>3.3 ± 2.3</td>
<td>0.78 (&lt;0.001)*</td>
</tr>
</tbody>
</table>

*Chlorophyll differences were evaluated with two-sample Welch test on the natural log transformed total and cyanobacterial chlorophyll concentrations due to non-normality of measurements.
Table 6.2. Chlorophyll fluoroprobe measurements for cyanobacterial specific chlorophyll for the largest 25\textsuperscript{th} quartile of blooms in Chautauqua Lake.

<table>
<thead>
<tr>
<th>Basin</th>
<th># of Samples</th>
<th>Range (µg/L)</th>
<th>Mean (µg/L)</th>
<th>Median (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>North</td>
<td>86</td>
<td>32.4-210,100</td>
<td>9591</td>
<td>212</td>
</tr>
<tr>
<td>South</td>
<td>49</td>
<td>97.4-29,680</td>
<td>2493</td>
<td>373</td>
</tr>
</tbody>
</table>
### Table 6.3. Occurrence and concentrations of microcystins, anatoxin-a, and paralytic shellfish poisoning toxins (PSTs) in Chautauqua Lake evaluated by year.

<table>
<thead>
<tr>
<th>Year</th>
<th>Date Range</th>
<th>Median Microcystins</th>
<th>Mean Microcystins</th>
<th>SD Microcystins</th>
<th>Median Anatoxin-a</th>
<th>Mean Anatoxin-a</th>
<th>SD Anatoxin-a</th>
<th># Toxic PSTs</th>
<th>Median Paralytic Shellfish Toxins</th>
<th>Mean Paralytic Shellfish Toxins</th>
<th>SD Paralytic Shellfish Toxins</th>
<th># Toxic PSTs</th>
</tr>
</thead>
<tbody>
<tr>
<td>2017</td>
<td>5/29 - 11/28</td>
<td>0.033</td>
<td>0.075</td>
<td>0.0097</td>
<td>0.59</td>
<td>1.1</td>
<td>0.23</td>
<td>28</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>2016</td>
<td>5/29 - 10/17</td>
<td>0.24</td>
<td>1.0</td>
<td>0.023</td>
<td>0.063</td>
<td>1.2</td>
<td>0.12</td>
<td>12</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>2015</td>
<td>6/7 - 10/19</td>
<td>0.058</td>
<td>0.073</td>
<td>0.011</td>
<td>0.058</td>
<td>0.12</td>
<td>0.012</td>
<td>22</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>2014</td>
<td>6/22 - 10/13</td>
<td>0.052</td>
<td>0.076</td>
<td>0.012</td>
<td>0.062</td>
<td>1.3</td>
<td>0.12</td>
<td>12</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0</td>
</tr>
</tbody>
</table>

Note: PSTs (µg STX eq/L), SD, standard deviation.
Three of eight samples that tested positive for PSTs by PCOX were confirmed to contain PSTs by STX-ELISA, but at a lower concentration than measured by PCOX (data not shown). Four of these eight samples were also evaluated by the receptor-binding assay, where none contained PSTs above the LOD of 2 μg/L.

### 6.4.3 Basin Wide Occurrence and Temporal Variation of Toxins

Cyanobacteria toxins were widespread in both basins of the Chautauqua Lake throughout multiple years (Figure 6.2). Blooms began to form and produce toxins starting in July, with toxins detectable through October. All three toxin classes were identified in both the North and South Basins, although not in all years.

MCs were identified at higher frequencies in the South Basin (20-40%). However, mean and median toxin concentrations were sometimes higher in the North Basin (Figure 6.2). The majority of samples for MCs contained between 1-100 μg/L of toxin, however some blooms contained much higher concentrations of toxin. Within the two basins, concentrations of toxin could vary significantly from year to year, with little MC detected in the South Basin in 2014, or in the North Basin in 2015, compared to other years. The majority of blooms exceeded the 4 μg/L recreational thresholds set by New York state [82], with far fewer exceeding the other 20 μg/L threshold of [83].
**Figure 6.2.** Box-and-whisker plots of microcystins, anatoxin-a and total paralytic shellfish poisoning toxins (PSTs) lakewide and in the North and South Basins of Chautauqua Lake over the years 2014-2017. Paralytic shellfish poisoning toxins (PSTs) were not analyzed in samples collected in 2014 and 2015. For missing box-and-whisker plots, no toxin was detected for that toxin and year. Dashed red lines indicate different recreational thresholds for each of the toxins set by several states and countries worldwide [83–88]. Upper and lower bounds of the boxes are the 25th and 75th percentiles, while the bar represents the mean. Whisker lengths are 1.5× the distance between the 25th and 75th percentiles, with any samples containing toxins outside this range are shown as points.
Anatoxin-a was mostly associated with the Southern Basin, with 80% of all anatoxin-a detections occurring in this basin. Over all four years, 70% anatoxin-a detections in Chautauqua lake occurred 2016, with this year being the only one where anatoxin-a was detected in the North Basin, where anatoxin-a was detected at one site. In the year that anatoxin-a was present in the North Basin, concentrations were 5-10-fold lower than those in the South Basin. None of the three recreational thresholds for anatoxin-a was exceeded [83–85].

Paralytic shellfish poisoning toxins (PSTs) were detected in both basins in 2016 and 2017. Concentrations of PSTs were slightly higher in the South Basin in 2016, but much higher in the North Basin in 2017. The number of PST-containing blooms was similar between the two basins over the two years. Most samples exceeded the 3 μg STX eq./L recreational guideline established by Ohio [83], however few samples exceeded the stringent 75 and 100 μg/L thresholds set by Oregon and Washington [86,88].

6.4.4 Site-Specific Occurrence of Toxins

MCs were detected regularly at the Whiteside, Bridge, and CLA sites during the summer and fall of 2014-2017 (Figure 6.3). Microcystins (MCs) were most commonly identified at the Bridge, located between the two basins (Table S6.3). The CLA site in the South Basin had slightly fewer MC detections compared to the Bridge, while the North
Figure 6.3. Box-and-whisker plots of microcystins, anatoxin-a and total paralytic shellfish poisoning toxins (PSTs) at the CLA, Bridge, and Whiteside over the years 2014-2017. Paralytic shellfish poisoning toxins (PSTs) were not analyzed in samples collected in 2014 and 2015. For missing box-and-whisker plots, no toxin was detected for that toxin and year. Dashed red lines indicate different recreational thresholds for each of the toxins set by several states and countries worldwide [83–88]. Upper and lower bounds of the boxes are the 25th and 75th percentiles, while the bar represents the mean. Whisker lengths are 1.5× the distance between the 25th and 75th percentiles, with any samples containing toxins outside this range are shown as points.
Basin site, Whiteside, had the fewest detections and lower average concentrations compared to the other two sites (Figure 6.3, Table S6.3). However, in 2017 an extreme bloom event occurred at Whiteside, where MC concentrations were greater than 100 μg/L in four of the five samples collected over a five-week period and maximum MC concentrations exceeded 4,000 μg/L. Detectable levels of MCs appeared earlier at the Bridge and CLA sites than Whiteside. Microcystins (MCs) were detected in all four years at the Bridge and CLA sites. Microcystin (MC) concentrations at the Bridge site were consistent between years. In contrast, concentrations at the CLA site were highly variable. Many blooms exceeded the 4 μg/L recreational guideline for MCs, although this heavily depended on the site and the year [82]. Far fewer exceeded the 20 μg/L guideline in all circumstances [83].

Anatoxin-a was found primarily at the CLA site. Nearly 50% of all anatoxin-a detections in these three sites occurred at the CLA in 2016. The highest detectable concentrations of toxin, ranging from <0.1-7.1 μg/L, also occurred that year. No blooms exceeded recreational guidelines for anatoxin-a [83–85].

PSTs were identified at all three sites more commonly than anatoxin-a, but much less frequently than the MCs. PST prevalence and concentrations were similar in 2016 and 2017. Several blooms in late July or early August contained high concentrations of total PSTs, exceeding 100 μg/L in several blooms. The majority of blooms exceeded a 3
μg/L recreational guideline [83], however few of the samples exceeded the larger 75 and 100 μg/L guidelines proposed by Washington and Oregon [86,88].

6.4.5 Co-occurrence of Multiple Cyanobacteria Toxins

MCs were detected with the most frequency at all scales, followed by PSTs and anatoxin-a. However, the toxin most commonly found co-occurring with multiple toxins were PSTs, followed by anatoxin-a and the MCs (Figure 6.3). This was the same for both basins, with the exception that anatoxin-a was largely undetected in the North Basin. MCs were found most often (70-85%) without other toxins. Although PSTs and anatoxin-a were less common than MCs, 30-60% of the samples containing these toxins contained other toxins.

The co-occurrence of the toxins depended on the individual site. Microcystins (MCs) rarely co-occurred with anatoxin-a at the Bridge and Whiteside sites, but co-occurred frequently with MCs at the CLA site. MCs had co-occurring PSTs at similar rates (23-33%) across all three sites, while PST containing samples had co-occurring MCs 50-71% of the time. Anatoxin-a co-occurrence varied significantly between the three sites.
**Figure 6.4.** Pie plots depicting the percent co-occurrence of multiple cyanotoxins at different scales. The dark grey slices represent the number of samples analyzed where the primary cyanobacteria toxin (labels on the left side of the figure) was not detected. The light grey slice plus the hatched slices represent total number of samples toxic with the primary toxin, while hatched slices represent the portion (with percentage) of samples containing two toxins. The outer pie chart includes all samples collected between 2014-2017 (analyzed for anatoxin-a and microcystins), with the inner pie chart includes all samples between 2016-2017 (analyzed for paralytic shellfish poisoning toxins). The co-occurrence of paralytic shellfish poisoning toxins (PSTs) with microcystins and anatoxin-a are shown clockwise from the light grey slice representing the number of samples containing PSTs.
6.4.6 Correlation of Cyanobacterial chlorophyll to Environmental Variables

Eleven environmental and physical parameters were used to model lakewide cyanobacterial chlorophyll. These terms produced a simplified ordinary least squares (OLS) model containing six parameters: PAR, AWS, pH, TP, conductivity, and TN (Figure S6.5A). Penalized regression models containing these six terms are shown in Figure S6.5B. All of the selective models from the least selective ridge regression ($\alpha = 0$), to the most selective LASSO ($\alpha = 1$), converged on selecting pH, TP, and TN as predictors (Table 6.4). The correlation of the parameters in the lakewide models for cyanobacterial chlorophyll were driven primarily by blooms in the North Basin, where 3/5 terms (AWS, pH, and TP) were selected by the OLS models lakewide and for the North Basin.

The parameters correlated to the cyanobacterial chlorophyll concentrations in the North and South basins were significantly different from one another. In the North Basin parameters AWS, pH, TP, TN, rainfall, and Secchi disk depth (Figure S6.5C) were all selected by OLS models, while in the South Basin, only TP was selected (Figure S6.5E) as a predictor of cyanobacterial chlorophyll. Ordinary least squares (OLS) bootstrapped models were not selective, where many terms in OLS models were not selected in further penalized models. For this reason, the selection of TP in the South Basin was not indicative of the term being a strong correlate to cyanobacterial chlorophyll. For the reduced model of the North Basin (Figure 6.5D), three of the terms
pH, TP, and Secchi disk depth were chosen most frequently, while a less stringent model also included rainfall in 50% of model iterations. No penalized models were obtained for cyanobacterial chlorophyll in the South Basin as only one term was selected.

6.4.7 Comparison Between Environmental Parameters for Cyanobacterial chlorophyll and the Three Cyanotoxins

The predictors for the three cyanotoxins were different from those predicting cyanobacterial chlorophyll (Table 6.4). For PSTs, the OLS model (Figure S6.6A) included cyanobacterial chlorophyll, AWS, pH, and conductivity as predictors, with pH dropping out when $\alpha = 0$ (Figure S6.6B), and no terms were selected when $\alpha > 0$. This was different from lakewide cyanobacterial chlorophyll models that selected pH, TP, and TN as important predictors.

The OLS model for anatoxin-a (Figure S6.6C) selected cyanobacterial chlorophyll, PAR, AWS, water temperature, TN, and rainfall as predictors. Cyanobacterial chlorophyll, PAR, and AWS were retained in the penalized models (Figure S6.6D), with cyanobacterial chlorophyll and AWS selected at all levels of $\alpha$, and PAR dropping out at $\alpha = 1$. The predictors for anatoxin-a were different from the lakewide and South Basin cyanobacterial chlorophyll predictors: pH, TP, and TN for the lakewide model and TP for the South Basin (OLS model).
**Table 6.4.** A summary of some of the models produced for cyanobacterial chlorophyll (Chl), microcystins, anatoxin-a, and paralytic shellfish poisoning toxins. Full model details are described in Figures S6.5, S6.6, and S6.7, while explanation of the parameter selection is described in the text and Technical Note S6.1. Abbreviations: TP, total phosphorus. TN, total nitrogen. PAR, photosynthetically active radiation. AWS, average wind speed. AWD, average wind direction.

<table>
<thead>
<tr>
<th></th>
<th>Cyanobacterial chlorophyll (Chl)</th>
<th>Microcysts</th>
<th>Anatoxin-a</th>
<th>Paralytic Shellfish Poisoning Toxins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lakewide</td>
<td>pH, TP, TN</td>
<td>None</td>
<td>Chl, PAR, AWS</td>
<td>Chl, AWS, conductivity</td>
</tr>
<tr>
<td>North Basin</td>
<td>pH, TP, Secchi</td>
<td>Chl, TP, water temp, rainfall</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>South Basin</td>
<td>None</td>
<td>Chl, PAR, AWD</td>
<td>Chl, PAR, AWS*</td>
<td>-</td>
</tr>
</tbody>
</table>

*As anatoxin-a was limited largely to the South Basin, the lakewide model is essentially a south basin model.*
Microcystins (MCs) had only two parameters selected by the OLS model (Figure S6.7A) for the entire lake: conductivity and water temperature. Neither of these terms were selected in penalized models. Similar to cyanobacterial chlorophyll, MCs in the two basins were correlated to different environmental parameters.

In the North Basin, cyanobacterial chlorophyll, TP, conductivity, water temperature, rainfall, and Secchi disk depth were correlated to MCs in the OLS model (Figure S6.7B). The penalized models selected cyanobacterial chlorophyll, TP, water temperature, and rainfall when $\alpha = 0$ (Figure S6.7C). These predictors were similar to those selected for cyanobacterial chlorophyll in the North Basin, which were pH, TP, Secchi, and rainfall.

In the South Basin, cyanobacterial chlorophyll, PAR, AWD, pH, and TP were selected by the OLS model (Figure S6.7D), with cyanobacterial chlorophyll, PAR and AWD all retained at $\alpha = 0$ (Figure S6.7E). Unlike the North Basin, penalized models up to $\alpha = 0.5$ still selected cyanobacterial chlorophyll as a predictor in the South Basin. Microcystins (MCs) and cyanobacterial chlorophyll were correlated to different predictors in the South Basin.

6.5 Discussion

The two basins of Chautauqua Lake were different morphologically, chemically, and biologically. The South Basin was more eutrophic than the North Basin over the
four-years of analysis, at least in part due to higher levels of TP, and slightly elevated surface water temperatures. This led to higher concentrations of cyanobacterial chlorophyll in the South Basin (Table 6.1). However, there were exceptionally large blooms in the North Basin, which were not observed in the South Basin (Table 6.2). Water temperature, while not found to correlated to bloom size, was related to the onset of blooms [89,90]. Blooms appeared several weeks earlier in the season in the warmer South Basin.

The two basins of Chautauqua Lake represent different types of lakes, where lake morphometry is highly influential with regards to cyanobacterial blooms. Blooms in the deeper North Basin responded more to nutrient inputs, with rainfall and phosphorus concentrations both correlating to cyanobacterial chlorophyll. In contrast, cyanobacterial chlorophyll in the shallow nutrient rich South Basin did not correlate well with any of our predictors, including nutrients, in the penalized regression models.

In addition to cyanobacterial chlorophyll, the MCs in Chautauqua Lake also correlated to different environmental predictors between the two basins. In the North Basin, MC concentrations correlated with nutrient parameters, while in the South Basin they were correlated with physical parameters including wind and light (Table 6.4). Anatoxin-a occurrence was largely restricted to the South Basin, where the environmental predictors included cyanobacterial chlorophyll, PAR, and AWS. Nutrients again were missing from the penalized model, reflecting the high levels of
nutrients in the South Basin. That anatoxin-a was rare in the North Basin may reflect environmental or physical differences between the North and South basins. There were insufficient data points to generate models for the PSTs in the individual basins.

Some lakes mimic the North Basin of Chautauqua Lake, where episodic large and highly toxic cyanobacteria blooms occur. Other lakes are comparable to the South Basin, and include shallow eutrophic water bodies. Nutrient management plans frequently are proposed to reduce populations of cyanobacteria in highly productive lakes [91], but nutrient reductions may have less of an impact when cyanobacteria are less responsive to nutrient inputs. A “one-size fits all” approach to nutrient management and cyanobacteria reduction may not always be effective.

The presence of cyanobacteria is necessary for the production of cyanotoxins, but environmental and physical variables may impact toxin occurrence differently than the cyanobacteria blooms themselves. Nutrient control can reduce cyanobacteria abundance [92], but may not necessarily reduce toxicity. Shoreline toxin concentrations in the South Basin depended more on physical parameters such as wind and light than on the endogenous nutrients, where changes in nutrients were not found to correlate with changes in bloom density or toxin concentrations.

The variation in toxins concentrations both between years and within years presents a significant problem for managing exposure to multiple toxins. Because of the variation between the basins and sites, extrapolations or assumptions about toxicity
from one site to the rest of the lake were not appropriate. The variation between years was often greater than the variation within years, and toxin concentrations from one year should not be extrapolated to concentrations in another year, even in highly eutrophic environments such as the South Basin. A similar high variability in toxin production has been observed in other shallow eutrophic lakes [93].

Co-occurrence of multiple cyanobacterial toxins has historically been uncommon. In Chautauqua Lake, each of the three cyanobacterial toxin classes co-occurred with one-other frequently (Figure 6.4). As risk assessments are often made using one cyanobacterial toxin, usually the MCs, the presence of other co-occurring toxins may increase the potential risks from exposure. Additionally, the PSTs and anatoxin-a were detected without any co-occurring MCs. Quantification of these neurotoxins will be important in future monitoring efforts, as MCs alone did not adequately evaluate the presence of multiple cyanobacterial toxins.

A sampling strategy should include samples collected from all sites where the risks to recreational users are highest. In Chautauqua Lake these were at shoreline sites where physical parameters may concentration the blooms. Many of the shoreline sites exceeded the 4 µg/L New York recreational guideline for MCs [82] and the 3 µg STX eq./L for PSTs set by Ohio [83].

Exposure to these toxins through drinking water is less likely. The drinking water intakes are located ~100 m away from the shore, limiting interaction with the
densest blooms. There were infrequent lakewide blooms in the North Basin [80], where MCs or PSTs produced from these blooms could enter the drinking water facility. However standard drinking water treatments are generally effective for MCs [9]. Some treatment strategies are effective for the ATXs or PSTs, however many remain untested [94].

While symptoms of acute exposure to cyanotoxins have been described [18,95], only MCs have been evaluated, and strongly linked, to long term chronic health impacts [96,97]. The toxicology and neurological effects from chronic exposure to either ATXs or PSTs have not been evaluated. Whether periodic exposure to these toxins in drinking water is cause for concern is unclear. Furthermore, the toxicological effects of cyanotoxins have been assessed individually. The synergistic or antagonistic effects of multi-cyanotoxin exposure has not been well evaluated [68]. Synergistic effects of these toxins could lead to underestimations of toxicity, as MCs frequently co-occurred with PSTs and anatoxin-a (Figure 6.4). Currently, most cyanobacteria toxin monitoring programs only incorporate the MCs. Microcystin (MC) monitoring alone may underestimate health risks from exposure to cyanobacteria toxins, as the presence of MCs was not indicative of the presence of either anatoxin-a or the PSTs.

The health risks from these neurotoxins is difficult to determine. For both the PSTs and anatoxin-a, guideline values for drinking water and recreational contact are highly variable. Anatoxin-a concentrations were generally low, but anatoxin-a was
found to be unstable in natural environmental conditions [57]. Therefore, any concentrations measured here may underestimate the concentrations of anatoxin-a in Chautauqua Lake. Paralytic shellfish poisoning toxins (PSTs) were commonly found in the lake. However, the receptor binding assay showed low to no toxicity [98], suggesting a dominance of congeners with little toxicity. A better understanding of the toxin profiles in these freshwater systems, and the potential for transformation [31,99] of freshwater PSTs to more toxic congeners is needed to evaluate the potential risk from these toxins.

Chautauqua Lake contained a complex mixture of cyanobacterial toxins. The presence and concentrations of these toxins depended on the basin and site (Figures 6.2 and 6.3). The heterogeneity of toxin concentrations within and between years is problematic for protecting lake users from exposure. Sampling of all sites where exposure is possible will be necessary, as concentrations of toxin frequently exceeded recreational guidelines. Monitoring should also include toxins other than the MCs. The neurotoxic PSTs and anatoxin-a were also found within Chautauqua Lake. While some information about the toxicity of these cyanotoxins has been described, there is still significant uncertainty about the chronic toxicity of both neurotoxins, including the parent compounds and their derivatives. These neurotoxins may pose a significant risk to human and environmental health.
6.6 References


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**Technical Note S6.1.** A description of the penalized regression models and a description of the challenges related to traditional statistical analysis of cyanobacterial chlorophyll and toxins in Chautauqua Lake.

The penalized regression modeling approach was chosen over more traditional methods as it resolved several common issues with regression that were observed in the Chautauqua Lake data-set. In the multiple regression MC models that contained many predictors, there were small but detectable amounts of multi-collinearity, as identified by variable inflation factor (VIF) and condition indices. Although simplified models did not appear to contain multicollinearity, standard model simplification approaches, as a side effect of their reliance on $p$-values, will be influenced by the instability of coefficients when collinear terms are included in the same model. For these reasons we determined that standard model simplification using stepwise removal or addition of predictors and F-tests or AIC for model selection would not be reliable when trying to answer the questions we wanted to explore in Chautauqua Lake.

To compare the results from the four response variables, the model selection approach for each response, cyanobacterial chlorophyll, MCs, ATX, and PSTs started with the same predictors. Initial model simplification was performed using bootstrap
enhanced OLS regression using 11 predictors for the toxins and 10 for cyanobacterial chlorophyll, with any of the parameters selected in 50% or more of the OLS models by non-parametric bootstrap quantile confidence interval selection (QNT) [78] included in a further set of penalized regression models. This initial simplification was performed because the inclusion of extraneous terms in the full models impacted the inclusion probability for all of the other parameters. Because the full models only selected a limited number of predictors, a reduction in the number of included terms to only those selected in the full model iteration of OLS led to better convergence at higher model restrictiveness levels in the penalized regression models.

Using the parameters selected by the bootstrapped OLS model, five penalized regression models ridge ($\alpha= 0$), elastic net ($\alpha= 0.25, 0.5, 0.75$) and LASSO ($\alpha = 1$) were produced at different levels of selectivity ($\alpha$), with larger $\alpha$ increasing the selectiveness criteria for the inclusion of parameters into each model. Parameter inclusion for each model was evaluated using QNT selection [78]. Because ideal model restrictiveness levels varied between response variables, each model from $\alpha = 0$ to $\alpha = 1$ is shown in Figures S6.5, S6.6 and S6.7. QNT parameter selection allowed for a meaningful interpretation of ridge regression, where unimportant parameters were eliminated due to their lack of contribution to the model. Rather than only reducing the impact of unimportant coefficients on ridge regression models, QNT selection produced models
with limited numbers of predictors. All models were used to examine the differences between the basins and between toxins only, not to produce models for prediction.
Table S6.2. The number of points, representing two-week averages for each parameter, used to produce each model shown in Table 6.4 and Figures S6.5, S6.6 and S6.7 following omission of any data points that contained any missing value. The maximum number of possible data points was 40. Chl, cyanobacterial chlorophyll.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Number of Data Points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chl All Lake</td>
<td>31</td>
</tr>
<tr>
<td>Chl OLS only</td>
<td>31</td>
</tr>
<tr>
<td>Chl North</td>
<td>30</td>
</tr>
<tr>
<td>Chl North OLS</td>
<td>30</td>
</tr>
<tr>
<td>Chl South</td>
<td>23</td>
</tr>
<tr>
<td>Chl South OLS only</td>
<td>NA</td>
</tr>
<tr>
<td>MC All Lake</td>
<td>19</td>
</tr>
<tr>
<td>MC OLS only</td>
<td>19</td>
</tr>
<tr>
<td>MC North</td>
<td>12</td>
</tr>
<tr>
<td>MC North OLS</td>
<td>13</td>
</tr>
<tr>
<td>MC South</td>
<td>14</td>
</tr>
<tr>
<td>MC South OLS only</td>
<td>16</td>
</tr>
<tr>
<td>PSTs</td>
<td>12</td>
</tr>
<tr>
<td>PSTs OLS only</td>
<td>12</td>
</tr>
<tr>
<td>ATX</td>
<td>12</td>
</tr>
<tr>
<td>ATX OLS Only</td>
<td>12</td>
</tr>
</tbody>
</table>
Table S6.3. Summary of the number of samples that contained microcystins (MCs), anatoxin-a, and paralytic shellfish poisoning toxins (PSTs) from the CLA, Bridge, and Whiteside sites between 2014-2017. NA, not tested.

<table>
<thead>
<tr>
<th>Year</th>
<th>CLA</th>
<th>Bridge</th>
<th>Whiteside</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># with MCs/total samples</td>
<td># with anatoxin-a</td>
<td># with PSTs</td>
</tr>
<tr>
<td>2014</td>
<td>1/12</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>2015</td>
<td>4/18</td>
<td>5</td>
<td>NA</td>
</tr>
<tr>
<td>2016</td>
<td>3/17</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>2017</td>
<td>8/17</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>
**Table S6.4.** GPS coordinates for all sites sampled on Chautauqua Lake for cyanobacteria toxins, as shown in Figure 6.1.

<table>
<thead>
<tr>
<th>Site Name</th>
<th>Site GPS Coordinates Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chautauqua CSLAP North</td>
<td>42°10'51.4&quot;N 79°25'50.5&quot;W</td>
</tr>
<tr>
<td>Chautauqua CSLAP South</td>
<td>42°07'23.8&quot;N 79°21'50.0&quot;W</td>
</tr>
<tr>
<td>Bemus Bay Shore Edge</td>
<td>42°09'38.3&quot;N 79°23'33.9&quot;W</td>
</tr>
<tr>
<td>Bemus Hotel Lenhart</td>
<td>42°09'31.7&quot;N 79°23'41.9&quot;W</td>
</tr>
<tr>
<td>Bemus Point DEC Launch Ramp</td>
<td>42°09'22.5&quot;N 79°23'36.5&quot;W</td>
</tr>
<tr>
<td>Boys &amp; Girls Club Children's Camp - Chautauqua Institution</td>
<td>42°12'14.6&quot;N 79°27'37.1&quot;W</td>
</tr>
<tr>
<td>Bridge</td>
<td>42°09'08.4&quot;N 79°23'06.6&quot;W</td>
</tr>
<tr>
<td>Celoron Launch/Lighthouse</td>
<td>42°06'40.7&quot;N 79°16'58.3&quot;W</td>
</tr>
<tr>
<td>Central Dock</td>
<td>42°12'33.7&quot;N 79°27'44.2&quot;W</td>
</tr>
<tr>
<td>Cheney Ave Dock</td>
<td>42°07'18.8&quot;N 79°19'31.4&quot;W</td>
</tr>
<tr>
<td>Children's Beach</td>
<td>42°12'39.3&quot;N 79°27'43.6&quot;W</td>
</tr>
<tr>
<td>CLA</td>
<td>42°06'09.5&quot;N 79°18'05.8&quot;W</td>
</tr>
<tr>
<td>Crosswinds Marina</td>
<td>42°13'31.8&quot;N 79°26'22.4&quot;W</td>
</tr>
<tr>
<td>Approximate Location of Drinking Water Intake</td>
<td>42°12'45.9&quot;N 79°27'56.8&quot;W</td>
</tr>
<tr>
<td>Heinz Beach</td>
<td>42°12'21.4&quot;N 79°27'38.8&quot;W</td>
</tr>
<tr>
<td>Lakewood Beach</td>
<td>42°06'24.4&quot;N 79°19'35.2&quot;W</td>
</tr>
<tr>
<td>Long Point Park Beach</td>
<td>42°10'19.7&quot;N 79°24'46.1&quot;W</td>
</tr>
<tr>
<td>Long Point Park Marina</td>
<td>42°10'19.8&quot;N 79°24'56.0&quot;W</td>
</tr>
<tr>
<td>Mayville Beach</td>
<td>42°14'31.3&quot;N 79°29'39.5&quot;W</td>
</tr>
<tr>
<td>Pier Beach</td>
<td>42°12'38.9&quot;N 79°27'44.8&quot;W</td>
</tr>
<tr>
<td>Prendergast Point</td>
<td>42°11'21.7&quot;N 79°26'27.0&quot;W</td>
</tr>
<tr>
<td>Stow Ferry</td>
<td>42°09'25.7&quot;N 79°24'01.9&quot;W</td>
</tr>
<tr>
<td>University Beach</td>
<td>42°12'49.3&quot;N 79°28'09.1&quot;W</td>
</tr>
<tr>
<td>Whiteside</td>
<td>42°11'38.5&quot;N 79°25'16.4&quot;W</td>
</tr>
<tr>
<td>Woodlawn Dock</td>
<td>42°10'16.5&quot;N 79°25'55.0&quot;W</td>
</tr>
</tbody>
</table>
**Figure S6.5.** Penalized regression models for cyanobacterial chlorophyll in the lakewide (A and B), the North Basin (C and D), and the South Basin (E). The models are shown as follows: **A**, Whole lakewide model; **B**, Whole lake with OLS terms selected in A; **C**, North Basin full model; **D**, North Basin with OLS terms selected in C; **E**, South Basin full model. As only one term was selected for the South Basin full model, no further analysis could be performed. Predictors used for **A, C, E**: 1 Photosynthetically Active Radiation (PAR), 2 Average Wind Speed (AWS), 3 Average Wind Direction (AWD), 4 pH, 5 Total Phosphorus (TP), 6 Conductivity, 7 Water Temperature, 8 Total Nitrogen (TN), 9 Rainfall, 10 Secchi Disk Depth. Predictors used for **B**: 1 PAR, 2 AWS, 3 pH, 4 TP, 5 Conductivity, 6 TN. Predictors for **D**: 1 AWS, 2 pH, 3 TP, 4 TN, 5 Rainfall, 6 Secchi Disk Depth.
Figure S6.6. Penalized regression models for paralytic shellfish poisoning toxins (PSTs) (A and B), and anatoxin-a (C and D) in both basins. The models are shown as follows, A, PSTs full model; B, PSTs with OLS terms selected in A; C, anatoxin-a full model; D, anatoxin-a terms selected in C. Predictors used for A and C: 1 Log Cyanobacterial Chlorophyll, 2 Photosynthetically Active Radiation (PAR), 3 Average Wind Speed (AWS), 4 Average Wind Direction (AWD), 5 pH, 6 Total Phosphorus (TP), 8 Conductivity, 9 Water Temperature, Total Nitrogen (TN), 10 Rainfall, 11 Secchi Disk Depth. Predictors used for B: 1 Log Cyanobacterial Chlorophyll, 2 AWS, 3 pH, 4 Conductivity. Predictors used for D: 1 Log Cyanobacterial Chlorophyll, 2 PAR, 3 AWS, 4 Water Temperature, 5 TN, 6 Rainfall.
Elastic-Net

OLS  Ridge (\(\alpha = 0\))  \(\alpha = 0.25\)  \(\alpha = 0.5\)  \(\alpha = 0.75\)  LASSO (\(\alpha = 1\))

A

Threshold \((1 - \alpha^*)\)

0.5  0.6  0.7  0.8  0.9

2  4  6  8  10

B

Threshold \((1 - \alpha^*)\)

0.5  0.6  0.7  0.8  0.9

1  2  3  4

C

Threshold \((1 - \alpha^*)\)

0.5  0.6  0.7  0.8  0.9

2  4  6  8  10

D

Threshold \((1 - \alpha^*)\)

0.5  0.6  0.7  0.8  0.9

1  2  3  4  5  6

Predictor

Predictor

Predictor

Predictor

Predictor
Figure S6.7. Penalized regression models for Microcystins (MCs) in the whole lake (A), the North Basin (B and C) and the South Basin (D and E). The models are shown as follows, A, whole lake full model; B, North Basin full model; C, North Basin with OLS terms selected in B; D, South Basin full model; E, South Basin with OLS terms selected in D. For brevity, the penalized models for the full lake are not shown as neither of the terms chosen in A were selected in the penalized models. Predictors used for A, B, and D: 1 Log Cyanobacterial Chlorophyll, 2 Photosynthetically Active Radiation (PAR), 3 Average Wind Speed (AWS), 4 Average Wind Direction (AWD), 5 pH, 6 Total Phosphorus (TP), 8 Conductivity, 9 Water Temperature, Total Nitrogen (TN), 10 Rainfall, 11 Secchi Disk Depth. Predictors used for C: 1 Log Cyanobacterial Chlorophyll, 2 TP, 3 Conductivity, 4 Water Temperature, 5 Rainfall, 6 Secchi Disk Depth. Predictors used for E: 1 Log Cyanobacterial Chlorophyll, 2 PAR, 3 AWS, 4 Water Temperature, 5 TN, 6 Rainfall.
Elastic-Net

OLS  Ridge (\(\alpha = 0\))  \(\alpha = 0.25\)  \(\alpha = 0.5\)  \(\alpha = 0.75\)  LASSO (\(\alpha = 1\))
7. Concluding Discussion

Cyanobacteria produce a wide range of toxins [1,2]. Most studies have focused on the occurrence of the hepatotoxic microcystins (MCs) because of their known toxicity [3,4], global distribution [5], and established regulatory guidelines in drinking water and for recreation [6,7]. Surveys evaluating the distribution of the neurotoxic paralytic shellfish poisoning toxins (PSTs) and anatoxins (ATXs) in lakes are limited. The hypothesis of this work was that neurotoxins were produced by benthic and planktonic cyanobacteria throughout New York and that the concentrations of these toxins exceeded recreational guidelines for the protection of human health frequently enough to be a concern. To evaluate this hypothesis, we measured the occurrence and distribution of these neurotoxins in New York lakes both regionally, using samples collected through the Citizens Statewide Lake Assessment Program, and locally, in two lakes to determine whether they impacted environmental and human health.

The neurotoxic PSTs represent a unique challenge as the methods for their analysis are complex. There are a large number of PST congeners, and the toxicity of the congeners can differ greatly. Freshwater PSTs are of emerging concern and effective analytical tools for their measurement are needed. Four analytical methods, originally developed for marine PSTs, were compared to evaluate the effectiveness of these methods for the analysis of freshwater PSTs. No one method emerged as the “best” method for freshwater PST analysis relative to the other three. Depending on the lake or
sample in question, each of the four methods had advantages and disadvantages over the others. There was significant disagreement between methods at estimating PST concentrations and on which samples contained toxins. Differences in PST quantification were not unexpected as the methods relied upon different properties of the PST backbone for detection and therefore the reactivity of the different congeners relative to saxitoxin (STX) were different. Additionally, the long-term stability of the freshwater PSTs has not been evaluated, and degradation of the PSTs over time may have also led to some of the differences in PSTs measured by the different methods.

To reduce the likelihood of false negatives in analysis of freshwater PSTs, we suggest a combination of two methods be used for PST monitoring. Of the four methods evaluated, the receptor-binding assay is the most effective at estimating exposure risks for humans as the receptor-binding assay utilizes sodium channels, the biological target for PSTs, to detect toxin. Therefore, to effectively protect human health other methods should equal or over-estimate toxin concentrations relative to the receptor-binding assay. For the majority of method combinations, one of the two methods equaled or over-estimated PST concentrations compared to the receptor-binding assay [8], whereas only one analytical method did not always quantify toxins at or above the concentration measured by the receptor-binding assay. Future work should include a greater number of samples from more environments to provide better clarity on how different methods compare when quantifying PSTs relative to the receptor-binding assay.
While estimates of total PSTs between methods varied, there was also significant variation in PSTs estimated by the three mass spectrometric analyses. These differences were partially due to methodology, such as with the ion suppression observed in raw extracts. However, analyst differences also led to large differences in toxin quantification. The analyst for method 1 utilized the retention time information provided by the HPLC with post-column oxidation, looking primarily for the molecular weight and fragmentation of PSTs as a confirmation of the results from oxidation. The analyst for method 2 did not utilize the information from other analytical methods, and therefore did not flag the same compounds as toxins. Part of the quality assurance protocol for established analytical methods is to minimize the differences between analysts. As the methods for freshwater PSTs are still emerging, and there are many variants of PSTs with similar structure, the analyst is a significant source of variability in the analysis of PSTs.

Unlike the PSTs, the methods for anatoxin-a are well established [9], with multiple studies having evaluated the distributions of anatoxin-a in freshwater environments [10–15]. However, these methods have mostly focused on a single compound. There are a number of other ATX variants, including dihydro-anatoxin and homo-anatoxin, that may be toxic. These variants were incorporated into an established tandem mass spectrometric analysis for anatoxins to determine the distribution in New York waters.
On a regional scale, PSTs were found in 15% of blooms collected from New York lakes between 2016-2018, and at much higher percentages in some lakes. Previous studies suggested PSTs occurred in low concentrations and in a limited number of waterbodies [11]. The widespread presence of the PSTs across New York is in agreement with the distributions of PSTs identified in Ohio [16].

Most PST exposure studies have focused on acute exposure to high concentrations of PSTs from consumption of shellfish [17,18], while there is little information about long term and chronic exposure to PSTs. The toxicities of the freshwater lyngbyatoxin (LWTX) PSTs congeners are lower relative to STX. However, sodium channel inhibition was still identified above the 3 µg/L recreational guideline established by Ohio [19]. This suggests that even with the reduced toxicity of some of the known freshwater PST congeners, there may still be the potential for harm in humans.

These toxins were consistently present in multiple lakes between years, leading to the possibility of chronic exposure. Recreational exposure was most likely as the majority of blooms were collected from shoreline sites. However, some PSTs may have been part of the dissolved phase, raising the risk of toxins entering drinking water facilities. Monitoring for PSTs across New York state will be important to minimize the impacts these toxins may have on humans and animals.
Anatoxins (ATX) were found in a similar number of samples as the PSTs (11%), but in much lower concentrations. Anatoxin-a was the predominant congener present, and dihydro-anatoxin and homo-anatoxin derivatives were not widespread in New York blooms. Anatoxins (ATX) are unstable in the environment [15], and therefore the levels measured may underestimate their true concentrations. While dihydro-anatoxin and homo-anatoxin were rare overall, there was a subset of blooms where the peak concentrations of these congeners were high (>50 µg/L), potentially exposing humans and animals to harmful concentrations of these toxins.

Anatoxin-a has been found worldwide [10,11,14,20], and the ATX congeners were widespread in rivers and streams in New Zealand [21,22], where ATXs have led to canine fatalities [23–25]. Anatoxins have been suggested to chronically contaminate drinking water in New Zealand [26]. In New York, exposure to these toxins via drinking water is unlikely, as the majority of the samples collected in this survey were from shoreline scums and blooms. However, exposure to the ATXs during recreational use of the contaminated lakes may occur. Unlike MCs, ATXs were poorly correlated with surface chlorophyll, and therefore users may be exposed to toxins when no visible threat is present. The combination of exposure potential, the potential for underestimation of the actual concentrations of ATXs, and the uncertainty of human recreational guidelines, leave conclusions about the risk from ATXs in New York decidedly uncertain. Risk assessments of ATXs are critically needed to produce
guidelines for anatoxin-a and the derivatives, including toxicological data from chronic
dosing of the toxin where long-term neurological damage was evaluated.

While the survey of neurotoxins across New York lakes gave a broad overview of
toxin occurrence, this assessment may miss the intricacies of neurotoxin occurrence in
individual lakes. Two lakes were examined in detail. One lake contained PSTs
produced by a benthic cyanobacterium. The other lake contained a mixture of MCs,
anatoxin-a, and PSTs, where these toxins frequently co-occurred. These two lakes
emphasize the challenges associated with risk assessment of toxin exposure in different
environments.

Paralytic shellfish poisoning toxins (PSTs) in Butterfield Lake were produced by
a benthic cyanobacterium, Microseira wollei. While intracellular toxin concentrations of
PSTs were high, the toxins were never detected in the surface water of Butterfield Lake.
However, if this toxin were to be released during a massive detachment event, such as
the one that occurred in Lake Erie in 2006 [27], significant quantities of toxin could be
released. A large amount of biomass accumulated on the shoreline during the Lake Erie
event. Using our preliminary estimates of the concentrations of toxin within the
cyanobacterium, the toxin load on 100 m of shoreline was ~40,000 kg of saxitoxin
equivalents. A similar event in Butterfield Lake could be disastrous to humans or other
animals that come into contact with the released algae. Management options to reduce
the potential for exposure are not obvious. Removal of the benthic material is unlikely
to be a viable approach as the cyanobacteria would simply regrow following removal.

As the cyanobacterium appeared to acquire much of their nutrients from the sediments, traditional nutrient management plans are unlikely to have a great impact.

The highest concentrations of toxin were found as water temperatures decreased in September and October, corresponding with less recreational use on the lake. Consumption of benthic material by pets was a possibility. Drinking water was an unlikely exposure vector as dissolved toxins were not detected in the water column. However, to reduce the potential for chronic consumption, private drinking water intakes should not be placed near the Microseira mats. Signage should be placed near the public access points in the lake to alert recreational users and pet owners to not approach dislodged benthic mat material as a precaution.

In Chautauqua Lake, the source(s) of both anatoxin-a and PSTs may have been planktonic cyanobacteria, benthic cyanobacteria, or a combination of the two. Exposure to these toxins could occur from recreational contact and/or through drinking water. The overall risk was increased by the high heterogeneity of the blooms and of their toxin concentrations. Combinatorial effects of multiple cyanotoxins present in these blooms may also change the risk(s) from exposure.

Recreational contact would be the most likely route of exposure to toxins in Chautauqua Lake. The majority of samples were collected from shoreline locations around the lake, including several swimming beaches. Toxin concentrations at these
locations could be in the hundreds of µg per liter, well above the recreational contact guidelines for both the MCs and PSTs. Further, the variability of toxin concentrations within and between years makes it difficult to predict the risk from a bloom, without direct toxin testing. The combination of high concentrations of toxin that far exceed recreational guidelines, easy access to the lake, and a large population of users who may not understand the risks that come from exposure to cyanobacteria toxins all combine to create a potentially dangerous circumstance. Monitoring the shoreline areas for cyanobacteria toxins in Chautauqua Lake is advised.

Exposure through public drinking water intake is an unlikely scenario in Chautauqua Lake. The drinking water intake was located ~100 m away from the shore, and away from the impact of most blooms. There were infrequent lakewide blooms in the North Basin. While toxins produced from these blooms could enter drinking water facilities [28], standard drinking water treatments are generally effective for MCs [29], and the risk of chronic exposure to MCs is low. Some treatment strategies are effective for the ATXs or PSTs, however many remain untested [30]. While ATXs were nearly undetected in the North Basin and therefore the risk from these toxins was minimal, there were high concentrations of PSTs. Infiltration of the PSTs into the drinking water plant in Chautauqua Lake may therefore be of concern. Response plans such as those in Ohio, may help minimize the impact in an event where cyanobacteria toxins contaminate the water treatment plants [31].
Butterfield and Chautauqua Lakes had different toxins and relationships with these toxins. While cyanobacteria in Butterfield Lake did differ at the two sites in how they responded to environmental variables, they responded to the same set of physical environmental parameters. This was not true in Chautauqua Lake, where the morphology of the lake significantly changed how the environment interacted with blooms and toxins. In the North Basin, nutrient inputs were most important for blooms and toxins, while in the South Basin, physical parameters were most important. In Chautauqua Lake, the environment produced a complex mixture of cyanobacteria genera that contained a mixture of cyanobacterial toxins, unlike Butterfield Lake which was dominated by PST-producing *Microseira*.

There are different challenges associated with managing human exposure to cyanobacterial toxins in Butterfield Lake and Chautauqua Lake. In Butterfield Lake, the population density was low and the cyanotoxin producing organisms located in only two sites, whereas in Chautauqua Lake is heavily populated and the toxins were produced by cyanobacteria blooms that at times spanned the entire lake. In Chautauqua Lake, signage and regular monitoring can help mitigate the potential for people to be exposed to high concentrations of toxins. However, these same strategies are likely to be ineffective in Butterfield Lake as the cyanobacteria rarely detached from the sediment surface. While this means the risks are lower overall, in event of detachment the concentrations of toxin could be exceptionally large and the risks from exposure
significantly increased. The studies in these two lakes emphasizes that cyanobacterial bloom and risk management strategies for different water bodies do not always overlap.

Most of our risk assessment for cyanotoxins has focused on the hepatoxic MCs. While the neurotoxins were less common than the MCs in New York, they were still present in dozens of lakes. Lack of toxicological information about these toxins makes it difficult to determine whether the concentrations of toxin observed represent a health hazard. However, the combination of easy access to many New York lakes, and measured toxin concentrations that exceeded current recreational guidelines, suggest that these toxins are a cause for concern. As there is significant diversity in the limnology and ecology between lakes, and as lakes are used for a number of different purposes, e.g. fishing, recreation, drinking water, etc., approaches toward managing the potential for exposure to cyanobacteria toxins may vary from lake to lake.

While the structures for many PSTs and ATXs were elucidated decades ago, little is known about their biochemistry and the fundamental biological purpose for the production. While the studies outlined here suggest that these toxins may be important with regard to human health, this study is limited by the lack of knowledge about the producing organisms and how they respond to nutrient and physical stimuli. The models for neurotoxins in both Butterfield Lake and Chautauqua Lake could be much improved by further in vivo studies, where most other models for these toxins have
been made using macroscopic observations, rather than from a fundamental heuristic understanding of how the cyanobacteria function. This is exemplified by the relationship of PSTs with temperature in Butterfield Lake, where temperature may be covariate with other environmental factors such as the amount of available light. Controlled laboratory studies with these organisms may help discern these relationships.

There is only a limited set of freshwater PST-producing cyanobacteria, and even fewer ATX producing cyanobacteria, in culture. As outlined, laboratory studies with these cyanobacteria may improve our understanding of the risks these organisms pose to human health. However, isolating the organisms producing the toxins is a crucial step in this process. As freshwater PST-producing planktonic cyanobacteria are more common than previously realized, these organisms should be isolated for further study.

This will be aided by improvements in analytical techniques for PSTs, which can be used to detect PSTs not commonly detected in marine environments. Improvement in the analytical methods for the PSTs will also be crucial for environmental analysis. While advances in mass spectrometry are responsible for the largest step-forward in decades in analytical methods for the PSTs, there are still many challenges with this method. The lack of agreement between the mass spectrometry methods and other older techniques for measuring PSTs, as well between the mass spectrometry methods themselves emphasizes that analytical techniques need significant refining to best
protect human health. Further advancements in these areas in the science of neurotoxins will be important for managing the impact these toxins in New York and the wider world.

7.1. References


Vita

Education

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List of Publications and Presentations

Publications:

Presentations:


Freshwater Paralytic Shellfish Toxin Analysis by HPLC-Fluorescence in New York State: Implications for Great Lakes Monitoring. International Association for Great Lakes Research 2017. Poster


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